IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF MASSACHUSETTS

JANSSEN BIOTECH, INC., and NEW YORK UNIVERSITY	)	
Plaintiffs,	)	
	)	
v.	)	**Civil Action No.**
	)	
CELLTRION HEALTHCARE CO., LTD., CELLTRION, INC., and HOSPIRA, INC.	)	
Defendants.	)	
	)	

COMPLAINT

Plaintiffs Janssen Biotech, Inc. ("Janssen") and New York University ("NYU") (together "Plaintiffs") for their Complaint against Defendants Celltrion Healthcare Co., Ltd., and Celltrion, Inc. ("Celltrion") and Hospira, Inc. ("Hospira") (together "Defendants") allege as follows.

NATURE OF THE ACTION

1. This is one of the first actions for patent infringement under 35 U.S.C. § 271(e)(2)(C), which was enacted in 2010 in the part of the Patient Protection and Affordable Care Act known as the Biologics Price Competition and Innovation Act ("BPCIA").

2. This is also an action to enforce the patent dispute resolution provisions of the BPCIA, which Defendants have refused to follow to date and in fact have repeatedly sought to circumvent. Had Defendants obeyed the statutory requirements, the patents asserted in this Complaint might never have needed to be asserted, or might not have needed to be asserted in their current form.

3. However, Defendants have insisted that Plaintiffs file this lawsuit before the conclusion of the BPCIA dispute resolution process and have threatened to seek penalties under the BPCIA if Plaintiffs fail to do so. Although Plaintiffs believe that Defendants' demand

violates the BPCIA, Plaintiffs file this suit to protect their rights and to obtain an order compelling Defendants to comply with the provisions of the BPCIA.

4. The BPCIA created an abbreviated regulatory pathway for the approval of biosimilar versions of biological medicines. The BPCIA pathway allows biosimilars makers to avoid the full complement of pre-clinical and clinical studies required for regulatory approval and instead rely on data supporting the safety and efficacy of the innovative biological product which the biosimilar mimics. By taking advantage of the BPCIA regulatory pathway, biosimilars makers can greatly reduce the time and expense of obtaining marketing approval.

5. In order to prevent the new biosimilar pathway from undermining the intellectual property rights of innovators and thereby deterring innovation, the BPCIA also created an intricate and carefully orchestrated set of dispute resolution procedures to facilitate the orderly resolution of patent disputes before a biosimilar product could enter the market.

6. Pursuant to the BPCIA, Defendants submitted an abbreviated Biologic License Application ("aBLA") seeking permission to market a proposed biosimilar version of Janssen's revolutionary biological medicine Remicade® (infliximab).

7. Defendants' aBLA was accepted for review by the Food and Drug Administration ("FDA"), but FDA has not yet approved the application or given any indication whether it will be approved, when it will be approved, or what the scope of any approval will be.

8. To avoid burdening the Court and parties with unnecessary disputes, the BPCIA requires a series of information exchanges and good-faith negotiations between the parties before the filing of a patent infringement lawsuit. Defendants, however, have refused to follow these procedures. After bringing two premature (now dismissed) declaratory judgment actions outside of the provisions of the BPCIA, Defendants have sought to short-circuit the BPCIA process by

withholding required information, refusing to participate in required procedures, and threatening to seek penalties if Plaintiffs did not file this action before the time called for by the BPCIA.

9. Defendants have further thwarted the BPCIA patent dispute resolution process by serving a premature "notice of commercial marketing." Under the BPCIA, a biosimilar applicant must serve a notice of commercial marketing at least 180 days before marketing a licensed biosimilar product. The purpose of this provision is to ensure adequate time to adjudicate a possible preliminary injunction motion before a licensed biosimilar product enters the market.

10. In serving a purported "notice of commercial marketing" before their biosimilar product is licensed and before the parties have engaged in the statutorily mandated good-faith negotiations regarding Plaintiffs' patents, Defendants effectively deprived Plaintiffs of the statutory time period for considering the need for, and adjudicating, a potential preliminary injunction motion.

11. Although an action for patent infringement is premature under the BPCIA, in light of Defendants' actions, Plaintiffs are asserting, in addition to their claims for violations of the BPCIA, claims for infringement of six patents under 35 U.S.C. § 271(e)(2)(C). Plaintiffs assert infringement under 35 U.S.C. § 271(e)(2)(C)(i) of three patents based on Defendants' submission of the aBLA for marketing approval of their proposed biosimilar product. Plaintiffs assert infringement under 35 U.S.C. § 271(e)(2)(C)(ii) of three additional patents based on Defendants' submission of the aBLA and their failure to provide manufacturing information in addition to the aBLA itself as required under the BPCIA.

PARTIES

12. Janssen Biotech, Inc. ("Janssen") is a company organized and existing under the laws of the Commonwealth of Pennsylvania, with a principal place of business in Horsham, Pennsylvania.

13. New York University ("NYU") is a research university organized as a corporation under the laws of the State of New York and having a place of business in New York, New York.

14. Upon information and belief, Celltrion Healthcare Co., Ltd. and Celltrion, Inc. are companies organized and existing under the laws of the Republic of Korea. Celltrion, Inc. is a biopharmaceutical company that specializes in research and development of antibody biosimilars and biopharmaceuticals. Celltrion Healthcare Co., Ltd. markets and distributes such biopharmaceutical products in the United Sates. Celltrion Healthcare Co., Ltd. maintains an office for U.S. business operations in Cambridge, Massachusetts.

15. Upon information and belief, Hospira, Inc., is a Delaware corporation having corporate offices and a principal place of business in Lake Forest, Illinois.

JURISDICTION AND VENUE

16. This is an action for violations of 42 U.S.C. § 262(l), and patent infringement under the patent laws of the United States, Title 35, United States Code. This Court has subject matter jurisdiction pursuant to 28 U.S.C. §§ 1331, 1338(a), 2201(a), and 2202.

17. On information and belief, Celltrion Healthcare Co., Ltd. intends to market and distribute its proposed biosimilar infliximab product in Massachusetts, through Hospira. On information and belief, Celltrion, Inc. will collaborate in the commercialization of the product in Massachusetts.

18. Celltrion Healthcare Co., Ltd. maintains an office for U.S. business operations in Cambridge, Massachusetts. On information and belief, among the purposes of this office is to market and distribute products manufactured by Celltrion, Inc. in collaboration with Celltrion, Inc.

19. Celltrion Healthcare Co., Ltd. is registered to do business in Massachusetts and has consented to be sued in Massachusetts. On information and belief, among the purposes of Celltrion Healthcare Co., Ltd.'s Massachusetts business is to market and distribute products manufactured by Celltrion, Inc. in collaboration with Celltrion, Inc.

20. Celltrion Healthcare Co., Ltd. and Celltrion, Inc. previously filed a Declaratory Judgment Complaint in this District against Janssen (*see Celltrion Healthcare Co., Ltd. and Celltrion, Inc. v. Janssen Biotech, Inc.*, No. 14-cv-11613 (D. Mass. filed Mar. 31, 2014)) involving the same proposed biosimilar product and two of the patents at issue in this Complaint. That declaratory judgment action was voluntarily dismissed by Celltrion on October 23, 2014 after Janssen's motion to dismiss was fully briefed.

21. This Court has personal jurisdiction over Celltrion Healthcare Co., Ltd. and Celltrion, Inc.

22. On information and belief, Hospira has been involved in an ongoing and continuing business relationship with Celltrion concerning their proposed biosimilar infliximab product since at least 2009. On information and belief, Hospira has been aware during that time that Celltrion maintains its office for U.S. business operations in Massachusetts.

23. On information and belief, Hospira entered into an exclusive license agreement and/or marketing agreement with Celltrion covering the proposed biosimilar infliximab.

24. On information and belief, Hospira is obligated, or entitled, to indemnify, defend, or participate in patent litigation brought against Celltrion related to the proposed biosimilar infliximab product. On information and belief, Hospira is actively collaborating with Celltrion in preparation for litigation concerning Defendants' proposed biosimilar infliximab product.

25. On information and belief, Hospira has collaborated with Celltrion in the submission of Defendants' aBLA and intends to market and distribute the proposed biosimilar infliximab product in Massachusetts.

26. On information and belief, Hospira is engaged in the distribution of generic pharmaceutical products throughout the world, including in Massachusetts.

27. On information and belief, Hospira has voluntarily and purposely directed its activities at residents of this forum, including by engaging in an ongoing and continuing business relationship with Celltrion, and by engaging in continuous and systematic activity in Massachusetts through its authorized distributors and a Customer Fulfillment Center in Massachusetts.

28. This Court has personal jurisdiction over Hospira.

29. Venue is proper in this judicial district under 28 U.S.C. §§ 1391(b), (c) and/or 1400(b).

REMICADE® (INFLIXIMAB)

30. Janssen is a pioneer and leader in the development of biologic drugs. Janssen's biologic drug Remicade® was one of the first drugs of its kind sold in the United States for treatment of a chronic disease.

31. Remicade® is a monoclonal antibody that binds to and neutralizes a substance in our bodies called TNFα. TNFα is an important player in our immune systems but, if it is over-produced, it can lead to chronic disease.

32. Scientists at NYU worked with scientists at Janssen's predecessor Centocor to develop the infliximab monoclonal antibody, also known as the "cA2" antibody.

33. Although the cA2 antibody had promising in vitro properties, given its complex structure and mechanism of operation it required extensive pre-clinical and clinical development before it could become a useful medicine for human beings.

34. From the time the infliximab antibody was first discovered, it took nearly a decade for Remicade® to be approved for sale in the United States. During that time, Centocor conducted dozens of clinical trials and spent tens of millions of dollars, with no guarantee of success.

35. Remicade® was first approved for the U.S. market in 1998. The first indication, or use, for which Remicade® was approved was the treatment of Crohn's disease, an inflammatory bowel disease that causes inflammation of the lining of the digestive tract. Remicade® was the first biological therapy approved for Crohn's disease in the United States.

36. After Remicade® entered the market, Centocor continued to pursue extensive clinical development efforts for the drug. These efforts led to the discovery that Remicade® is safe and effective for a number of additional diseases and indications other than Crohn's disease.

37. Janssen's extensive development efforts have led to 16 FDA approvals for Remicade®, including indications for use in the treatment of Crohn's disease (1998), rheumatoid arthritis (1999), ankylosing spondylitis, a chronic inflammatory disease of the axial skeleton

(2004), psoriatic arthritis (2005), and ulcerative colitis, an inflammatory bowel disease (2006). Remicade® has changed the standard of care for the treatment of these diseases.

38. In total, Janssen has sponsored more than 170 clinical trials for Remicade®. Janssen has spent hundreds of millions of dollars in research and development of the drug.

39. Remicade® had been used to treat and improve the lives of more than 2.2 million patients suffering from chronic disease.

PLAINTIFFS' PATENTS ON INFLIXIMAB AND RELATED TECHNOLOGY

40. In the course of developing Remicade®, Janssen has obtained or exclusively licensed a number of patents related to infliximab, its uses in treating disease, and the processes for manufacturing infliximab. Plaintiffs assert six of these patents in this action.

The Antibody Patent (the 471 Patent)

41. Janssen and NYU jointly own United States Patent No. 6,284,471 ("the 471 patent"), which covers the infliximab cA2 monoclonal antibody itself. The cA2 antibody is a highly complex biological molecule that took years to develop and has highly potent healing properties.

42. On September 4, 2001, the United States Patent and Trademark Office ("PTO") issued the 471 patent, which is titled "Anti-TNFα Antibodies and Assays Employing Anti-TNFα Antibodies." A true and correct copy of the 471 patent is attached as Exhibit A.

43. The 471 patent is jointly assigned to Janssen (through its predecessors Centocor, Inc. and Centocor Ortho Biotech, Inc.) and NYU.

44. The 471 patent will expire on September 4, 2018.

45. In a prior proceeding, Defendants did not dispute that their proposed biosimilar infliximab product practices claims of the 471 patent.

46. The 471 patent is currently undergoing reexamination by the PTO.

47. On information and belief, one or more of the Defendants, or an agent or affiliate of one or more of the Defendants, initiated the PTO reexamination proceeding challenging the validity of the 471 patent.

48. In the reexamination, the specification of the 471 patent was amended at Janssen's request. A PTO examiner has rejected the claims of the 471 patent. Janssen is currently addressing the rejection.

The Fistulizing Crohn's Patent (the 396 Patent)

49. Janssen and NYU jointly own United States Patent No. 7,223,396 ("the 396 patent"). In contrast to the 471 patent, which covers the infliximab antibody itself, the claims of the 396 patent cover novel uses of infliximab to treat disease. In particular, the 396 patent covers specific methods of using infliximab to treat fistulas – abnormal connections or openings between two organs that are not normally connected – in patients with Crohn's disease.

50. On May 29, 2007, the PTO issued the 396 patent, which is titled "Methods of Treatment of Fistulas in Crohn's Disease with Anti-TNF Antibodies." A true and correct copy of the 396 patent is attached as Exhibit B.

51. The 396 patent is jointly assigned to Janssen (through its predecessors Centocor, Inc. and Centocor Ortho Biotech, Inc.) and NYU.

52. The 396 patent will expire on June 29, 2016.

53. In a prior proceeding, Defendants did not dispute that the proposed use of their product would practice the claims of the 396 patent.

54. It is unknown whether a U.S. approval of Defendants' proposed biosimilar product, if any, would include an indication for treating fistulas in Crohn's disease, as claimed by the 396 patent.

The Methods of Producing Antibodies Patent (the 715 Patent)

55. Janssen has exclusively licensed from Board of Trustees of the Leland Stanford Junior University ("Stanford") and the Trustees of Columbia University in the City of New York ("Columbia") U.S. Patent No. 5,807,715 ("the 715 patent"), which covers methods of producing functional antibodies that are capable of specifically binding antigens.

56. On September 15, 1998, the PTO issued the 715 patent, which is entitled "Methods And Transformed Mammalian Lymphocyte Cells For Producing Functional Antigen-Binding Protein Including Chimeric Immunoglobulin." A true and correct copy of the 715 patent is attached as Exhibit C.

57. Stanford and Columbia hold title to the 715 patent.

58. Janssen holds all substantial rights in the 715 patent, including the sole and exclusive right to initiate, control, and defend any patent infringement litigation under the BPCIA involving the 715 patent.

59. The 715 patent will expire on September 15, 2015.

The Chemical Cell Growth Media Patents (the 083 Patent and the 056 Patent)

60. Janssen owns U.S. Patent No. 7,598,083 ("the 083 patent") and U.S. Patent No. 6,900,056 ("the 056 patent"), which cover cell growth media for use in growing biological products, including infliximab.

61. On October 6, 2009, the PTO issued the 083 patent, entitled "Chemically Defined Media Compositions." A true and correct copy of the 083 patent is attached as Exhibit D.

62. On May 31, 2005, the PTO issued the 056 patent, entitled "Chemically Defined Medium for Cultured Mammalian Cells." A true and correct copy of the 056 patent is attached as Exhibit E.

63. The 083 patent will expire on February 7, 2027.

64. The 056 patent will expire on October 5, 2022.

The Purification Patent (the 600 Patent)

65. Janssen owns U.S. Patent No. 6,773,600 ("the 600 patent"), which covers novel methods of purifying biological products such as infliximab so that they are suitable for use in human medicines.

66. On August 10, 2004, the PTO issued the 600 patent, entitled "Use of Clathrate Modifier, To Promote Passage of Proteins During Nanofiltration." A true and correct copy of the 600 patent is attached as Exhibit F.

67. The 600 patent will expire on June 4, 2023.

BIOLOGICS, BIOSIMILARS, AND THE BPCIA

Biologics

68. Biological medicines, or biologics, are complex biological molecules that need to be grown in living cultures rather than chemically synthesized, as are the more familiar pharmaceutical products known as chemical or small-molecule drugs. Because the biologic manufacturing process is complex and uses living organisms, the structural features of a biologic drug can vary based on the precise manner in which the drug is made. Unlike small-molecule drugs, moreover, biological molecules generally cannot be completely characterized.

69. Because of the differences between biological and small-molecule drugs, biological and small-molecule pharmaceutical products are approved for sale in the United States

through different regulatory pathways. Whereas small-molecule drugs are approved based on the submission of a New Drug Application ("NDA") (*see* 21 U.S.C. § 355), biological products are assessed pursuant to a Biological License Application ("BLA") (*see* 42 U.S.C. § 262(a)).

The BPCIA Pathway for Biosimilar Approval

70. Although Congress created an abbreviated regulatory pathway for the approval of generic small-molecule drugs in the Hatch-Waxman Act of 1984, no abbreviated pathway for approval of follow-on biologics products existed until the enactment of the BPCIA, as part of the Patient Protection and Affordable Care Act, in 2010. Before the enactment of the BPCIA, the only way to obtain U.S. approval of a biological product was through an original BLA supported by a full complement of pre-clinical and clinical data.

71. The BPCIA creates an abbreviated approval pathway for FDA licensure of biological products upon a determination that the biological product is "biosimilar" to a previously licensed "reference product." 42 U.S.C. § 262(k). The BPCIA defines a "biosimilar" as a biological product that is (1) "highly similar to the reference product notwithstanding minor differences in clinically inactive components"; and (2) has "no clinically meaningful differences between the biological product and the reference product in terms of the safety, purity, and potency of the product." 42 U.S.C. §§ 262(i)(2)(A), (B). The BPCIA defines a "reference product" to be a "single biological product licensed under subsection (a) against which a biological product is evaluated in an application submitted under subsection (k)." 42 U.S.C. § 262(i)(4).

72. Under the BPCIA, biosimilar applicants are permitted to make use of FDA's prior determinations as to the safety, purity, and potency of the reference product that was already approved by FDA. In particular, a biosimilar applicant must identify a single reference product

that has already been approved by FDA and submit to FDA "publicly-available information regarding the Secretary's previous determination that the reference product is safe, pure, and potent." 42 U.S.C. § 262(k)(2)(A)(iii)(I). Consequently, the § 262(k) pathway created by the BPCIA allows the biosimilar applicant to reduce the time, expense, and risks of research and development and the full complement of pre-clinical and clinical testing, and gain licensure to commercialize its biological product in the market as a biosimilar sooner and more cheaply than it could have done through the submission of an original BLA.

The BPCIA's Patent Dispute Resolution Procedures

73. As Congress expressly indicated, the purpose of the BPCIA is to establish "a biosimilars pathway balancing innovation and consumer interests." Biologics Price Competition and Innovation Act of 2009, Pub. L. No. 111-148, § 7001(b), 124 Stat. 119, 804 (2010).

74. To further this goal, Congress created a set of mandatory procedures for addressing patent disputes relating to prospective biosimilar drugs. These procedures are set forth in 42 U.S.C. § 262(l) and in corresponding amendments to the patent infringement statute, 35 U.S.C. § 271. The procedures are intended to ensure that the maker of an innovative biological product that is the subject of a biosimilar application will have sufficient time and opportunity to enforce its patent rights before a biosimilar product enters the United States market. The BPCIA's patent dispute resolution procedures are also intended to ensure that disputes over patent rights will take place in an orderly fashion, with the least possible uncertainty, brinksmanship, and burden on the parties and the courts.

75. The BPCIA patent dispute resolution procedures require the biosimilar applicant and reference product sponsor to undertake a series of specific steps before any patent action is filed. Reflecting their importance to the BPCIA, most of these steps are mandatory and

unconditional: the statute states that the parties "shall" undertake them. *See* 42 U.S.C. §§ 262(l)(2)(A), (l)(3)(A), (l)(3)(B), (l)(3)(C), & (l)(4)(A). Where a specific action is optional or conditional, the statute makes this clear, stating that the parties "may" take such action, *see* 42 U.S.C. § 262(l)(2)(B), or that they shall do so only "[i]f" a specified condition precedent occurs, *see* 42 U.S.C. § 262(l)(4)(B).

76. The BPCIA dispute resolution process begins when a biosimilar application is accepted for review by FDA. Within twenty days thereafter, the biosimilar applicant "shall provide" the reference sponsor with confidential access to "a copy of the application submitted . . . under subsection (k), ***and*** such other information that describes the process or processes used to manufacture the biological product that is the subject of such application." 42 U.S.C. § 262(l)(2)(A) (emphasis added). This step initiates a series of pre-litigation exchanges of information and positions so that the parties may engage in mandatory good-faith negotiations regarding what patents should be litigated prior to the approval of the biosimilar product. *See* 42 U.S.C. § 262(l)(2)-(l)(6).

77. The requirement that biosimilars applicants provide pre-litigation manufacturing information to reference product sponsors, which does not exist for generic small-molecule drugs under the Hatch-Waxman Act, reflects the complexity of manufacturing processes for biologics and their importance to innovation in the field. To ensure that full application and manufacturing information be provided without prejudice or delay, the BPCIA sets forth a detailed set of confidential access provisions governing the reference product sponsor's use of the required information. 42 U.S.C. § 262(l)(1).

78. The next step in the statutory process, section 262(l)(3)(A), states that within 60 days the reference product sponsor "shall provide" the biosimilar applicant a list of patents that

the reference product sponsor "believes a claim of patent infringement could reasonably be asserted" against the proposed biosimilar product or the uses or manufacture of such product. 42 U.S.C. § 262(l)(3)(A)(i). The reference product sponsor is also required to indicate whether it is willing to license any of these patents. 42 U.S.C. § 262(l)(3)(A)(ii).

79. The next statutory step, section 262(l)(3)(B), states that within 60 days the biosimilar applicant "shall provide" a "detailed statement" of its non-infringement, invalidity, and unenforceability defenses with respect to the listed patents, or a statement that the subsection (k) applicant "does not intend to bring commercial marketing of the biological product before the date that such patent expires." 42 U.S.C. § 262(l)(3)(B)(ii).

80. The next step in the statutory process, section 262(l)(3)(C), states that within 60 days the reference product sponsor "shall provide" a "detailed statement" of its infringement positions and "a response to the statement concerning validity and enforceability provided" by the biosimilar applicant. 42 U.S.C. § 262(l)(3)(C).

81. The next step in the statutory process states that the parties "shall engage in good faith negotiations" to agree on patents that will be subject to an action for patent infringement prior to the approval of the biosimilar application. 42 U.S.C. § 262(l)(4)(A).

82. If the parties agree on the patents that will be subject to an immediate action for infringement, then the reference product sponsor "shall bring an action for patent infringement" within thirty days of the agreement. 42 U.S.C. § 262(l)(6)(A). If the parties fail to reach agreement, they proceed to a further exchange process that will identify one or more patents for immediate litigation. 42 U.S.C. § 262(l)(4)(B) & (l)(5). As in the case of agreement, the reference product sponsor "shall bring an action for patent infringement" within thirty days after patents are selected for litigation through this process. 42 U.S.C. § 262(l)(6)(B).

83. If the reference product sponsor fails to bring suit within thirty days of the selection of patents for immediate litigation, its "sole and exclusive remedy" will be "a reasonable royalty." 35 U.S.C. § 271(e)(6)(B).

Notice of Commercial Marketing

84. In addition to the pre-litigation procedures described above, the BPCIA addresses litigation regarding a "biological product licensed under subsection (k)" – i.e., a biosimilar product that has been approved for marketing. The BPCIA requires the biosimilar maker to provide "notice to the reference product sponsor not later than 180 days before the date of the first commercial marketing of the biological product licensed under subsection (k)." 42 U.S.C. § 262(1)(8)(A).

85. Upon receipt of a notice of commercial marketing, the reference product sponsor may move for a preliminary injunction on patents that the sponsor identified as potentially infringed under section 262(l)(3)(A) of the pre-litigation dispute resolution procedures, but which the parties have not selected for litigation pursuant to these procedures. 42 U.S.C. § 262(1)(8)(B).

86. In addition, the notice of commercial marketing permits the reference product sponsor to bring a declaratory judgment action with respect to such patents that have been identified but not selected for immediate litigation. 42 U.S.C. § 262(1)(9)(A). Before the notice of commercial marketing, such declaratory judgments are prohibited. *Id.*

CELLTRION'S PROPOSED BIOSIMILAR PRODUCT

87. On information and belief, Celltrion has undertaken the development of a proposed biosimilar to Janssen's Remicade® infliximab product. The trade name for the Celltrion proposed biosimilar product is Remsima®.

88. On information and belief, in 2009, Hospira entered into an agreement with Celltrion, pursuant to which Hospira obtained the rights to exclusively market biosimilar infliximab in the United States. The trade name for the proposed biosimilar infliximab product to be marketed by Hospira is Inflectra®.

89. On information and belief, Defendants submitted an Investigational New Drug ("IND") application for their proposed biosimilar to Janssen's infliximab product under section 505(i) of the Federal Food, Drug, and Cosmetic Act on October 2, 2013, and the FDA accepted the IND on November 18, 2013. Defendants submitted an aBLA for this proposed biosimilar product on or about August 8, 2014 and the FDA accepted that application for review on or about October 7, 2014.

90. On information and belief, the proposed indications (uses) for which Defendants seek approval of its biosimilar product are:

> (1) Reducing signs and symptoms and inducing and maintaining clinical remission in adult patients with moderately to severely active Crohn's disease who have had an inadequate response to conventional therapy; (2) reducing the number of draining enterocutaneous and rectovaginal fistulas and maintaining fistula closure in adult patients with fistulizing Crohn's disease; (3) reducing signs and symptoms and inducing and maintaining clinical remission in pediatric patients 6 years of age and older with moderately to severely active Crohn's disease who have had an inadequate response to conventional therapy; (4) reducing signs and symptoms, inducing and maintaining clinical remission and mucosal healing, and eliminating corticosteroid use in adult patients with moderately to severely active ulcerative colitis who have had an inadequate response to conventional therapy; (5) reducing signs and symptoms and inducing and maintaining clinical remission in pediatric patients 6 years of age and older with moderately to severely active ulcerative colitis who have had an inadequate response to conventional therapy; (6) in combination with methotrexate, reducing signs and symptoms, inhibiting the progression of structural damage, and improving physical function in patients with moderately to severely active rheumatoid arthritis; (7) reducing signs and symptoms in patients with active ankylosing

> spondylitis; (8) reducing signs and symptoms of active arthritis, inhibiting the progression of structural damage, and improving physical function in patients with psoriatic arthritis; and (9) treatment of adult patients with chronic severe (i.e., extensive and/or disabling) plaque psoriasis who are candidates for systemic therapy and when other systemic therapies are medically less appropriate.

(U.S. Food and Drug Administration, POSTPONED: March 17, 2015: Arthritis Advisory Committee Meeting Announcement http://www.fda.gov/advisorycommittees/ucm433919.htm).

91. These proposed indications for Defendants' proposed biosimilar product are all indications for which Remicade® has been approved. Each of these nine indications results from extensive research and development by Plaintiffs, culminating in successful clinical trials demonstrating that Remicade® is safe and effective for these indications.

92. Based on publicly available information from Defendants' regulatory submissions in other countries, and from the clinicaltrials.gov database of clinical trials, Defendants have completed two Phase III clinical trials regarding the safety and efficacy of their proposed biosimilar product. One of these trials involved patients with rheumatoid arthritis and the other involved patients with ankylosing spondylitis. Neither of these two completed Phase III clinical trials for Defendants' proposed biosimilar product involved patients with Crohn's disease.

93. Defendants' proposed biosimilar infliximab product has been approved for sale in other jurisdictions, including Canada. In approving Defendants' product, the Canadian health authorities elected not to approve an indication for the treatment of Crohn's disease. The Canadian health authorities stated that "extrapolation of data from the settings of rheumatoid arthritis and [ankylosing spondylitis] to adult and pediatric inflammatory bowel diseases (Crohn's disease, ulcerative colitis) was not recommended." (Summary Basis for Decision, Remsima, http://www.hc-sc.gc.ca/dhp-mps/prodpharma/sbd-smd/drug-med/sbd_smd_2014_remsima_160195-eng.php).

94. On information and belief, FDA has not yet decided whether to approve Defendants' proposed biosimilar product or what indications to approve it for. Nor has FDA identified any timetable for a decision on Defendants' aBLA.

95. FDA had planned to consider Defendants' proposed product in a public, full-day meeting of its Arthritis Advisory Committee that was originally scheduled for March 17, 2015. *See* http://www.fda.gov/advisorycommittees/ucm433919.htm. Typically, FDA advisory committees vote whether to recommend approval, and what indications to recommend approval for, on the day of their meeting. Although these recommendations are not binding on FDA, they are usually followed.

96. On February 25, 2015, however, FDA postponed the scheduled advisory committee meeting on Defendants' proposed biosimilar product indefinitely. FDA's postponement announcement reads in its entirety: "The Food and Drug Administration (FDA) is postponing the meeting of the Arthritis Advisory Committee scheduled for March 17, 2015. The postponement is due to information requests pending with the sponsor of the application. A future meeting date will be announced in the Federal Register." *See* http://www.fda.gov/advisorycommittees/ucm433919.htm.

DEFENDANTS' EFFORTS TO AVOID THE BPCIA'S PATENT DISPUTE RESOLUTION PROCEDURES

97. From the time they began the process of seeking approval for their proposed biosimilar product, Defendants have sought to avoid the mandatory patent dispute resolution procedures of the BPCIA. Having failed in their initial efforts to bypass the BPCIA procedures altogether by filing premature declaratory judgment actions, Defendants have now proceeded to short-circuit the statutory process by withholding required information, by refusing to participate to date in required statutory procedures, and by serving a premature notice of commercial

marketing that, unless declared ineffective, would thwart the statutory purpose of litigating actual disputes and burden the parties and the Court with unnecessary litigation.

98. On information and belief, Defendants have participated jointly in formulating Defendants' pre-litigation and litigation strategy under the BPCIA.

Defendants Try Unsuccessfully to Bypass the BPCIA's Procedures

99. As alleged above, the BPCIA's dispute resolution procedures are triggered by FDA's acceptance of a biosimilar application for review. 42 U.S.C. § 262(1)(2)(A). Before Celltrion's application was accepted for review by FDA, Celltrion and Hospira each tried to circumvent the BPCIA by filing separate declaratory judgment actions seeking declarations of noninfringement or invalidity of patents that Celltrion and Hospira unilaterally identified as potentially relevant to their proposed biosimilar product. *See Celltrion Healthcare Co., Ltd., and Celltrion, Inc., v. Janssen Biotech, Inc.*, No. 14-cv-11613 (D. Mass.); *Hospira, Inc. v. Janssen Biotech, Inc. et al.*, No. 14-cv-07049 (S.D.N.Y.).

100. In both actions, Janssen moved to dismiss on the grounds that a declaratory judgment action was prohibited since Defendants could not seek a declaratory judgment before producing their aBLA and without following the requirements of the BPCIA. In response, Defendants contended that the BPCIA procedures have no effect prior to FDA's acceptance for review of the aBLA. According to Defendants, a prospective biosimilar applicant is entitled to bypass the BPCIA procedures by filing a declaratory judgment action concerning the patents it believes the patent holder will assert against the proposed biosimilar product, so long as the action is filed before the aBLA is accepted for review. Hospira also argued that the BPCIA litigation procedures were not applicable to it at any point.

101. Judge Crotty of the U.S. District Court for the Southern District of New York granted Janssen's motion to dismiss Hospira's declaratory judgment action. Judge Crotty rejected Hospira's argument that it was entitled to bring a declaratory judgment action without following the BPCIA patent dispute resolution procedures.

102. Judge Crotty reasoned that Hospira "seeks to utilize the BPCIA pathway for approval of its biosimilar drug, yet disavows the BPCIA's authority over patent disputes. Despite Hospira's best attempts to twist the BPCIA to serve its interests without hindering its pursuit of litigation, this effort fails." *Hospira, Inc. v. Janssen Biotech, Inc*., 113 U.S.P.Q.2d 1260, 1262 (S.D.N.Y. 2014). Judge Crotty stated that the "BPCIA purposefully ties the dispute resolution process to events throughout the biosimilar approval process, ensuring that full information exchange occurs at relevant and crucial periods during the approval process." *Id.* Judge Crotty concluded that permitting Hospira's declaratory judgment action to proceed would allow biosimilar applicants to "skirt the dispute resolution procedures Congress purposefully enacted" for patent disputes arising from the filing of a biosimilar application under the BPCIA. *Id.*

103. Celltrion voluntarily withdrew its declaratory judgment action after Janssen's motion to dismiss was fully briefed. *See Celltrion Healthcare Co., Ltd., and Celltrion, Inc., v. Janssen Biotech, Inc.*, No. 14-cv-11613 (D. Mass.) (Dkt. No. 33) (Oct. 23, 2014).

Defendants Refuse to Provide Required Manufacturing Information

104. Pursuant to section 262(l)(2)(A) of the BPCIA, Defendants began to provide Janssen with a copy of their aBLA (No. 125544) twenty days after the application was accepted for review by FDA. However, Defendants have refused to prove "such other information that describes the process or processes used to manufacture the biological product that is the subject

of such application" as required by the statute. Defendants provided Janssen only with their aBLA and nothing else.

105. On December 16, 2014, before its time for providing its section 262(l)(3)(A) listing of potentially infringed patents, Janssen asked Defendants to provide the manufacturing information required by the statute. Janssen also asked specific, detailed questions about Celltrion's manufacturing processes.

106. On December 23, 2014, Defendants responded by refusing to provide the requested information. Despite the statutory requirement that biosimilar applicants provide their aBLA ***and*** additional manufacturing information, Defendants asserted that "[a]ll relevant information needed to generate a list of patents for which a claim of patent infringement can reasonably be asserted by Janssen is included in Celltrion's [a]BLA." Defendants did not provide any additional manufacturing information.

107. On December 26, 2014, Janssen provided Defendants a list of patents for which a claim of infringement could reasonably be asserted pursuant to section 262(l)(3)(A) of the BPCIA patent dispute resolution process. 42 U.S.C. § 262(l)(3)(A). This list included the cell growth media patents (the 083 patent and the 056 patent) and the purification patent (the 600 patent).

108. On February 5, 2015, Defendants provided a statement of defenses pursuant to 42 U.S.C. § 262(l)(3)(B). Defendants did not produce any documentation of their manufacturing processes as required by 42 U.S.C. § 262(l)(2)(A).

109. On February 25, 2015, Janssen asked Defendants again for the manufacturing information that is required by the statute, and which Janssen had previously requested on December 16, 2014.

110. On March 4, 2015, Defendants responded by asserting once again that the aBLA contained all the information to which Plaintiffs were entitled, notwithstanding the BPCIA's unambiguous requirement that manufacturing information in addition to the aBLA be provided. Defendants refused to provide documentation of their manufacturing processes prior to any lawsuit as required under the BPCIA, contending that "Celltrion does not have the authority" to disclose certain information about the ingredients, such as the cell media, used in its product manufacture, and instead insisting that Janssen's request for such information "be addressed after suit is filed."

Defendants Attempt to Circumvent Mandatory BPCIA Procedures

111. As of their statement of defenses pursuant to 42 U.S.C. § 262(l)(3)(B), Defendants have refused to participate in further BPCIA patent dispute resolution procedures including the good-faith negotiations regarding patents to be included in immediate litigation pursuant to 42 U.S.C. § 262(l)(4).

112. Defendants assert that they have "consented to Janssen's patent list" and that as a result the remainder of the statutorily required patent-exchange procedures – namely Janssen's mandatory responses to Defendants' defenses pursuant to section 262(1)(3)(C) , the parties' mandatory good-faith negotiations under section 262(1)(4), and the procedures for identifying the patents to be immediately litigated in the absence of an agreement – are moot. *See* 42 U.S.C. § 262(1)(3)-(l)(5). Defendants have stated that they will not engage in good-faith negotiations with Janssen as required by 42 U.S.C. § 262(1)(4).

113. Defendants further assert that Janssen is required to file a lawsuit on all six listed patents within thirty days of Defendants' "detailed statement," i.e., by March 7, 2015, rather than within thirty days after the completion of the statutory pre-litigation procedures, as the BPCIA

requires. Defendants have asserted that if Janssen did not file suit within this time, its remedy in any later suit would be limited to reasonable royalty damages pursuant to 35 U.S.C. § 271(e)(6)(B).

114. Defendants' assertion that Janssen needed to file suit immediately or be limited to reasonable royalties was a clear threat to take this position in future litigation, and to require Janssen, if it did not meet Defendants' demands, to litigate the issue at the risk of losing its right to injunctive relief or lost profits. On information and belief, Defendants have repudiated their obligations under the BPCIA and made this legally baseless threat in the hope of compelling Janssen to file this action within the time period Defendants demanded, rather than at the time required by the BPCIA.

115. On February 25, 2015, Janssen asked Defendants to withdraw their threat and comply with the BPCIA's mandatory procedures.

116. On March 4, 2015, Defendants responded by reaffirming their position that Janssen is required to file suit by March 7, 2015 and reserving the "the right to limit Janssen's remedy for any judgment of infringement to a reasonable royalty" if "Janssen elects not to bring suit by March 7, 2015." Defendants offered to refrain from doing so as a "compromise" if "and only if" Janssen agreed to bring suit by April 6, 2015, prior to the mandatory good-faith negotiations that are a prerequisite to filing suit under the BPCIA. This proposed "compromise" was just another refusal to comply with mandatory BPCIA procedures. Defendants coupled that proposal with a variety of other unacceptable demands that violate the BPCIA. These included the insistence that Plaintiffs seek preliminary injunctions on patents that are premature to litigate.

117. Given Defendants' threat to Plaintiffs' intellectual property rights and refusal to comply with the provisions of the BPCIA, Plaintiffs have filed this Complaint to protect their

interests and enforce the mandatory statutory provisions which Defendants seek to bypass. As a direct result of Defendants' wrongful conduct, Plaintiffs are being compelled to assert patent infringement claims that might never have needed to be litigated or, with respect to the 471 patent, would have been litigated in a different form had Defendants complied with the BPCIA.

118. Had Defendants complied with the BPCIA, the parties' mandatory good-faith negotiations (42 U.S.C. § 262(l)(4)) might have led to an agreement to postpone litigation of the 471 patent, covering the cA2 antibody, until the conclusion of the PTO reexamination proceeding that, on information and belief, was brought by one or more of Defendants or their agents or affiliates. If the 471 patent is upheld in reexamination (as Janssen and NYU believe it will be), it would be litigated in a form that is different from the patent today since the 471 patent specification has been amended in the reexamination proceeding.

119. Had Defendants complied with the BPCIA, the parties' mandatory good-faith negotiations might have led to an agreement to postpone litigation of the 396 patent, covering methods for treating fistulas in Crohn's disease, until FDA determined whether Defendants' proposed biosimilar product would receive an indication for treating fistulas in Crohn's disease. If FDA determines that Defendants' product should not receive such an indication – a significant possibility since the Canadian health authorities recently arrived at that very conclusion – the 396 patent would never need to be litigated.

120. Had Defendants complied with the BPCIA, the parties' mandatory good-faith negotiations might have led to an agreement to avoid litigating the 715 patent, which expires on September 15, 2015. In light of FDA's recent decision to indefinitely postpone the advisory committee meeting on Defendants' proposed biosimilar product, it is unlikely that Defendants' proposed product will even be approved, much less ready to be marketed, by September 15,

2015. Defendants have all but acknowledged this, asserting on March 4, 2015 that they would agree to "delay commercial marketing of the infliximab biosimilar product pursuant to aBLA 125544 until after September 15, 2015" – but only as part of a "compromise" in which Janssen would agree to disregard mandatory requirements of the BPCIA and burden the Court with premature and wasteful preliminary injunction motions on patents that might never need to be litigated, or might never need to be litigated in their current form.

121. Had Defendants complied with the BPCIA, the parties' mandatory good-faith negotiations might have led to the production of information that would have avoided the need to litigate Janssen's manufacturing patents – the cell growth media patents (the 083 patent and the 056 patent) and the purification patent (the 600 patent). Instead, in violation of the BPCIA, Defendants have proposed providing further information only after Janssen files suit.

122. Defendants' violations of the BPCIA have caused and will cause unnecessary burdens to Plaintiffs and the Court. Defendants' violations of the BPCIA have caused Plaintiffs irreparable harm for which they have no adequate remedy at law, and will continue unless these statutory requirements are enforced by this Court. Plaintiffs have been and will continue to be injured by Defendants' actions.

Defendants Serve a Premature Notice of Commercial Marketing

123. On February 5, 2015, the same day they provided their "detailed statement" to Janssen, Defendants compounded their violations of the BPCIA by serving a premature notice of commercial marketing, purportedly pursuant to 42 U.S.C. § 262(l)(8)(A). Defendants asserted that they would begin commercial marketing of their proposed biosimilar product "as early as 180 days from the date of this notice," i.e., August 4, 2015.

124. Prior to their purported "notice of commercial marketing" of February 5, 2015, Defendants had previously asserted that a different document constituted a notice of commercial marketing under the BPCIA. In briefing in its unsuccessful declaratory judgment action, Hospira asserted that Celltrion's declaratory judgment complaint alleging that it intended to sell its proposed biosimilar infliximab product in the United States "should satisfy the Act's notice provision, which does not prescribe any particular form." *See Hospira, Inc. v. Janssen Biotech, Inc.*, No. 14-cv-7059 (S.D.N.Y. Oct. 16, 2014) (Dkt. No. 42 at 22).

125. In their latest purported notice of commercial marketing of February 5, 2015, Defendants asserted that the BPCIA does not "include a condition precedent to providing notice." But, to the contrary, the BPCIA includes a clear condition precedent to providing a notice of commercial marketing. The statutorily required notice is "of the first commercial marketing of the biological product licensed under subsection (k)." 42 U.S.C. § 262(l)(8)(A). The grant of a license under subsection (k) is a statutory prerequisite to providing a notice of commercial marketing.

126. As Defendants are aware, this was precisely the holding of the sole reported case to address this issue to date. *See Sandoz Inc. v. Amgen Inc.*, No. C-13-2904 MMC, 2013 U.S. Dist. LEXIS 161233 (N.D. Cal. Nov. 12, 2013). As Judge Chesney of the United States District Court for the Northern District of California concluded, a biosimilar applicant "cannot, as a matter of law, have provided a 'notice of commercial marketing'" prior to obtaining a biological license because until that time the biosimilar product "is not 'licensed under subsection (k).'" *Id.* at *6.

127. Defendants have not yet received a license to market their proposed biosimilar product under subsection (k). As a result, Defendants' proposed product is not a "biological

product licensed under subsection (k)" and cannot be the subject of a valid notice of commercial marketing pursuant to the BPCIA.

128. The purpose of the notice of commercial marketing provision is to provide the parties and the Court with sufficient time – 180 days – to resolve any disputes that need to be resolved before commercial launch of a biosimilar product. If Defendants are allowed to proceed based on their invalid notice of commercial marketing, the 180 day period would run during a time when the precise nature of the dispute between the parties, and even the need for litigation on certain patents, has not yet crystallized.

129. With respect to the 471 patent, there is a pending reexamination proceeding in which the specification of the patent has been amended. By the time Defendants' product is licensed and a notice of commercial launch is permitted under the BPCIA, the 471 patent may have emerged from the reexamination proceeding and would be litigated in its amended form.

130. With respect to the 396 patent, there is significant uncertainty whether Defendants' product, even if approved, will be approved for the method of treating fistulizing Crohn's disease claimed in the patent. If Defendants do not obtain an indication for fistulizing Crohn's disease, as they failed to do in Canada, by the time that Defendants' proposed biosimilar is licensed and a notice of commercial launch is permitted under the BPCIA, the 396 patent may never need to be litigated.

131. With respect to the 715 patent, the patent's September 15, 2015 expiration date makes any litigation almost certainly unnecessary since it is highly unlikely that Defendants' product will be approved 180 days prior to the expiration of the 715 patent.

132. With respect to the manufacturing patents, litigation may never need to have been brought because the production of manufacturing information as required under the BPCIA may reveal that the patents are not infringed.

133. By filing a premature notice of commercial marketing, Defendants have burdened the parties and the Court with premature litigation. They also deprived Plaintiffs of the orderly and certain process for protecting their patent rights under the BPCIA procedures.

134. On February 25, 2015, Janssen asked Defendants to withdraw their premature notice of commercial marketing.

135. On March 4, 2015, Defendants refused to withdraw the notice of commercial marketing. Defendants offered, as part of a "compromise," to agree to an accelerated schedule for litigating the parties' dispute over the effectiveness of Defendants' purported notice of commercial marketing.

136. Defendants' premature notice of commercial marketing has caused Plaintiffs irreparable harm for which they have no adequate remedy at law, and will continue unless the notice of commercial marketing is declared invalid by this Court. Plaintiffs have been and will continue to be injured by Defendants' actions.

COUNT 1: VIOLATION OF MANDATORY PROCEDURES UNDER 42 U.S.C. § 262(l)

137. Plaintiffs incorporate by reference paragraphs 1-136 as if fully set forth herein.

138. This claim arises under the BPCIA, 42 U.S.C. § 262(l), and the Declaratory Judgment Act, 28 U.S.C. §§ 2201(a) & 2202.

139. The BPCIA, 42 U.S.C. § 262(l), requires Plaintiffs and Defendants to follow mandatory procedures to resolve patent disputes related to the filing of an aBLA under 42 U.S.C. § 262(k).

140. Defendants have failed to comply with the mandatory requirements of the BPCIA. Defendants' violations of the BPCIA have injured Plaintiffs by depriving them of the procedural protections of the statute and by subjecting them to the burden of unnecessary litigation.

141. Under 42 U.S.C. § 262(l)(2)(A), Defendants were required to provide Janssen, within twenty days of when Defendants' aBLA was accepted for review, with a copy of the aBLA "and such other information that describes the process or processes used to manufacture the biological product that is the subject of such application." Defendants failed to provide such information in violation of the BPCIA.

142. Under 42 U.S.C. § 262(l)(4), Defendants were required to engage in good-faith negotiations with Janssen concerning which patents should be subject to immediate litigation. Defendants were required to engage in these good-faith negotiations after Janssen provided the information required by 42 U.S.C. § 262(l)(3)(C). Defendants are trying to circumvent the requirement for good-faith negotiations in violation of the BPCIA.

143. Under 42 U.S.C. § 262(l)(6), any patent litigation must be brought within 30 days of the completion of the good-faith negotiations, at a time when both parties have a better understanding of their respective positions and so do not burden the courts with unnecessary litigation. Defendants have forced Plaintiffs into filing premature patent claims by making baseless arguments that the failure to do so will cause Plaintiffs to lose valuable statutory rights.

144. Defendants' violations of the BPCIA's mandatory procedures, individually and collectively, have caused and will cause Plaintiffs injury, including irreparable harm for which Plaintiffs have no adequate remedy at law, and will continue unless the statutory requirements are declared and enforced by this Court.

COUNT 2: VIOLATION OF 42 U.S.C. § 262(l)(8)(A)

145. Plaintiffs incorporate by reference paragraphs 1-144 as if fully set forth herein.

146. This claim arises under the BPCIA, 42 U.S.C. § 262(l), and the Declaratory Judgment Act, 28 U.S.C. §§ 2201(a) & 2202.

147. Under 42 U.S.C. § 262(l)(8)(A), Defendants are required to provide notice to Janssen "not later than 180 days before the date of the first commercial marketing of the biological product licensed under subsection (k)." Defendants have violated this provision by purporting to serve a notice of commercial marketing even though their proposed biological product is not "licensed under subsection (k)."

148. Defendants' violation of 42 U.S.C. § 262(l)(8)(A) has caused and will cause Plaintiffs injury, including irreparable harm for which Plaintiffs have no adequate remedy at law, and will continue unless the statutory requirements are declared and enforced by this Court.

* * *

As a result of Defendants' violations of the BPCIA alleged above, Plaintiffs assert the following claims of patent infringement, which Plaintiffs believe are premature under the BPCIA, in order to preserve their rights to seek lost profits and injunctive relief.

COUNT 3: INFRINGEMENT OF THE 471 PATENT

149. Plaintiffs incorporate by reference paragraphs 1-148 as if fully set forth herein.

150. Upon information and belief, Defendants have been aware of the 471 patent since a time before Defendants filed their aBLA.

151. Defendants' submission of their aBLA was an act of infringement of the 471 patent under 35 U.S.C. § 271(e)(2)(C)(i), literally or under the doctrine of equivalents.

152. Defendants do not dispute that they infringe claims of the 471 patent.

153. Upon information and belief, Celltrion's and/or Hospira's commercial manufacture, use, sale, offer for sale and/or importation of their proposed biosimilar infliximab would infringe, contribute to the infringement of, and/or induce the infringement of claims 1, 3, and 5-7 of the 471 patent, literally or under the doctrine of equivalents.

154. Upon information and belief, Defendants' infringement of claims 1, 3, and 5-7 of the 471 patent would be objectively reckless and would make this case exceptional entitling Plaintiffs to attorneys' fees.

155. Unless Defendants are enjoined from infringing claims 1, 3, and 5-7 of the 471 patent, Plaintiffs will suffer irreparable injury for which damages are an inadequate remedy.

COUNT 4: INFRINGEMENT OF THE 396 PATENT

156. Plaintiffs incorporate by reference paragraphs 1-155 as if fully set forth herein.

157. Upon information and belief, Defendants have been aware of the 396 patent since a time before Defendants filed their aBLA.

158. Defendants' submission of their aBLA was an act of infringement of the 396 patent under 35 U.S.C. § 271(e)(2)(C)(i), literally or under the doctrine of equivalents.

159. Defendants do not dispute that they infringe claims of the 396 patent.

160. Upon information and belief, Celltrion's and/or Hospira's commercial manufacture, use, sale, offer for sale and/or importation of their proposed biosimilar infliximab would infringe, contribute to the infringement of, and/or induce the infringement of claims 5, 7-9 and/or 29 of the 396 patent, literally or under the doctrine of equivalents.

161. Through their intended labelling, product inserts, publications, websites and/or promotional materials, Defendants will instruct customers to use their proposed biosimilar infliximab in an infringing manner. Specifically, Defendants will encourage infringement of

claims 5, 7-9 and/or 29 of the 396 patent. Defendants have knowledge of the 396 patent and know or are willfully blind to the possibility that the uses indicated and promoted on their intended labeling, product inserts, publications, websites and/or promotional materials encourage infringement of the aforementioned claims of the 396 patent.

162. Upon information and belief, Defendants' infringement of claims 5, 7-9 and/or 29 of the 396 patent would be objectively reckless and would make this case exceptional entitling Plaintiffs to attorneys' fees.

163. Unless Defendants are enjoined from infringing claims 5, 7-9 and/or 29 of the 396 patent, Plaintiffs will suffer irreparable injury for which damages are an inadequate remedy.

COUNT 5: INFRINGEMENT OF THE 715 PATENT

164. Janssen incorporates by reference paragraphs 1-163 as if fully set forth herein.

165. On information and belief, Defendants have been aware of the 715 patent since a time before Defendants filed their aBLA.

166. Defendants' submission of their aBLA was an act of infringement of the 715 patent under 35 U.S.C. § 271(e)(2)(C)(i), literally or under the doctrine of equivalents.

167. Upon information and belief, Celltrion's and/or Hospira's commercial manufacture, use, sale, offer for sale and/or importation of their proposed biosimilar infliximab would infringe, contribute to the infringement of, and/or induce the infringement of claims of the 715 patent, literally or under the doctrine of equivalents.

168. Upon information and belief, Defendants' infringement of claims of the 715 patent would be objectively reckless and would make this case exceptional entitling Janssen to attorneys' fees.

169. Unless Defendants are enjoined from infringing claims of the 715 patent, Janssen will suffer irreparable injury for which damages are an inadequate remedy.

COUNT 6: INFRINGEMENT OF THE 083 PATENT

170. Janssen incorporates by reference paragraphs 1-169 as if fully set forth herein.

171. On information and belief, Defendants have been aware of the 083 patent since a time before Defendants filed their aBLA.

172. Defendants' submission of their aBLA was an act of infringement of the 083 patent under 35 U.S.C. § 271(e)(2)(C)(ii), literally or under the doctrine of equivalents.

173. Upon information and belief, Defendants' infringement of the 083 patent would be objectively reckless and would make this case exceptional entitling Janssen to attorneys' fees.

174. Unless Defendants are enjoined from infringing the 083 patent, Janssen will suffer irreparable injury for which damages are an inadequate remedy.

COUNT 7: INFRINGEMENT OF THE 056 PATENT

175. Janssen incorporates by reference paragraphs 1-174 as if fully set forth herein.

176. On information and belief, Defendants have been aware of the 056 patent since a time before Defendants filed their aBLA.

177. Defendants' submission of their aBLA was an act of infringement of the 056 patent under 35 U.S.C. § 271(e)(2)(C)(ii), literally or under the doctrine of equivalents.

178. Upon information and belief, Defendants' infringement of the 056 patent would be objectively reckless and would make this case exceptional entitling Janssen to attorneys' fees.

179. Unless Defendants are enjoined from infringing the 056 patent, Janssen will suffer irreparable injury for which damages are an inadequate remedy.

COUNT 8: INFRINGEMENT OF THE 600 PATENT

180. Janssen incorporates by reference paragraphs 1-179 as if fully set forth herein.

181. On information and belief, Defendants have been aware of the 600 patent since a time before Defendants filed their aBLA.

182. Defendants' submission of their aBLA was an act of infringement of the 600 patent under 35 U.S.C. § 271(e)(2)(C)(ii), literally or under the doctrine of equivalents.

183. Upon information and belief, Defendants' infringement of the 600 patent would be objectively reckless and would make this case exceptional entitling Janssen to attorneys' fees.

184. Unless Defendants are enjoined from infringing the 600 patent, Janssen will suffer irreparable injury for which damages are an inadequate remedy.

PRAYER FOR RELIEF

WHEREFORE, Plaintiffs respectfully request that this Court enter judgment in their favor against Defendants and grant the following relief:

(a) a declaration that Defendants have failed to comply with the requirements of the BPCIA patent dispute resolution process, including 42 U.S.C. § 262(l)(2)(A) and 42 U.S.C. § 262(l)(4);

(b) an order compelling Defendants to comply with the BPCIA patent dispute resolution process set forth in 42 U.S.C. § 262(l);

(c) a declaration that the "notice of commercial marketing" provided by Defendants on February 5, 2015 is not an effective "notice of commercial marketing" within the meaning of 42 U.S.C. § 262(l)(8)(A) and that Defendants may not begin the commercial marketing of their proposed biosimilar to Janssen's Remicade® infliximab product until at least 180 days after Defendants provide Janssen with proper notice pursuant to 42 U.S.C. § 262(l)(8)(A) that Defendants have received a license for and intend to begin commercial marketing of the product;

(d) preliminary and/or permanent equitable relief, including but not limited to a preliminary and permanent injunction that enjoins Defendants, their officers, partners, agents, servants, employees, parents, subsidiaries, divisions, affiliate corporations, other related business entities and all other persons acting in concert, participation, or in privity with them and/or their successors or assigns, from any commercial manufacture, use, offer to sell or sale within the United States, of Defendants' proposed biosimilar to Janssen's Remicade® infliximab product, until 180 days after Defendants provide Janssen with proper notice pursuant to 42 U.S.C. § 262(l)(8)(A) that Defendants have received a license for and intend to begin commercial marketing of the product;

(e) a judgment that Defendants have infringed under 35 U.S.C. § 271(e)(2)(C)(i):

(1) the 471 patent;
(2) the 396 patent; and
(3) the 715 patent;

(f) a judgment that Defendants have infringed under 35 U.S.C. § 271(e)(2)(C)(ii):

(1) the 083 patent;
(2) the 056 patent; and
(3) the 600 patent;

(g) a judgment declaring that the making, using, selling, offering to sell, or importing of the proposed biosimilar to Janssen's Remicade® infliximab product described in aBLA No. 125544 would constitute infringement of:

(1) the 471 patent;
(2) the 396 patent;
(3) the 715 patent;
(4) the 083 patent;
(5) the 056 patent; and
(6) the 600 patent;

(h) preliminary and/or permanent equitable relief, including but not limited to a preliminary and/or permanent injunction that enjoins Defendants, their officers, partners, agents,

servants, employees, parents, subsidiaries, divisions, affiliate corporations, other related business entities and all other persons acting in concert, participation, or in privity with them and/or their successors or assigns, from any commercial manufacture, use, offer to sell or sale within the United States, or importation into the United Sates, of any product that infringes, or the use or manufacture of which infringes:

(1) the 471 patent;
(2) the 396 patent;
(3) the 715 patent;
(4) the 083 patent;
(5) the 056 patent; or
(6) the 600 patent;

(i) an order compelling Defendants to compensate Plaintiffs for and awarding damages incurred as a result of Defendants' actions or inactions;

(j) a declaration that this is an exceptional case and an award to Plaintiffs of their attorneys' fees, costs and expenses pursuant to 35 U.S.C. § 271(e)(4) and 35 U.S.C. § 285; and

(k) such other relief as this Court may deem just and proper.

Dated: March 6, 2015

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EXHIBIT A

US006284471B1

(12) **United States Patent**
Le et al.

(10) **Patent No.: US 6,284,471 B1**
(45) **Date of Patent: Sep. 4, 2001**

(54) **ANTI-TNFA ANTIBODIES AND ASSAYS EMPLOYING ANTI-TNFA ANTIBODIES**

(75) Inventors: **Junming Le**, Jackson Heights; **Jan Vilcek**, New York, both of NY (US); **Peter Dadonna**, Palo Alto, CA (US); **John Ghrayeb**, Thorndale; **David Knight**, Berwyn, both of PA (US); **Scott A. Siegel**, Westborough, MA (US)

(73) Assignees: **New York University Medical Center**, New York, NY (US); **Centocor, Inc.**, Malvern, PA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **08/192,093**

(22) Filed: **Feb. 4, 1994**

Related U.S. Application Data

(63) Continuation-in-part of application No. 08/010,406, filed on Jan. 29, 1993, now abandoned, and a continuation-in-part of application No. 08/013,413, filed on Feb. 2, 1993, now abandoned, which is a continuation-in-part of application No. 07/943,852, filed on Sep. 11, 1992, now abandoned, which is a continuation-in-part of application No. 07/853,606, filed on Mar. 18, 1992, now abandoned, which is a continuation-in-part of application No. 07/670,827, filed on Mar. 18, 1991, now abandoned.

(51) **Int. Cl.**[7] **C07K 16/24**; A61K 39/395; C12N 15/00; G01N 33/53

(52) **U.S. Cl.** **435/7.1**; 435/69.6; 435/70.21; 530/387.3; 530/388.23; 530/391.3; 424/133.1; 424/139.1; 424/141.1

(58) **Field of Search** 530/387.3, 388.23, 530/391.3; 435/7.1, 240.27, 172.2, 70.21

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Primary Examiner—Anthony C. Caputa
Assistant Examiner—Karen A. Canella
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(57) **ABSTRACT**

Anti-TNF antibodies and anti-TNF peptides, specific for tumor necrosis factor (TNF) are useful for in vivo diagnosis and therapy of a number of TNF-mediated pathologies and conditions, as well as polynucleotides coding for anti-TNF murine and chimeric antibodies, peptides, methods of making and using the antibody or peptides in immunoassays and immuno-therapeutic approaches are provided, where the anti-TNF peptide is selected from a soluble portion of TNF receptor, an anti-TNF antibody or structural analog thereof.

9 Claims, 36 Drawing Sheets

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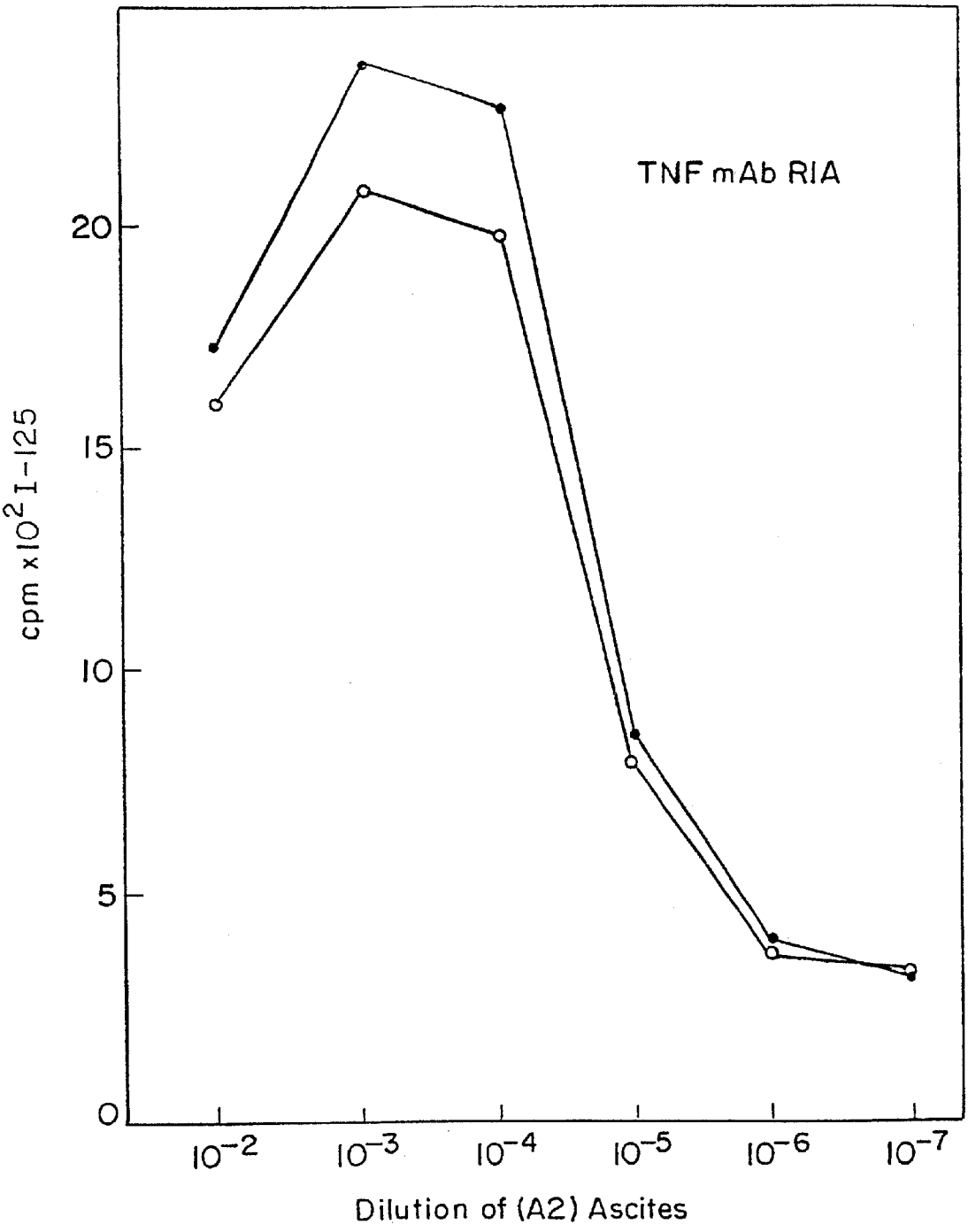

TNF mAb RIA
cpm x10² I-125
20
15
10
5
0
10⁻²
10⁻³
10⁻⁴
10⁻⁵
10⁻⁶
10⁻⁷
Dilution of (A2) Ascites

FIG. 1

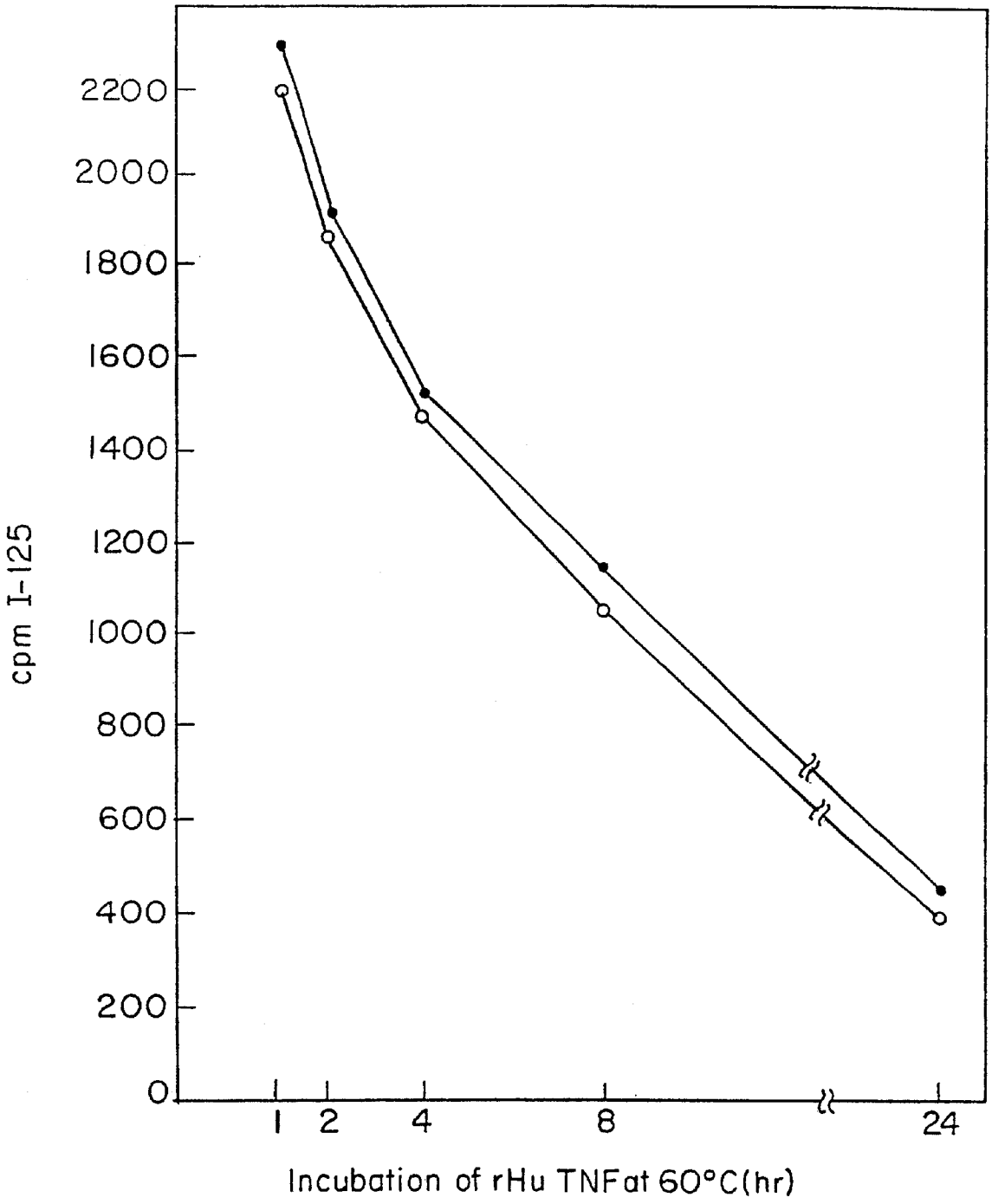

2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
cpm I-125
1
2
4
8
24
Incubation of rHu TNF at 60°C (hr)

FIG. 2

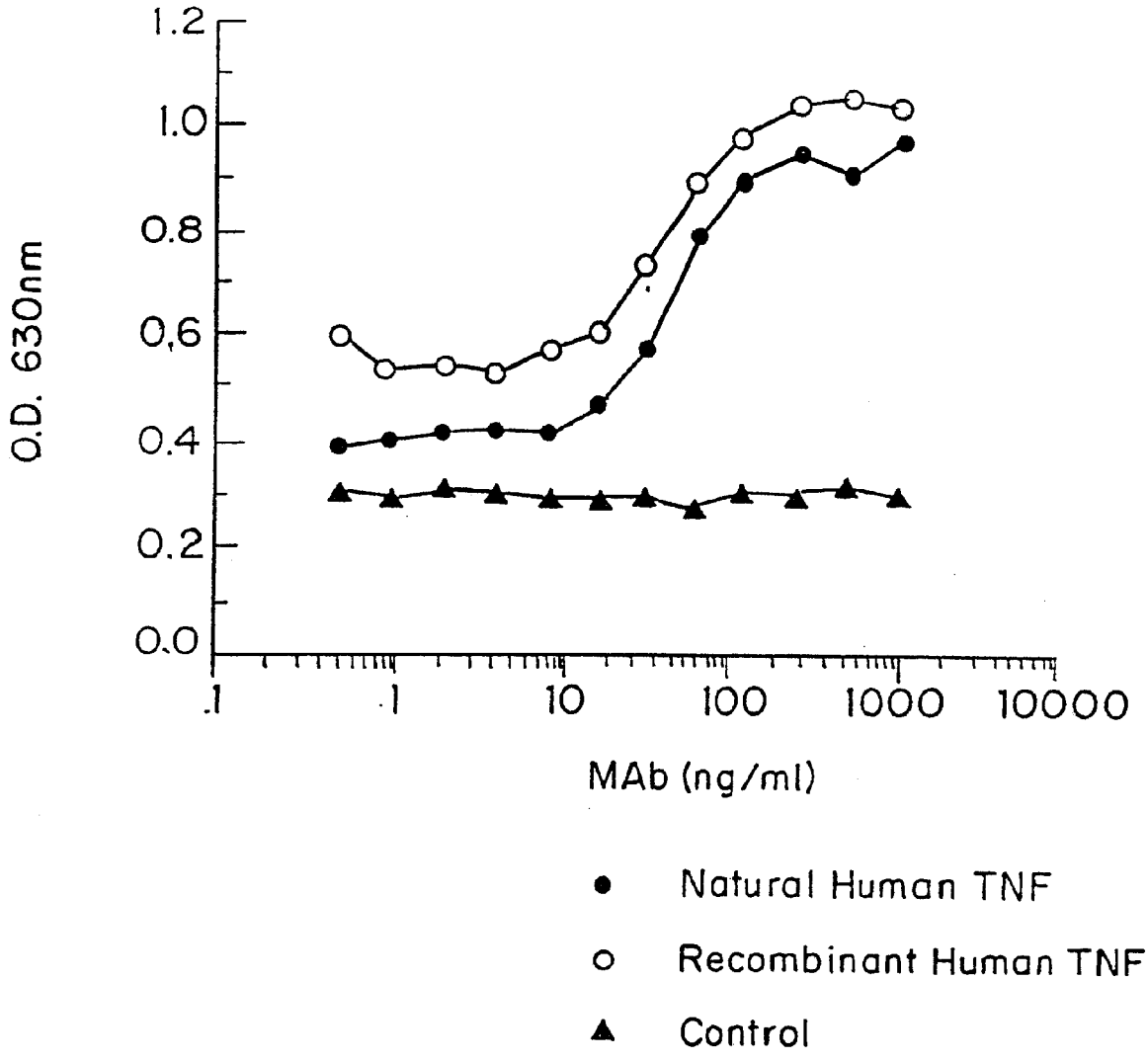

1.2
1.0
0.8
0.6
0.4
0.2
0.0
O.D. 630nm
.1
1
10
100
1000
10000
MAb (ng/ml)
Natural Human TNF
Recombinant Human TNF
Control

FIG. 3

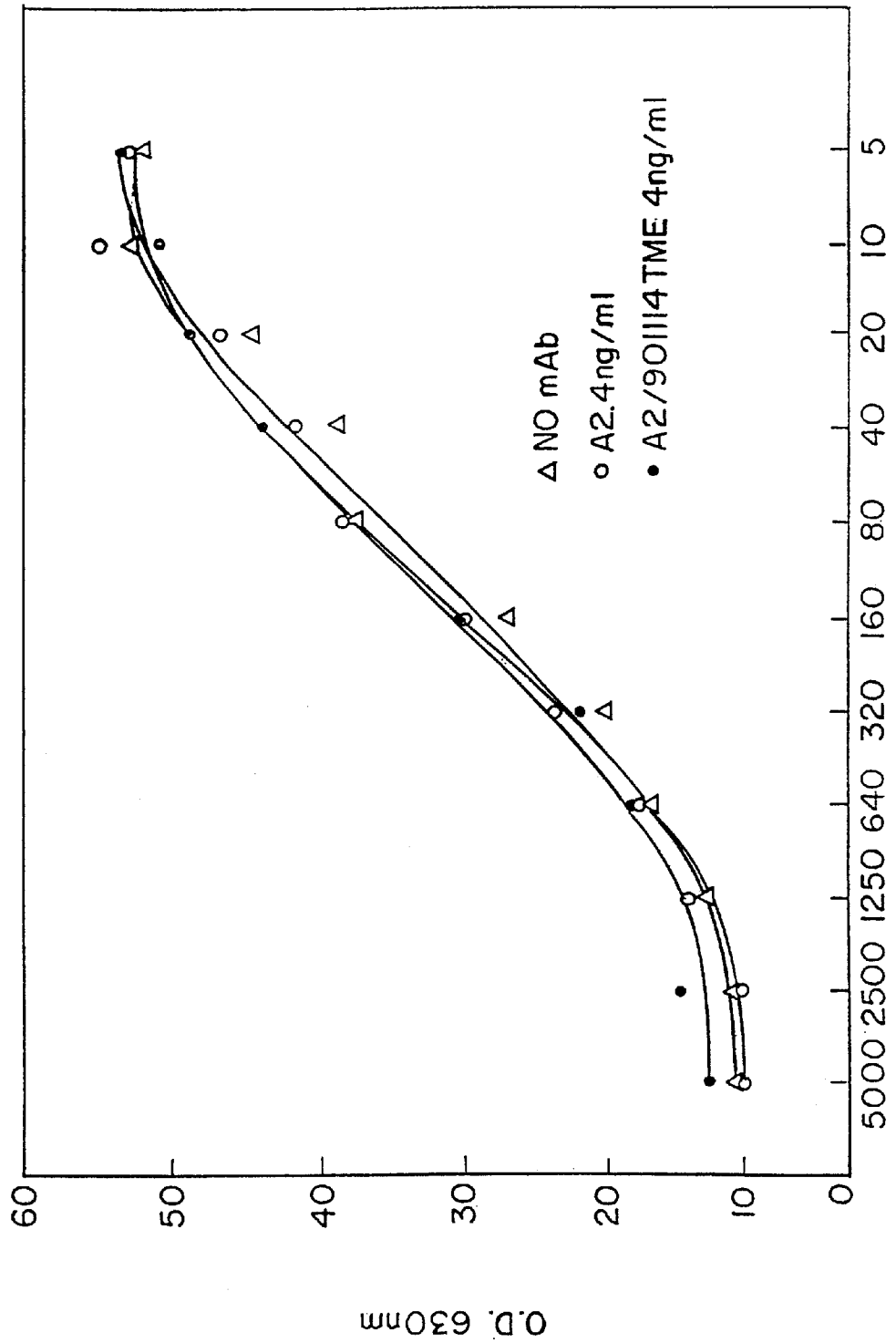

O.D. 630nm
0
10
20
30
40
50
60
5000
2500
1250
640
320
160
80
40
20
10
5
rHuLT (pg/ml)
Δ NO mAb
o A2.4ng/ml
• A2/9O1114TME 4ng/ml

FIG. 4

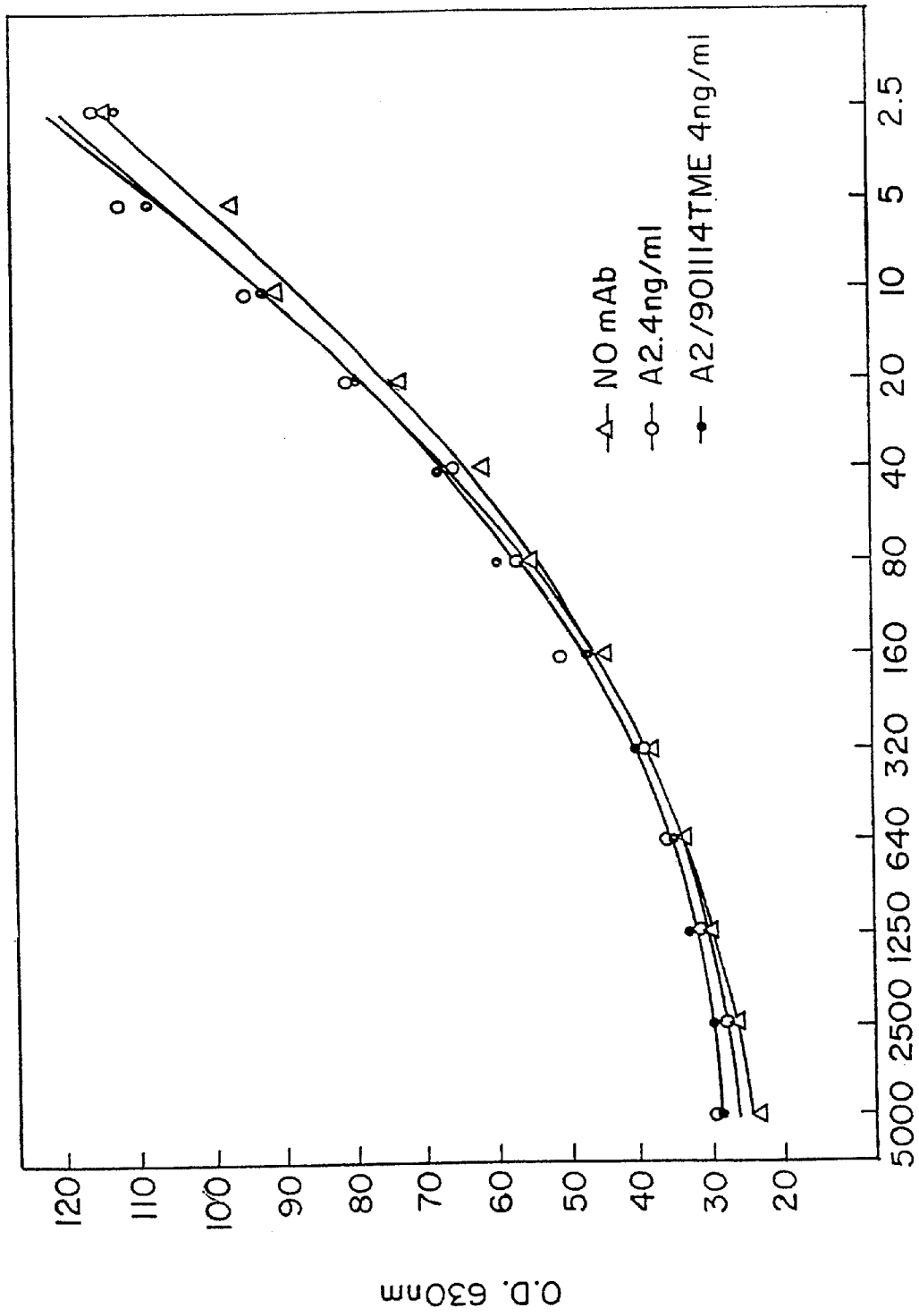

NO mAb
A2.4ng/ml
A2/9011I4TME 4ng/ml
murine TNF (pg/ml)
5000 2500 1250 640 320 160 80 40 20 10 5 2.5
O.D. 630nm
120 110 100 90 80 70 60 50 40 30 20

FIG. 5

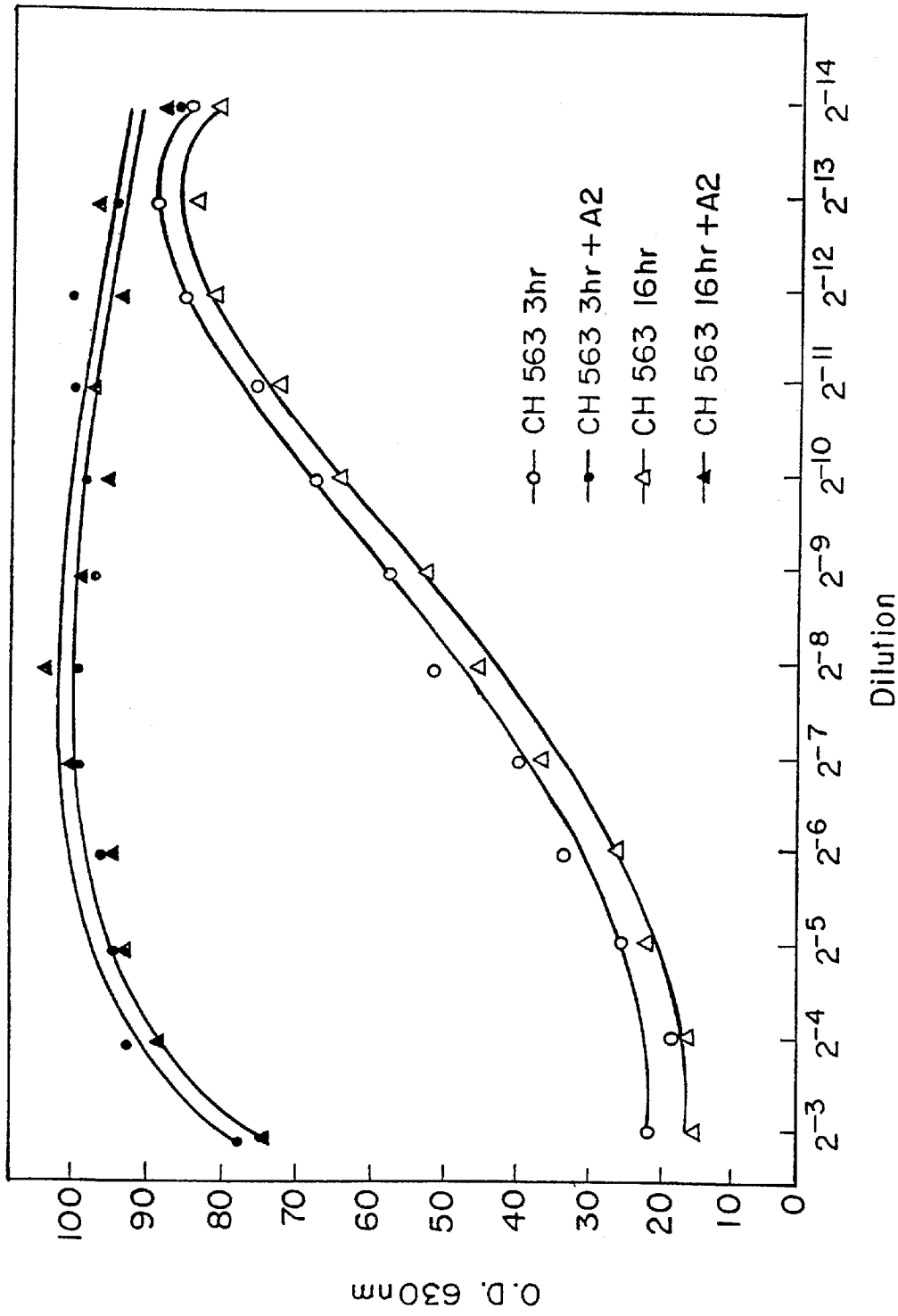

O.D. 630nm
100
90
80
70
60
50
40
30
20
10
0
2^{-3} 2^{-4} 2^{-5} 2^{-6} 2^{-7} 2^{-8} 2^{-9} 2^{-10} 2^{-11} 2^{-12} 2^{-13} 2^{-14}
Dilution
CH 563 3hr
CH 563 3hr + A2
CH 563 16hr
CH 563 16hr + A2

FIG. 6

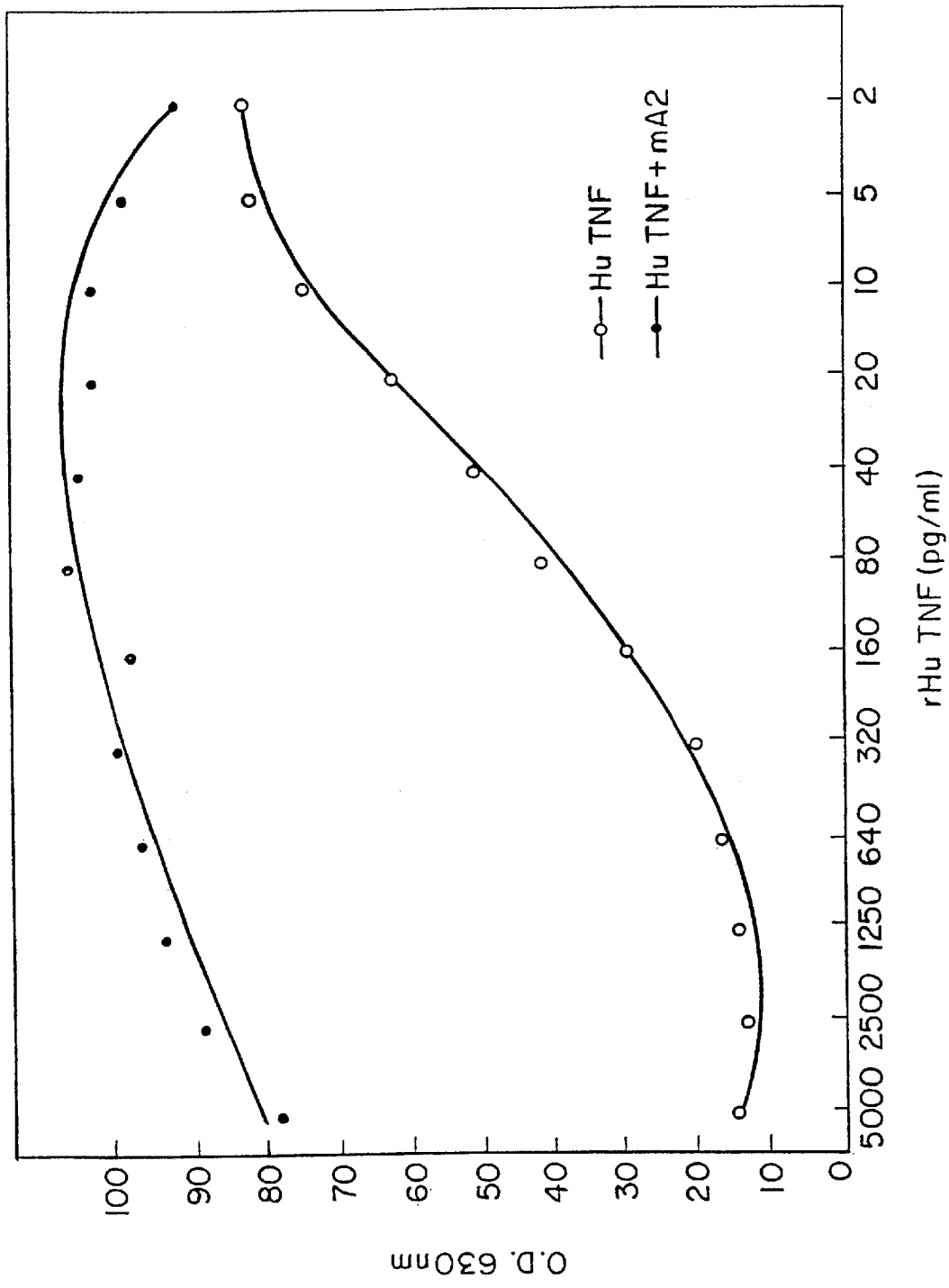
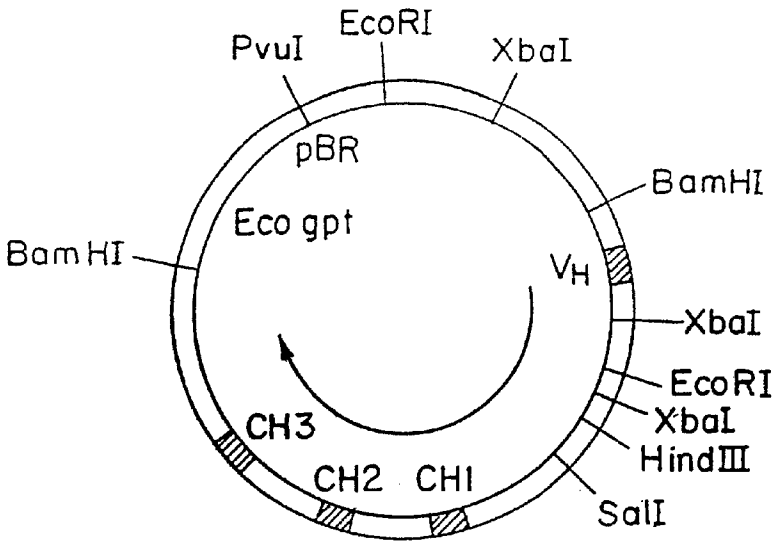
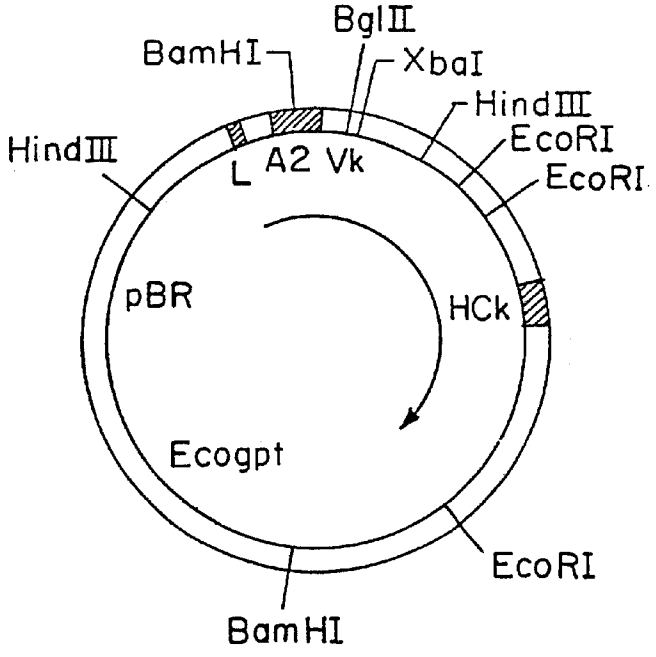

O.D. 630nm
100
90
80
70
60
50
40
30
20
10
0
5000 2500 1250 640 320 160 80 40 20 10 5 2
rHu TNF (pg/ml)
Hu TNF
Hu TNF + mA2

FIG. 7



pA2HG1apgpt

FIG. 8A



pA2HuKapgpt

FIG. 8B

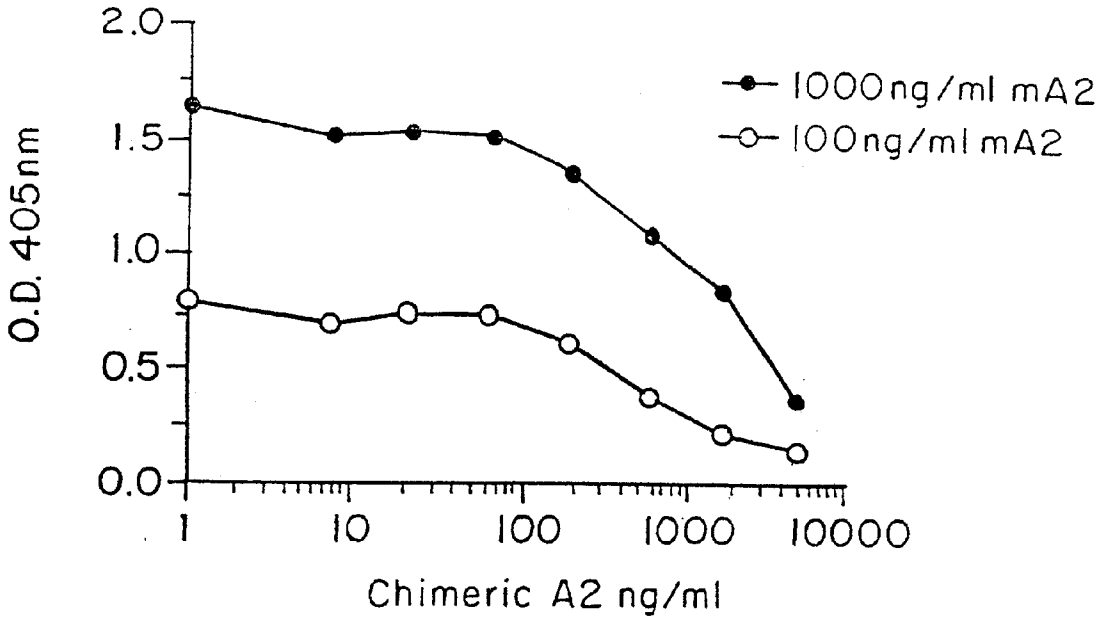

2.0
1.5
1.0
0.5
0.0
O.D. 405nm
1000ng/ml mA2
100ng/ml mA2
1
10
100
1000
10000
Chimeric A2 ng/ml

FIG. 9A

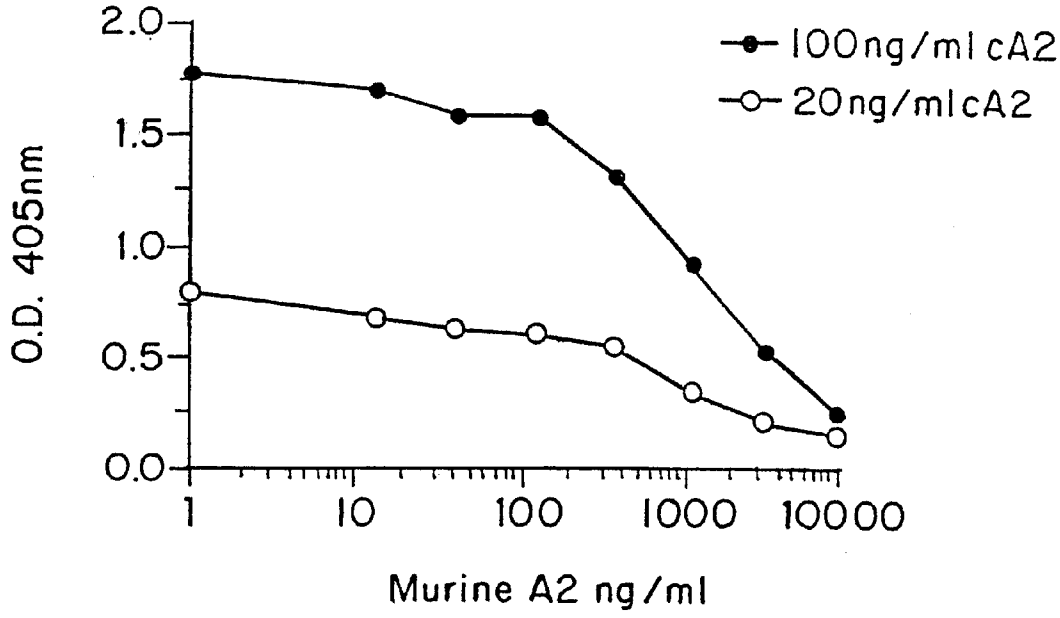

2.0
1.5
1.0
0.5
0.0
O.D. 405nm
100ng/ml cA2
20ng/ml cA2
1
10
100
1000
10000
Murine A2 ng/ml

FIG. 9B

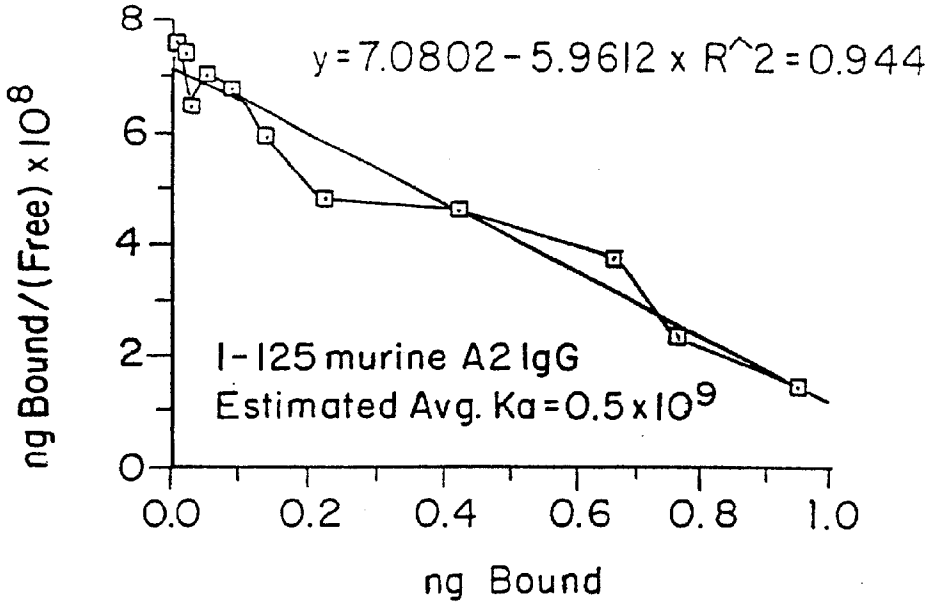


FIG. 10A

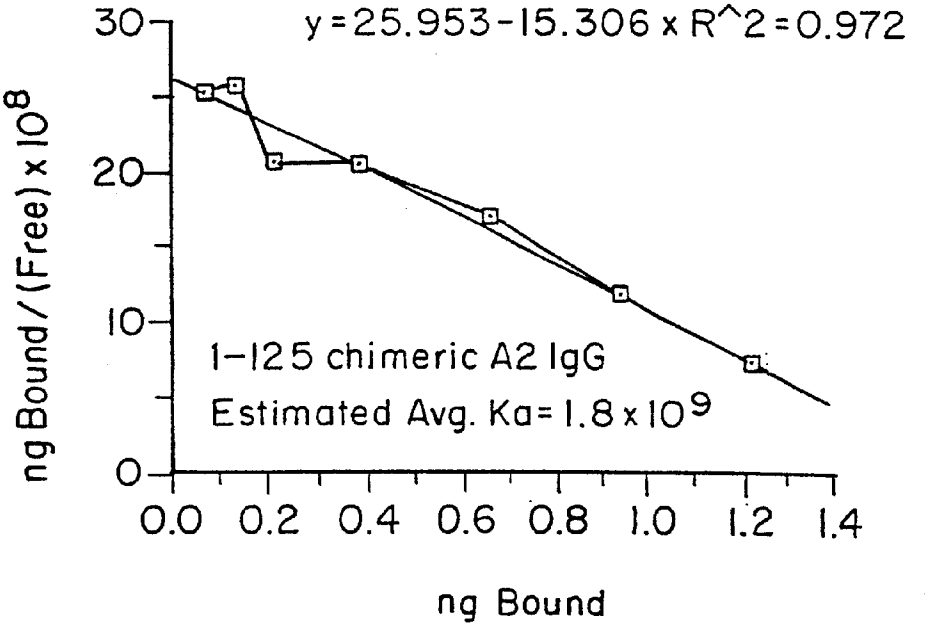


FIG. 10B

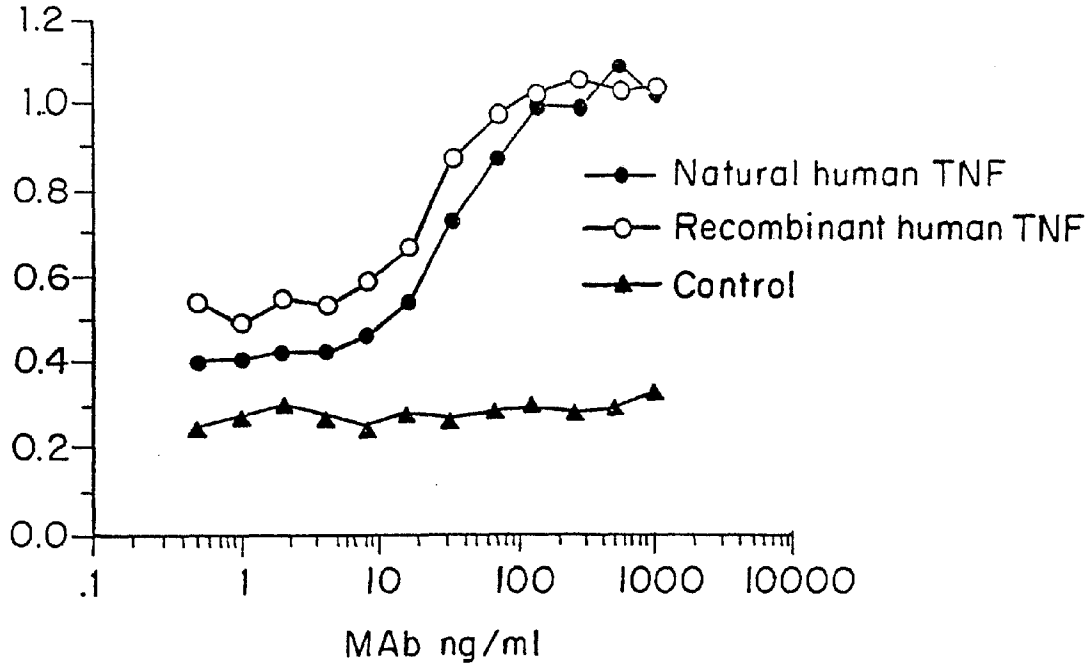

1.2
1.0
0.8
0.6
0.4
0.2
0.0
.1
1
10
100
1000
10000
MAb ng/ml
Natural human TNF
Recombinant human TNF
Control

FIG. 11

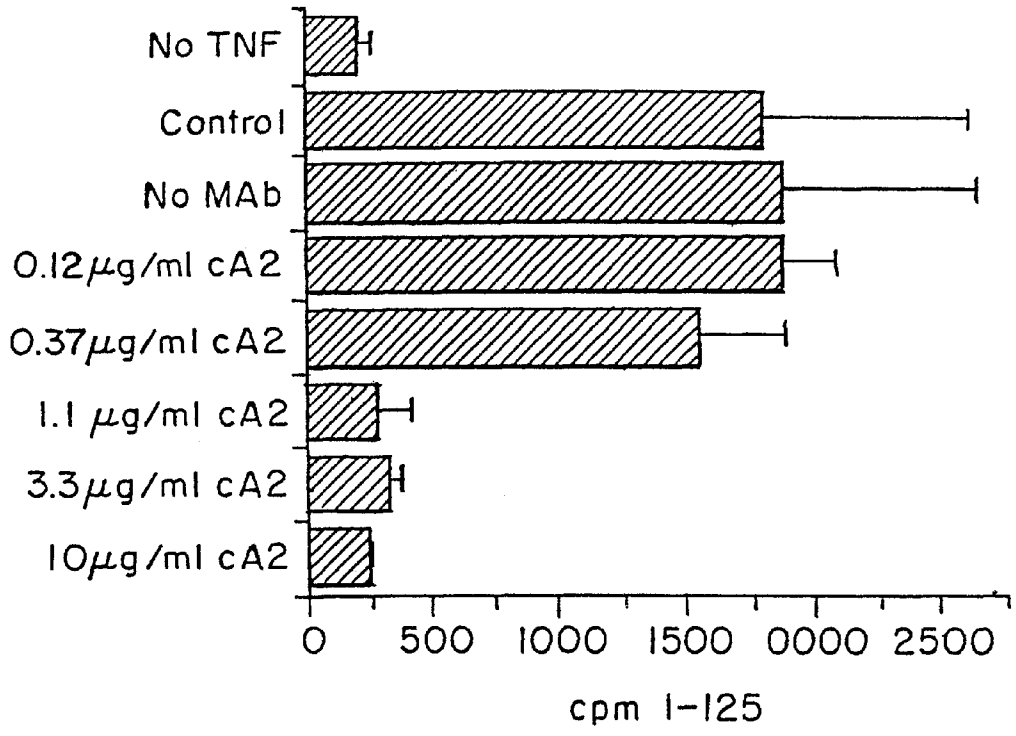

No TNF
Control
No MAb
0.12μg/ml cA2
0.37μg/ml cA2
1.1 μg/ml cA2
3.3μg/ml cA2
10μg/ml cA2
0
500
1000
1500
0000
2500
cpm I-125

FIG. 12

```
                                                  10
1   Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val Val Ala Asn Pro

                                                  30
21  Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg Ala Asn Ala Leu Leu Ala Asn Gly

                                                  50
41  Val Glu Leu Arg Asp Asn Gln Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser

                                                  70
61  Gln Val Leu Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile

                                                  90
81  Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala Ile Lys Ser Pro

                                                 110
101 Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys Pro Trp Tyr Glu Pro Ile Tyr Leu

                                                 130
121 Gly Gly Val Phe Gln Leu Glu Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp

                                                 150
141 Tyr Leu Asp Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu
```

FIG. 13

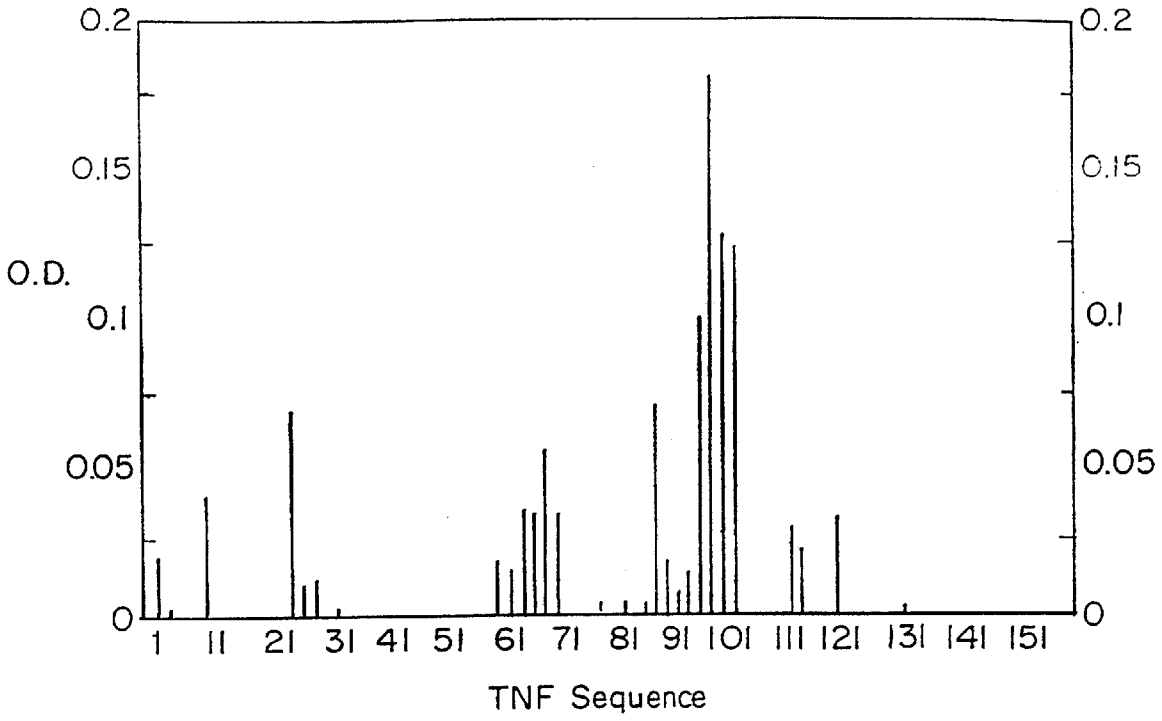


FIG. 14A

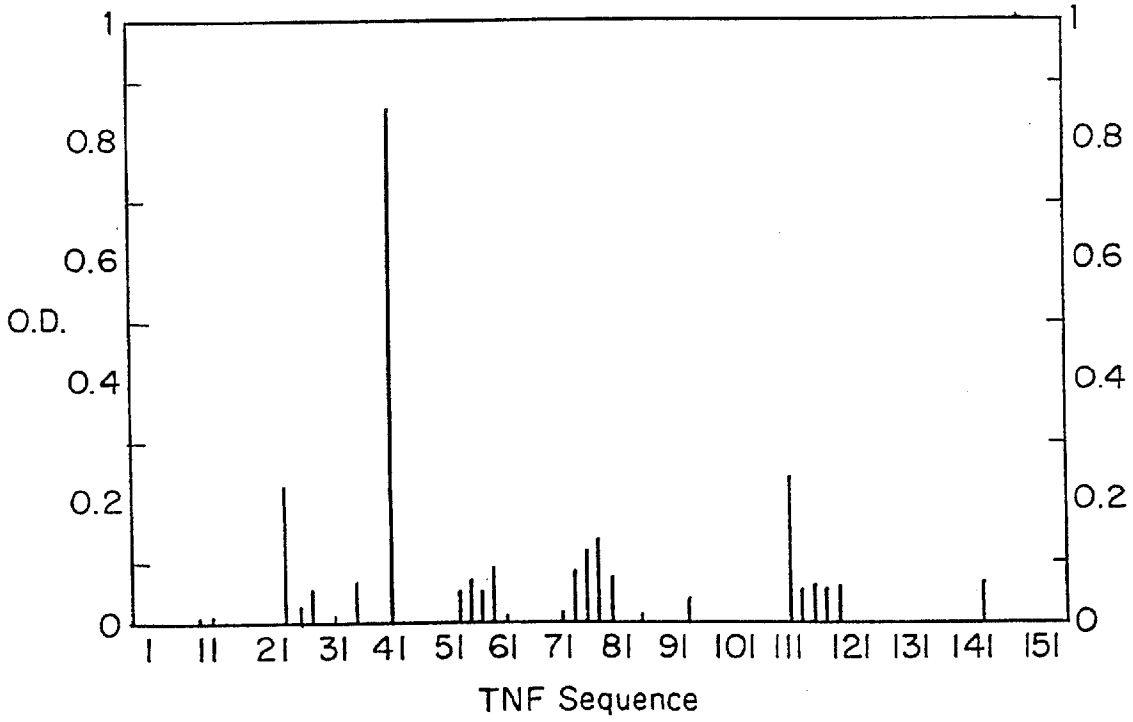


FIG. 14B

```
1                                                  10
Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val Val Ala Asn Pro

21                 30
Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg Ala Asn Ala Leu Leu Ala Asn Gly

41                 50
Val Glu Leu Arg Asp Asn Gln Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser

61                 70
Gln Val Leu Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile

81                 90
Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala Ile Lys Ser Pro

101                110
Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys Pro Trp Tyr Glu Pro Ile Tyr Leu

121                130
Gly Gly Val Phe Gln Leu Glu Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp

141                150
Tyr Leu Asp Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu
```

FIG. 15

```
GACATCTTGCTGACTCAGTCTCCAGCCATCCTGTCTGTGAGTCCAGGAGAAAGAGTCAGT
AspIleLeuLeuThrGlnSerProAlaIleLeuSerValSerProGlyGluArgValSer

TTCTCCTGCAGGGCCAGTCAGTTCGTTGGCTCAAGCATCCACTGGTATCAGCAAAGAACA
PheSerCysArgAlaSerGlnPheValGlySerSerIleHisTrpTyrGlnGlnArgThr

AATGGTTCTCCCAAGGCTTCTCATAAAGTATGCTTCTGAGTCTATGTCTGGGATCCCTTCC
AsnGlySerProArgLeuLeuIleLysTyrAlaSerGluSerMetSerGlyIleProSer

AGGTTTAGTGGCAGTGGATCAGGGACAGATTTTACTCTTAGCATCAACACTGTGGAGTCT
ArgPheSerGlySerGlyThrAspPheThrLeuSerIleAsnThrValGluSer

GAAGATATTGCAGATTTATTACTGTCAAGAAAGTCATAGCTGGCCATTCACGTTCGGCTCG
GluAspIleAlaAspTyrTyrCysGlnGlnSerHisSerTrpProPheThrPheGlySer

GGGACAAATTTGGAAGTAAAA
GlyThrAsnLeuGluValLys
```

FIG. 16A

```
GAAGTGAAGCTTGAGGAGTCTGGAGGAGGCTTGGTGCAACCTGGAGGATCCATGAAACTC
GluValLysLeuGluGluSerGlyGlyGlyLeuValGlnProGlyGlySerMetLysLeu

TCCTGTGTTGCCTCTGGATTCATTTTCAGTAACCACTGGATGAACTGGGTCCGCCAGTCT
SerCysValAlaSerGlyPheIlePheSerAsnHisTrpMetAsnTrpValArgGlnSer

CCAGAGAAGGGGCTTGAGTGGGTTGCTGAAATTAGATCAAATTCTATTAATTCTGCAACA
ProGluLysGlyLeuGluTrpValAlaGluIleArgSerLysSerIleAsnSerAlaThr

CATTATGCGGAGTCTGTGAAAGGGAGGTTCACCATCTCAAGAGATGATTCCAAAAGTGCT
HisTyrAlaGluSerValLysGlyArgPheThrIleSerArgAspAspSerLysSerAla

GTGTACCTGCAAATGAACCGACTTAAGAACTGAAGACACTGGCGTTTATTACTGTTCCAGG
ValTyrLeuGlnMetThrAspLeuArgThrGluAspThrGlyValTyrTyrCysSerArg

AATTACTACGGTAGTACCTACGACTACTGGGGCCAAGGCACCACTCTCACAGTGTCC
AsnTyrTyrGlySerThrTyrAspTyrTrpGlyGlnGlyThrThrLeuThrValSer
```

FIG. 16B

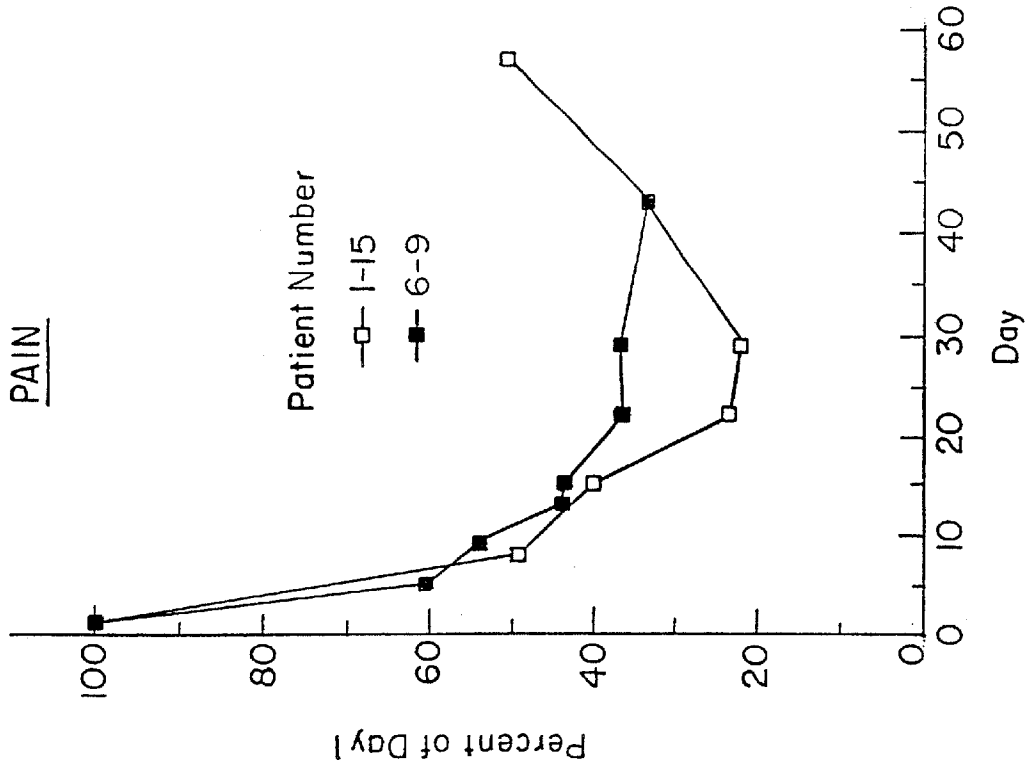

PAIN
Patient Number
1-15
6-9
Percent of Day 1
0
20
40
60
80
100
0
10
20
30
40
50
60
Day

FIG. 18

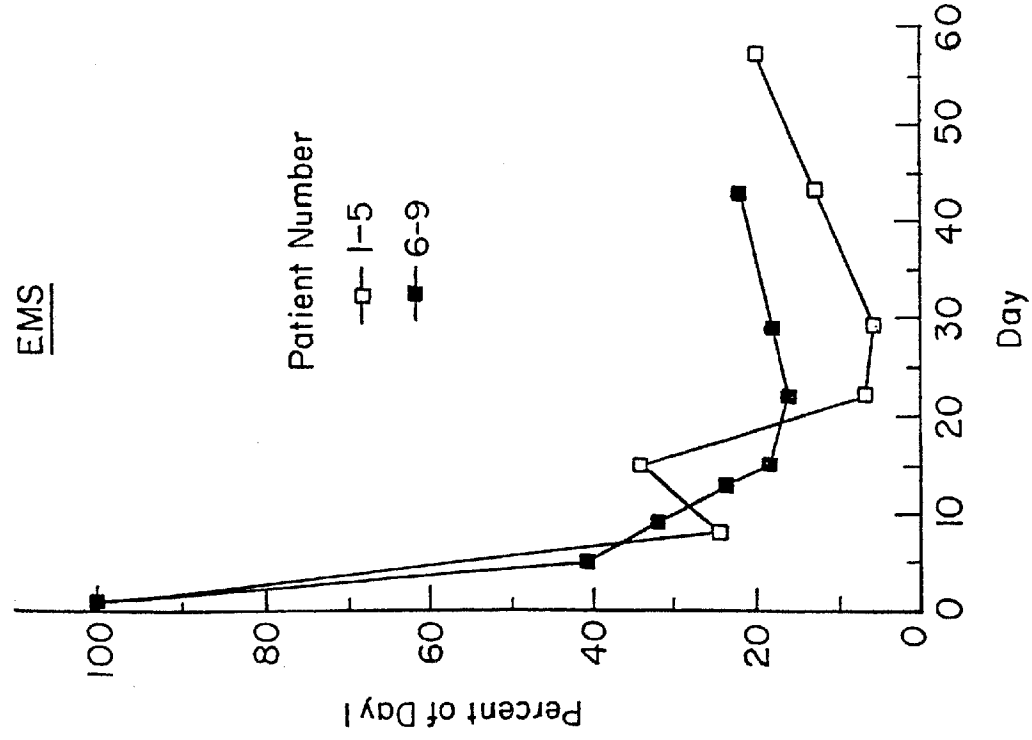

EMS
Patient Number
1-5
6-9
Percent of Day 1
0
20
40
60
80
100
0
10
20
30
40
50
60
Day

FIG. 17

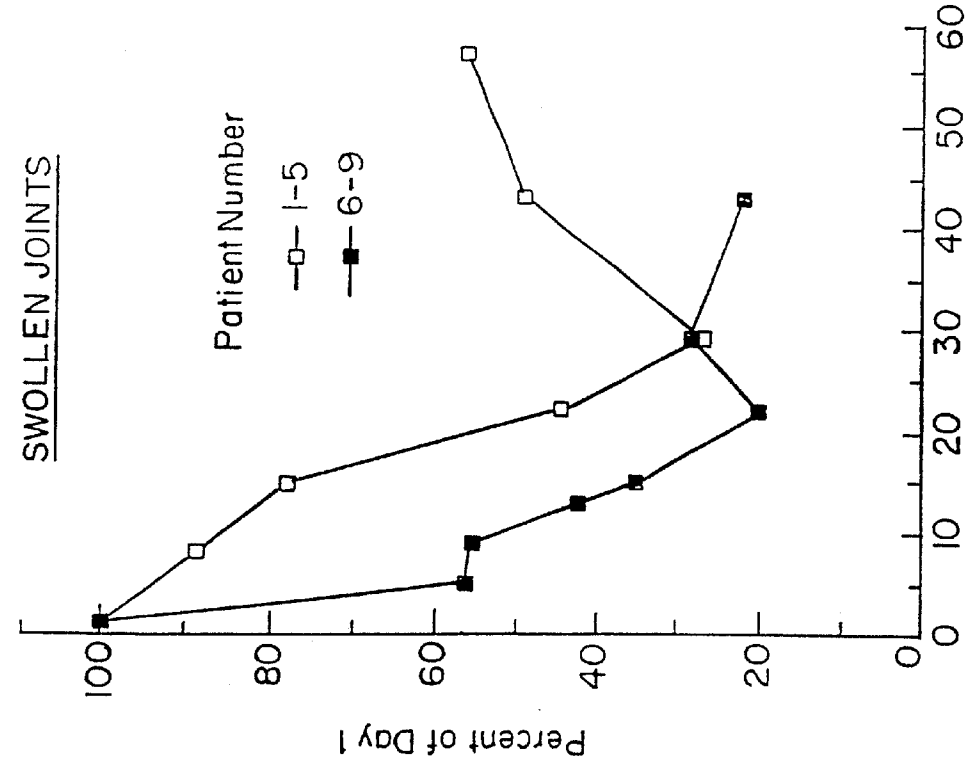

SWOLLEN JOINTS
Patient Number
1-5
6-9
Percent of Day 1
Day
0
20
40
60
80
100
0
10
20
30
40
50
60

FIG. 20

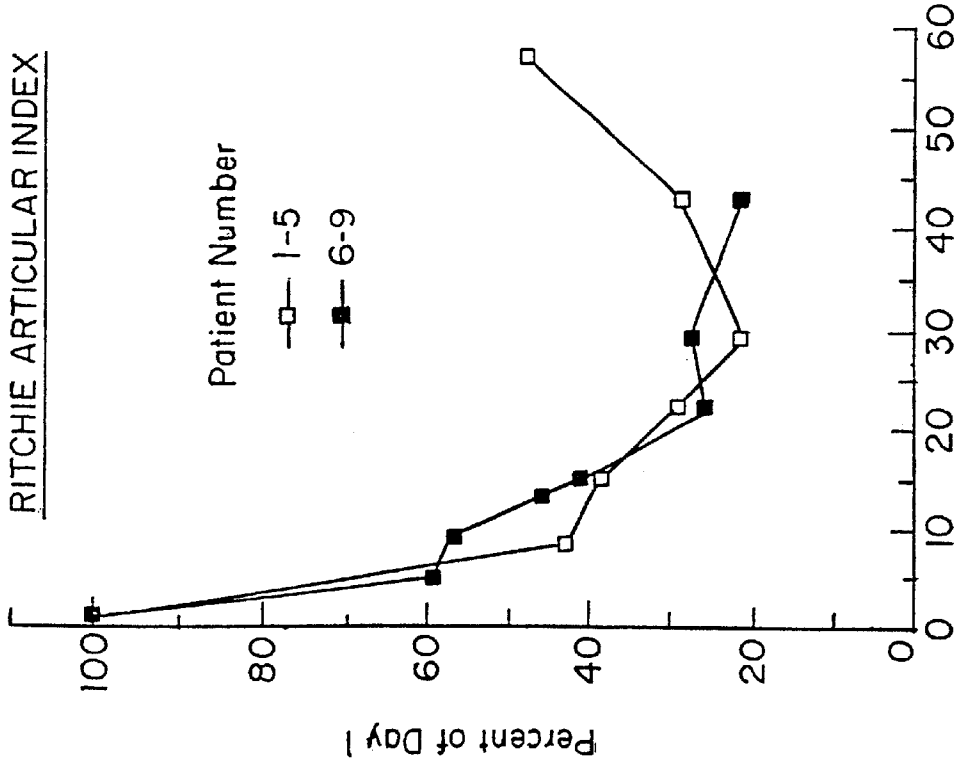

RITCHIE ARTICULAR INDEX
Patient Number
1-5
6-9
Percent of Day 1
Day
0
20
40
60
80
100
0
10
20
30
40
50
60

FIG. 19

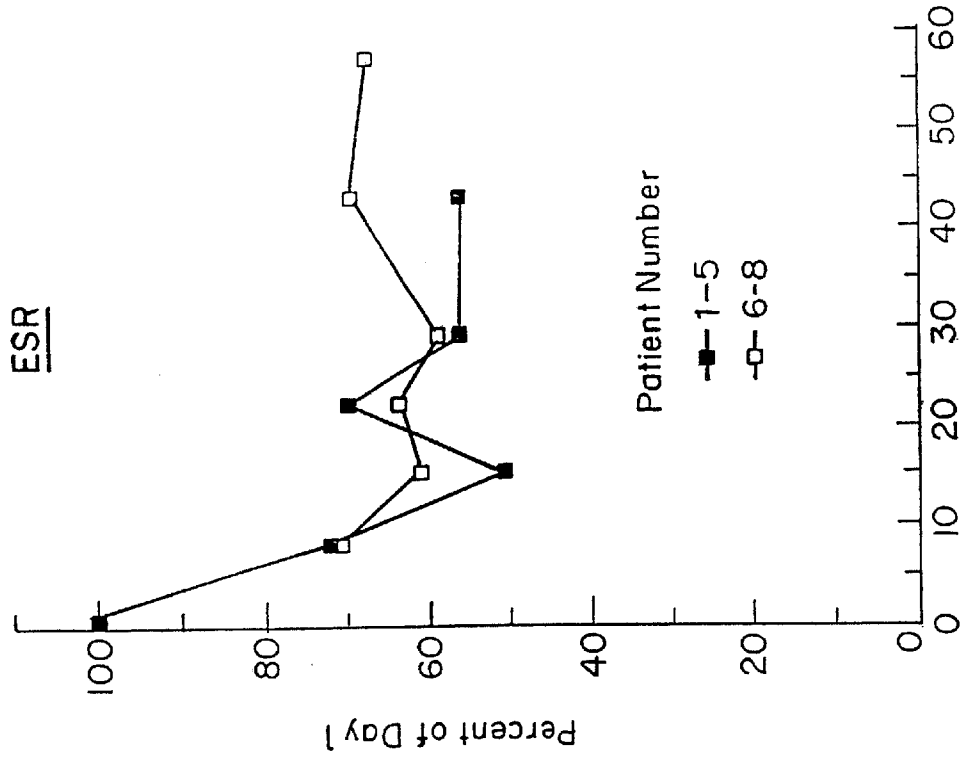

ESR
Patient Number
1-5
6-8
Day
Percent of Day 1
0
10
20
30
40
50
60
20
40
60
80
100

FIG. 22

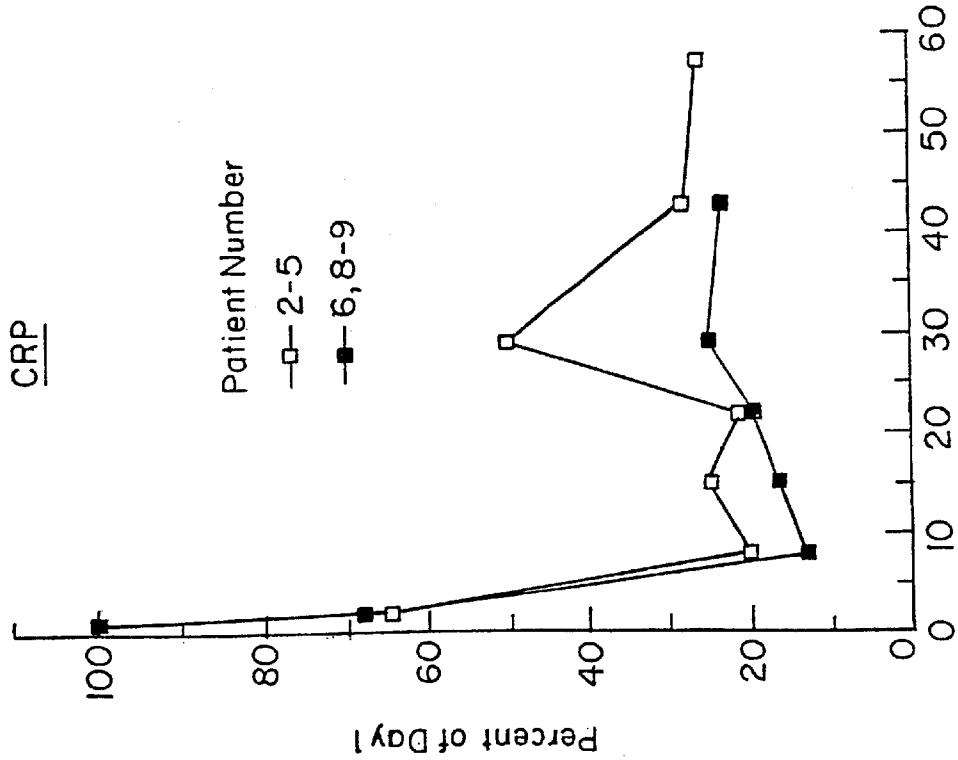

CRP
Patient Number
2-5
6,8-9
Day
Percent of Day 1
0
10
20
30
40
50
60
20
40
60
80
100

FIG. 21

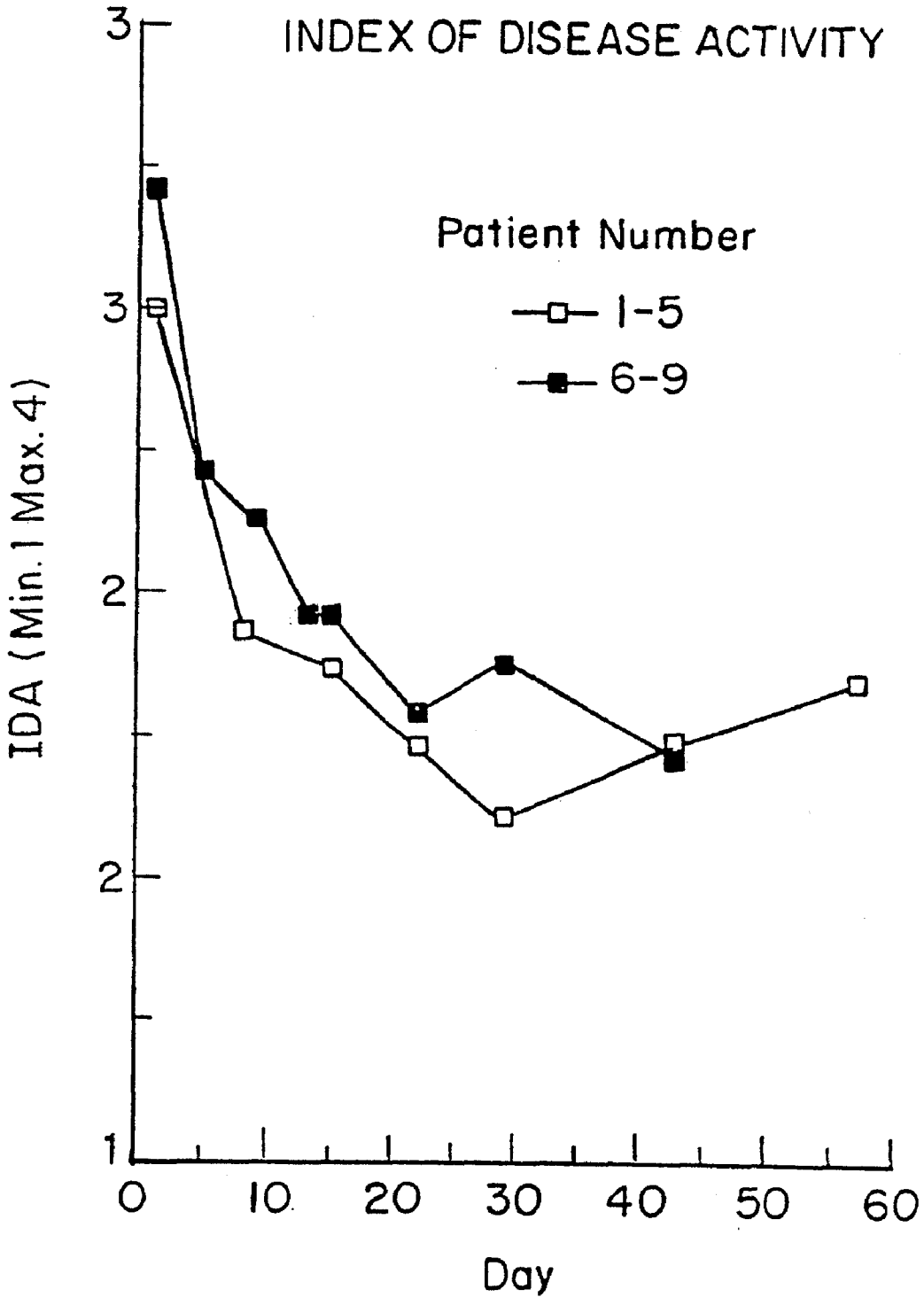

INDEX OF DISEASE ACTIVITY
Patient Number
1-5
6-9
IDA (Min. 1 Max. 4)
3
3
2
2
1
0
10
20
30
40
50
60
Day

FIG. 23

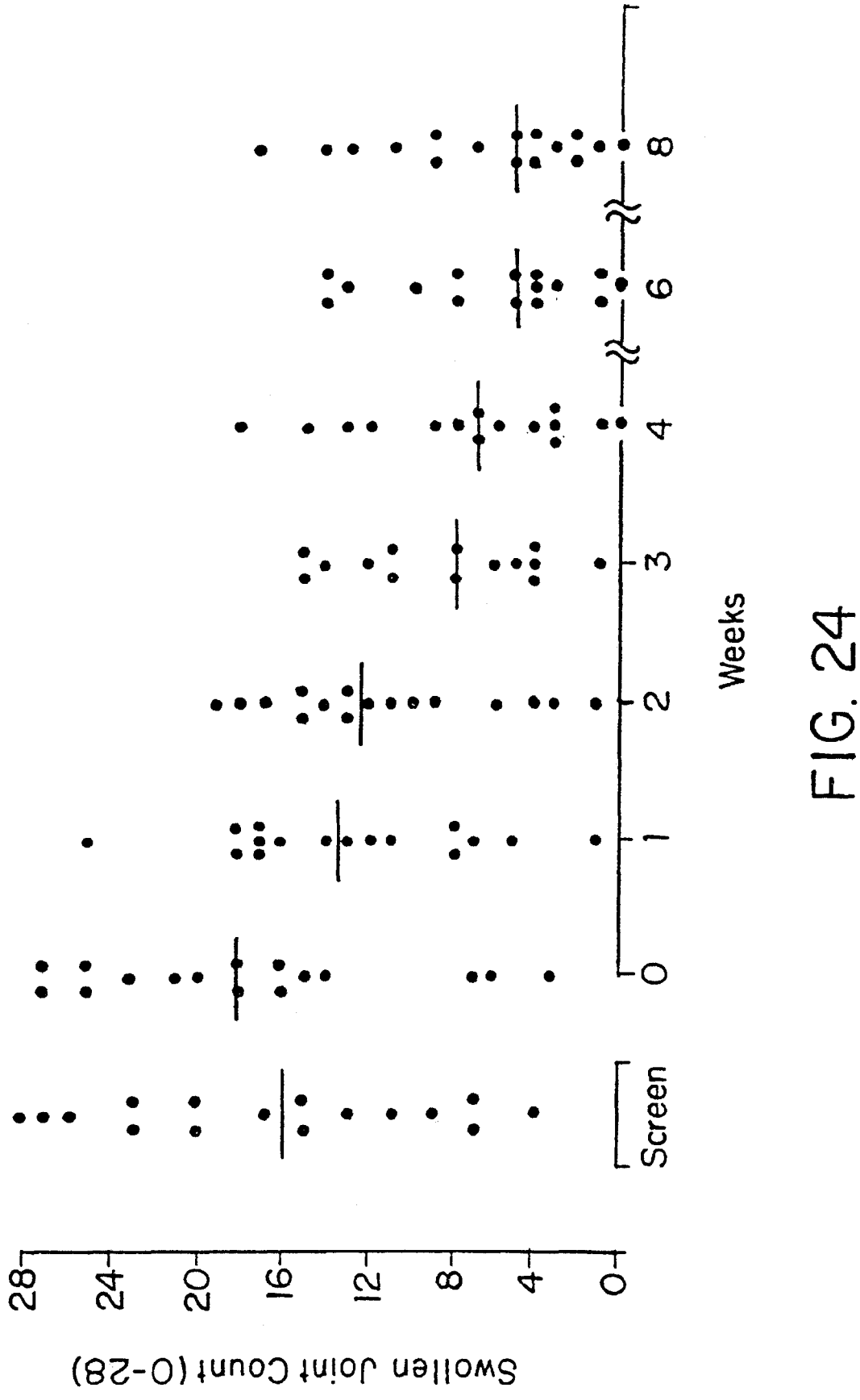

Swollen Joint Count (0-28)
28
24
20
16
12
8
4
0
Screen
0
1
2
3
4
6
8
Weeks

FIG. 24

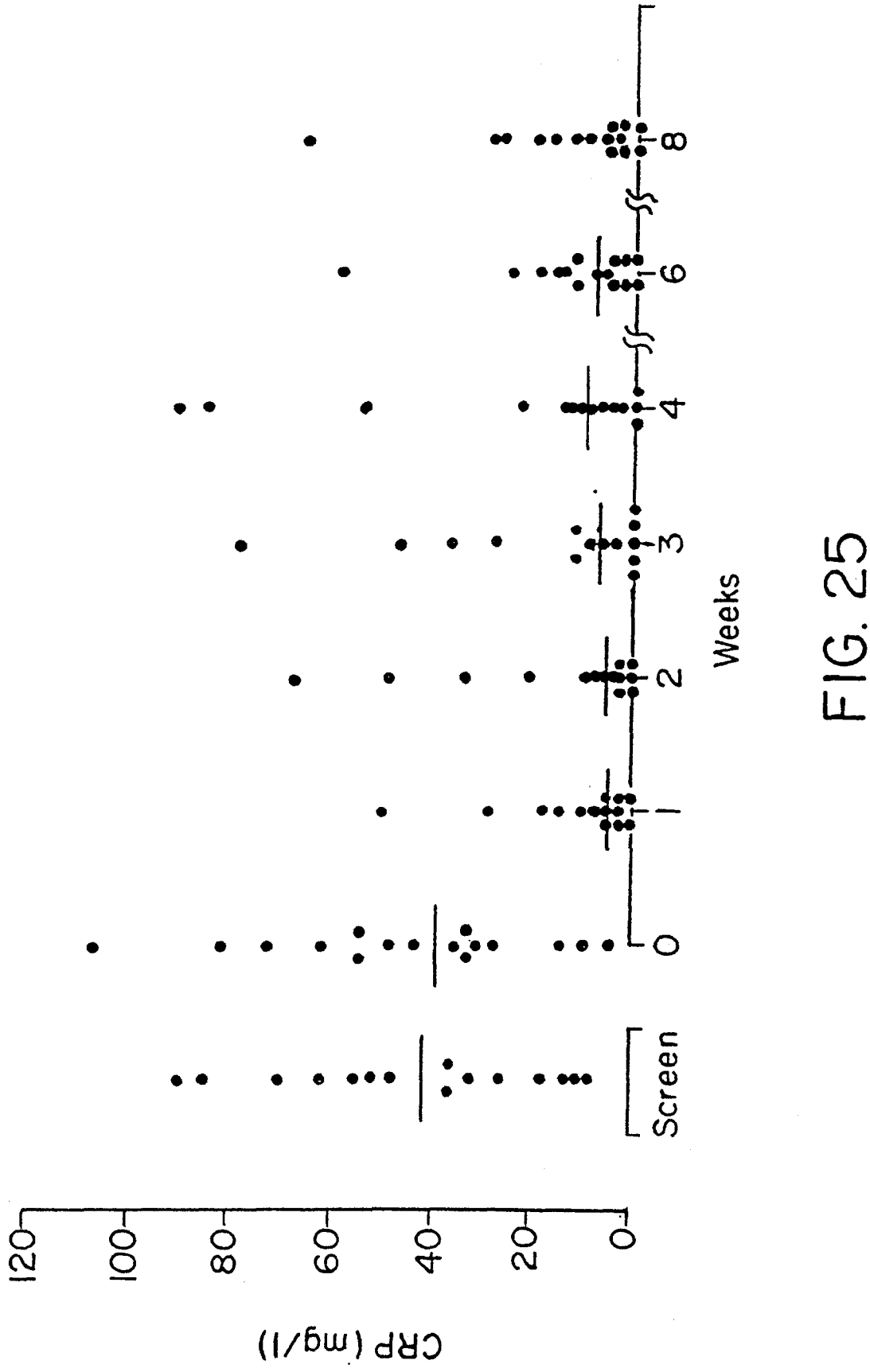
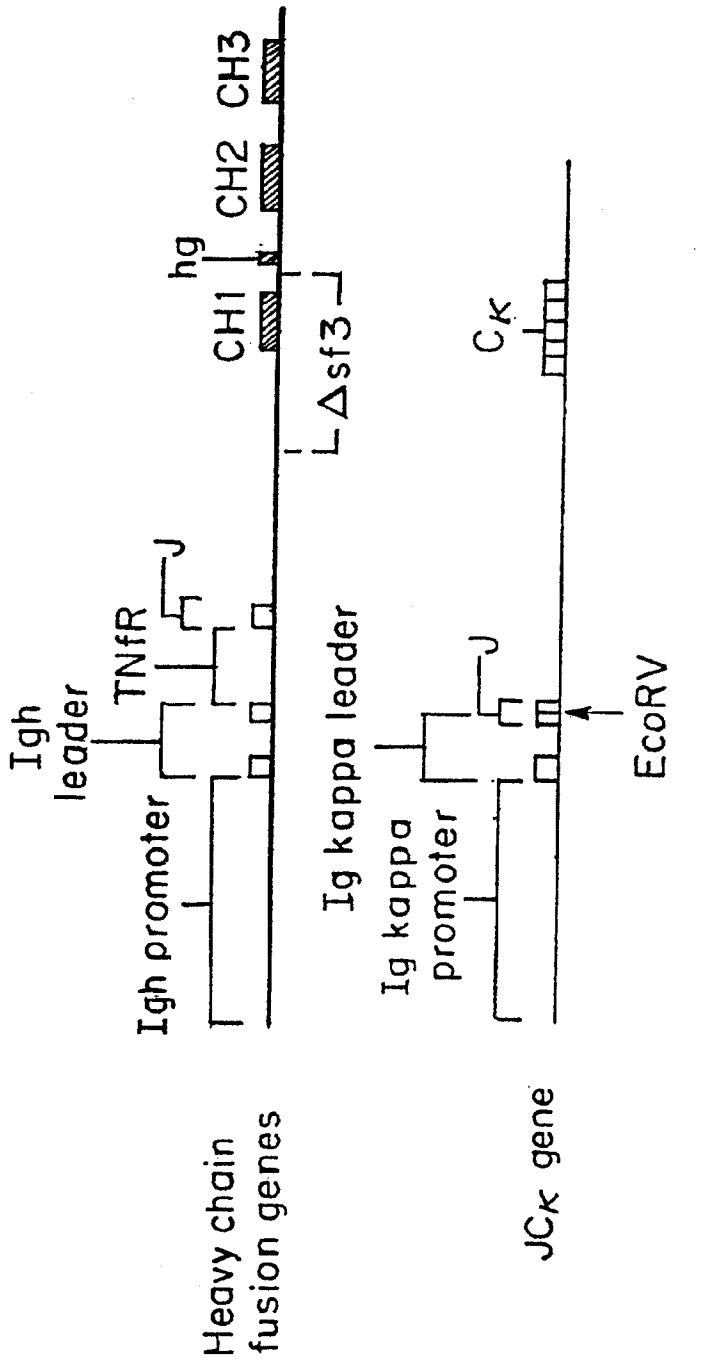


FIG. 25

FIG. 26A


Heavy chain fusion genes
Igh promoter
Igh leader
TNfR
J
hg
CH1
CH2
CH3
Δsf3
Ig kappa promoter
Ig kappa leader
J
Cκ
EcoRV
JCκ gene

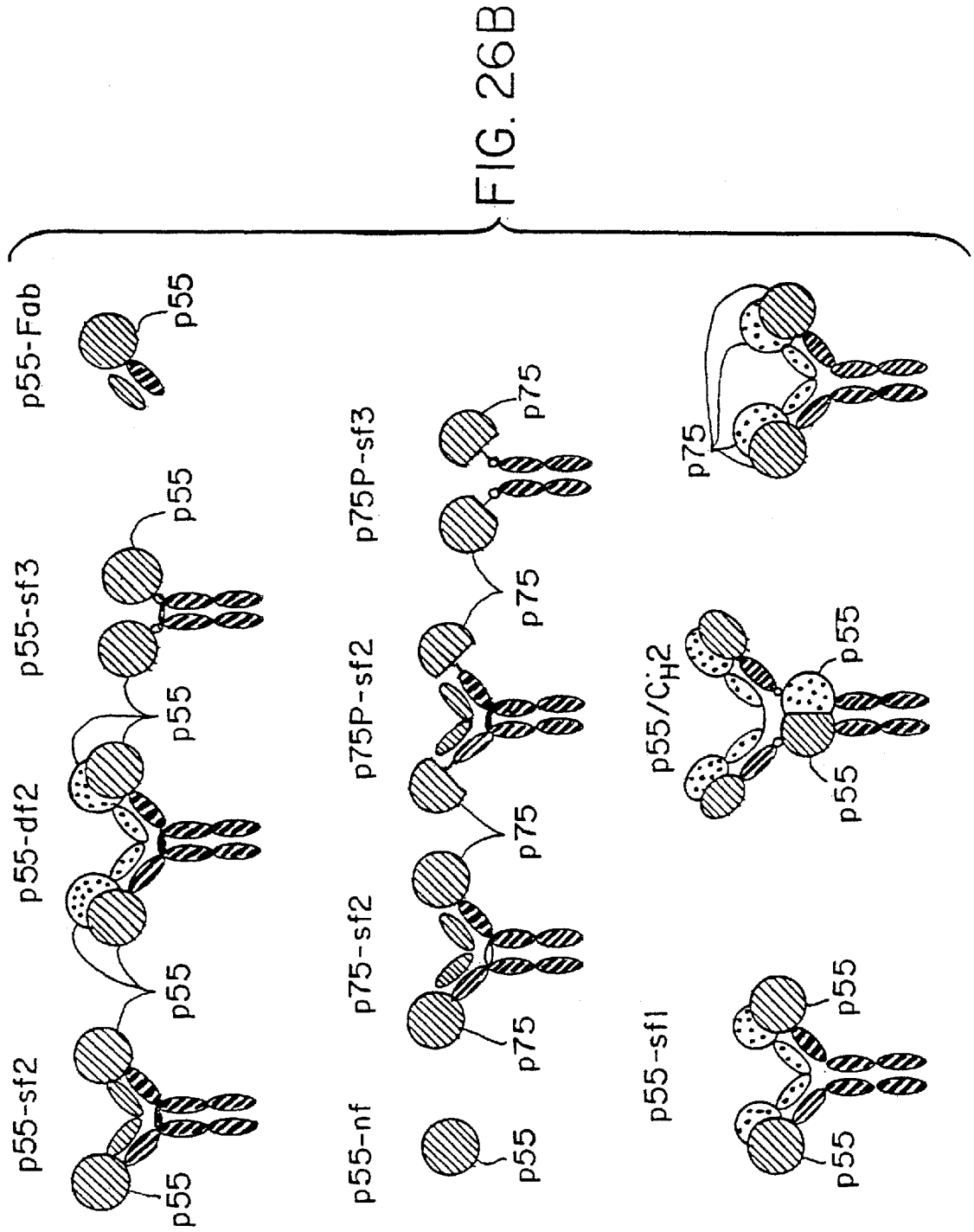
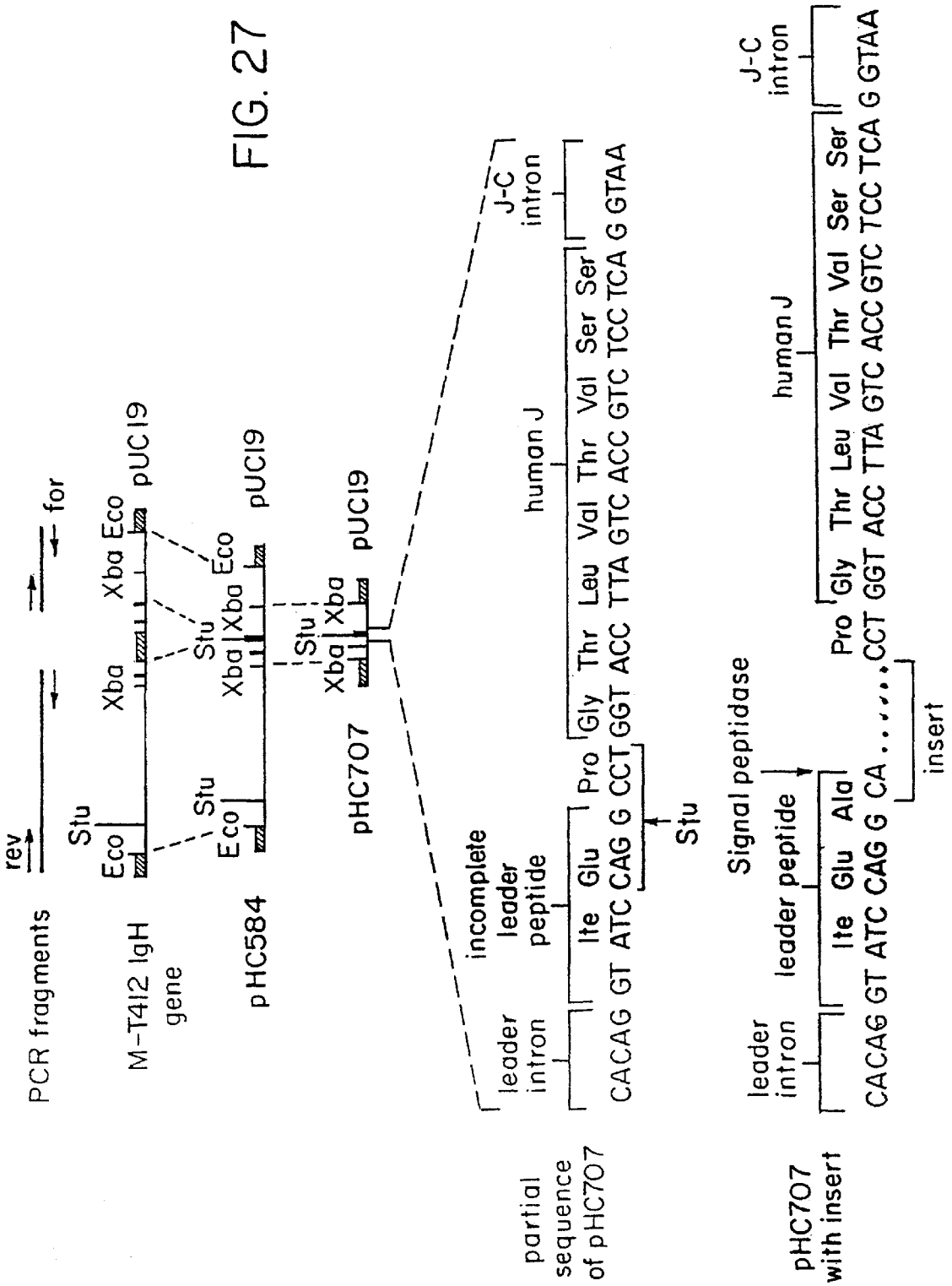

p55-Fab
p55
p55-sf3
p55
p55
p55-df2
p55
p55-sf2
p55
p75P-sf3
p75
p75
p75P-sf2
p75
p75-sf2
p75
p75
p55-nf
p55
p75
p55/C$_{H2}$
p55
p55
p55-sf1
p55
p55

FIG. 26B

FIG. 27


PCR fragments
rev
for
Stu
Eco
Xba
Xba Eco
pUC19
M-T412 IgH gene
Stu
Eco
Xba
Xba Eco
pUC19
pHC584
Stu
Xba
Xba
pUC19
pHC707
J-C intron
human J
incomplete leader peptide
leader intron
Ile Glu Pro Gly Thr Leu Val Thr Val Ser Ser
CACAG GT ATC CAG G CCT GGT ACC TTA GTC ACC GTC TCC TCA G GTAA
Stu
partial sequence of pHC707
Signal peptidase
leader peptide
Ile Glu Ala Pro Gly Thr Leu Val Thr Val Ser Ser
CACAG GT ATC CAG G CA ..... CCT GGT ACC TTA GTC ACC GTC TCC TCA G GTAA
insert
leader intron
human J
J-C intron
pHC707 with insert

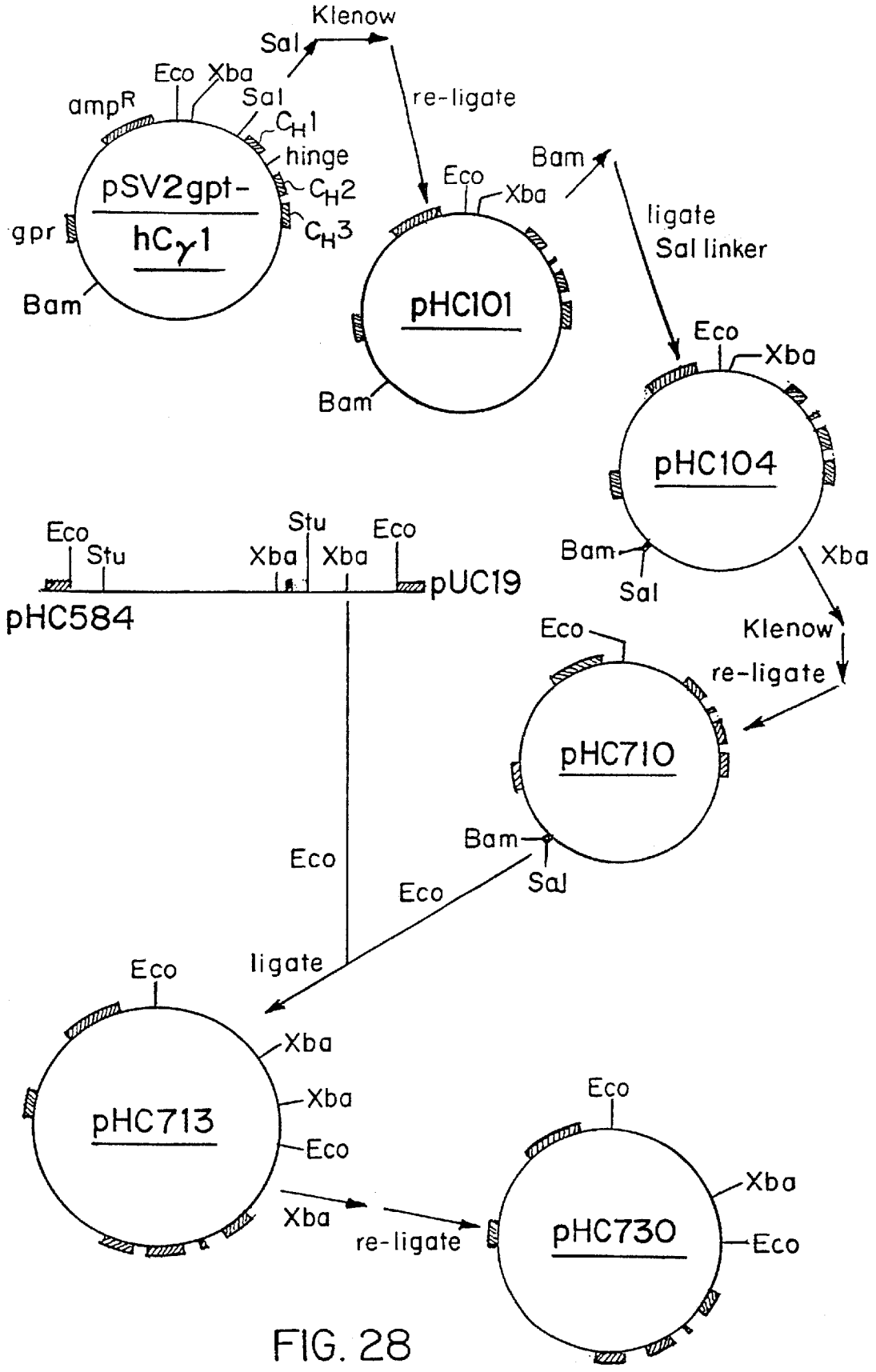

Klenow
Sal
Eco Xba
Sal
ampR
C_H1
hinge
C_H2
C_H3
pSV2gpt-
hCγ1
gpr
Bam
re-ligate
Eco
Xba
pHC101
Bam
Bam
ligate
Sal linker
Eco
Xba
pHC104
Bam
Sal
Xba
Eco
Stu
Xba
Xba
Eco
pUC19
pHC584
Eco
Klenow
re-ligate
pHC710
Bam
Sal
Eco
Eco
ligate
Eco
Xba
Xba
Eco
pHC713
Xba
re-ligate
Eco
Xba
Eco
pHC730

FIG. 28

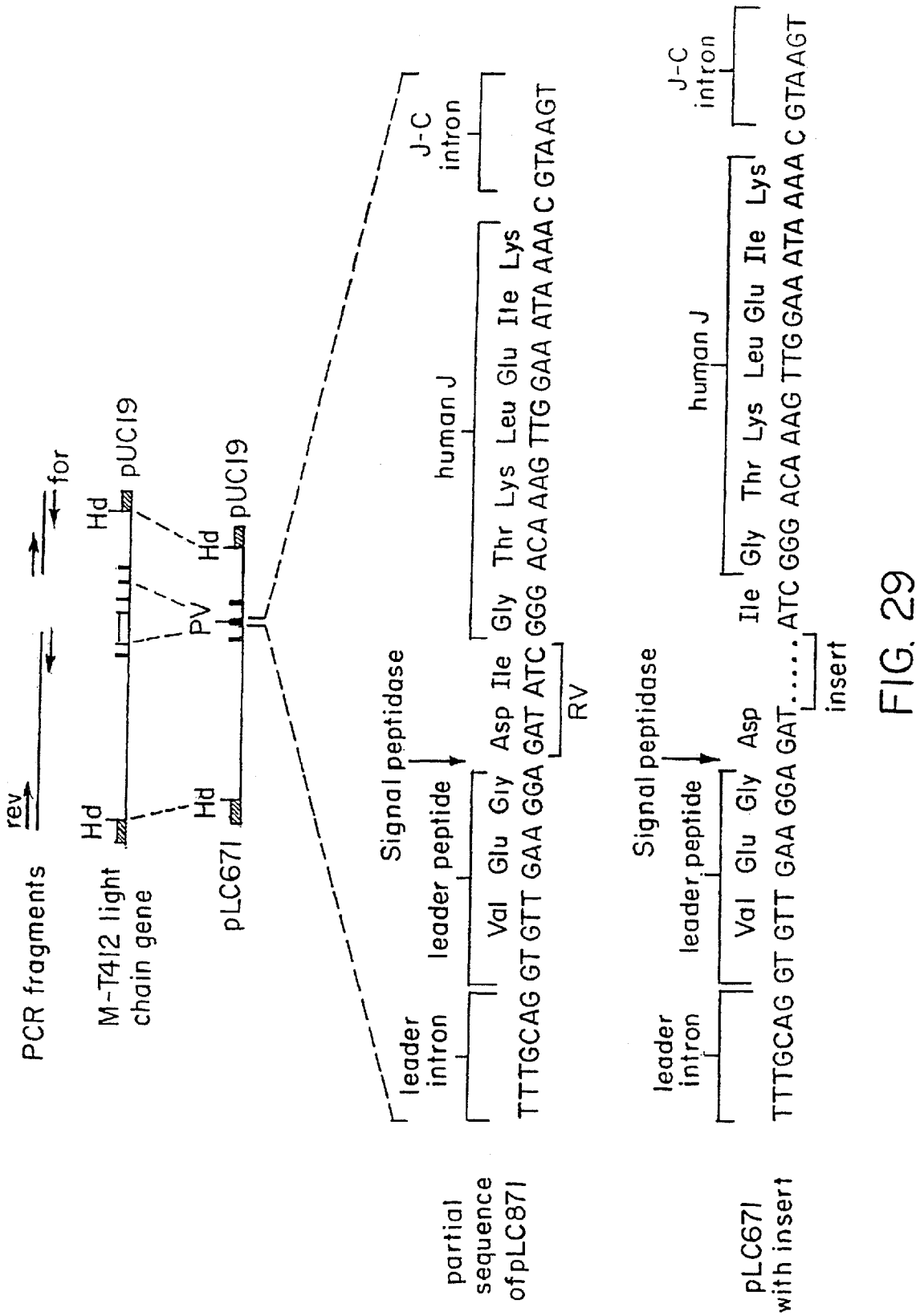

PCR fragments
rev
for
M-T412 light chain gene
Hd
Hd
pUC19
PV
Hd
pUC19
pLC671
partial sequence of pLC871
leader intron
Signal peptidase
leader peptide
human J
J-C intron
TTTGCAG GT GTT GAA GGA GAT ATC GGG ACA AAG TTG GAA ATA AAA C GTAAGT
Val Glu Gly Asp Ile Gly Thr Lys Leu Glu Ile Lys
RV
pLC671 with insert
leader intron
Signal peptidase
leader peptide
human J
J-C intron
TTTGCAG GT GTT GAA GGA GAT.....ATC GGG ACA AAG TTG GAA ATA AAA C GTAAGT
Val Glu Gly Asp Ile Gly Thr Lys Leu Glu Ile Lys
insert

FIG. 29

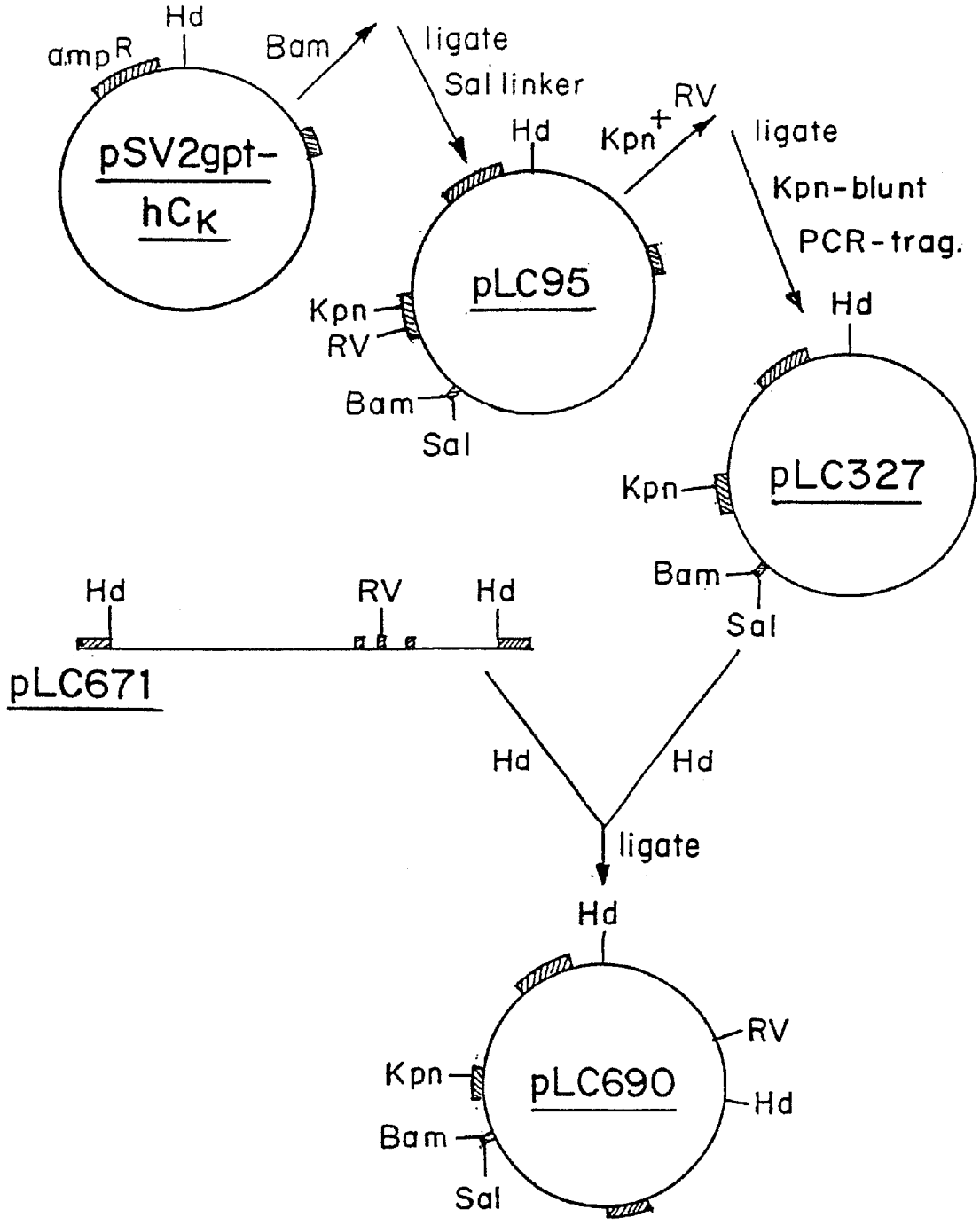

Hd
ampR
Bam
ligate
Sal linker
pSV2gpt-
hCK
Hd
Kpn+
RV
ligate
Kpn-blunt
PCR-frag.
pLC95
Kpn
RV
Bam
Sal
Hd
pLC327
Kpn
Bam
Sal
Hd
RV
Hd
pLC671
Hd
Hd
ligate
Hd
RV
Kpn
pLC690
Hd
Bam
Sal

FIG. 30

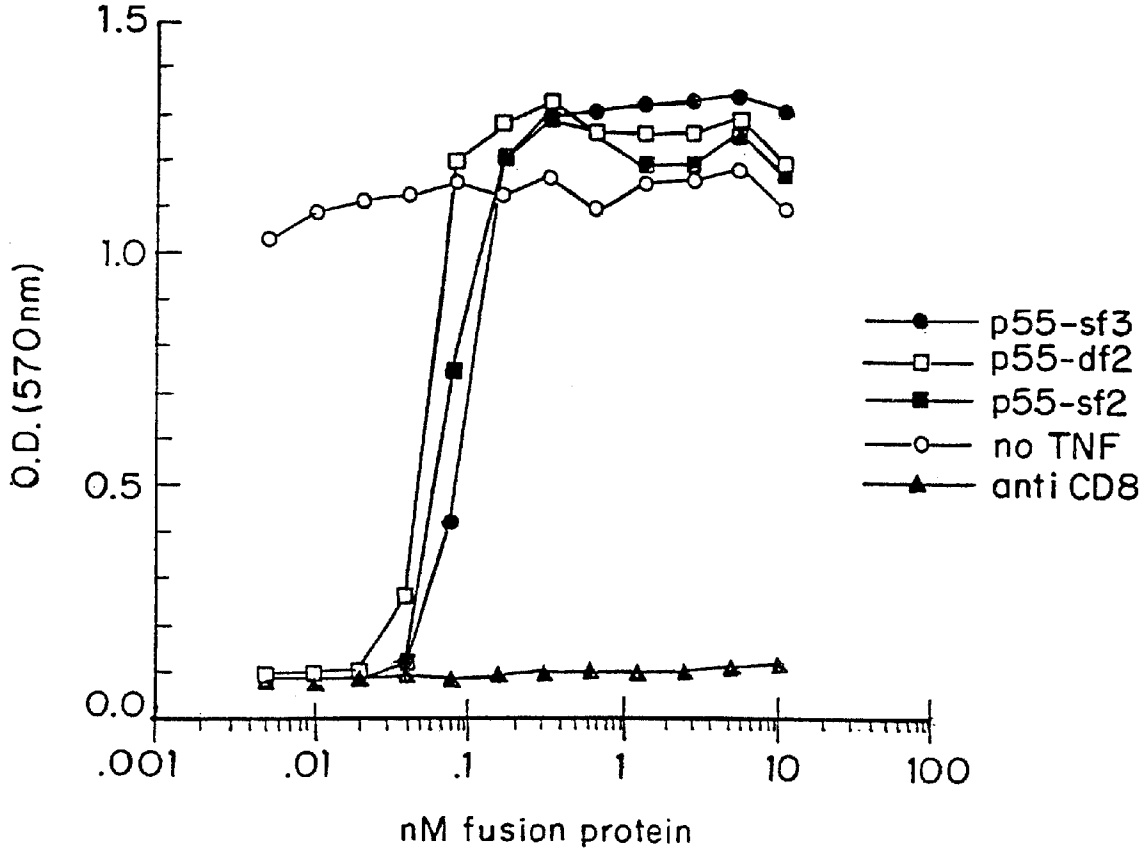

1.5
1.0
0.5
0.0
O.D.(570nm)
.001
.01
.1
1
10
100
nM fusion protein
p55-sf3
p55-df2
p55-sf2
no TNF
anti CD8

FIG. 31A

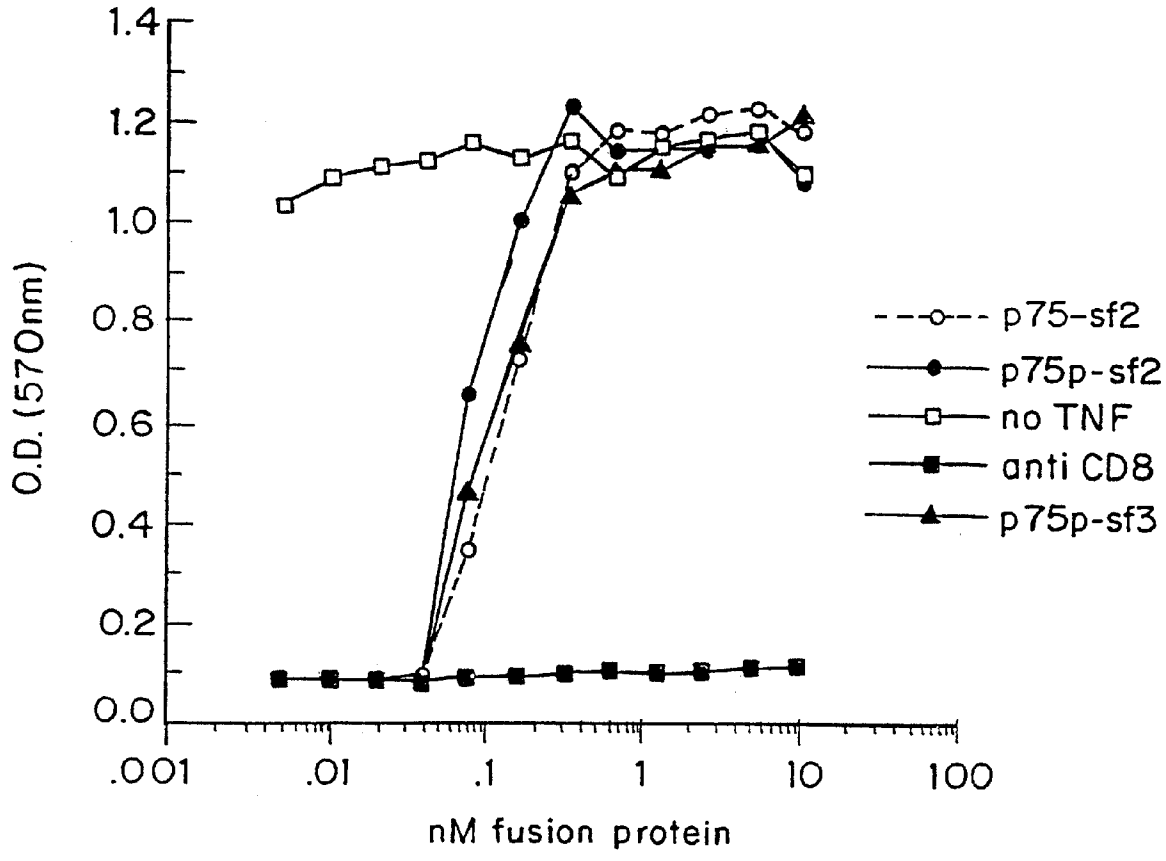

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
O.D. (570nm)
.001
.01
.1
1
10
100
nM fusion protein
p75-sf2
p75p-sf2
no TNF
anti CD8
p75p-sf3

FIG. 31B

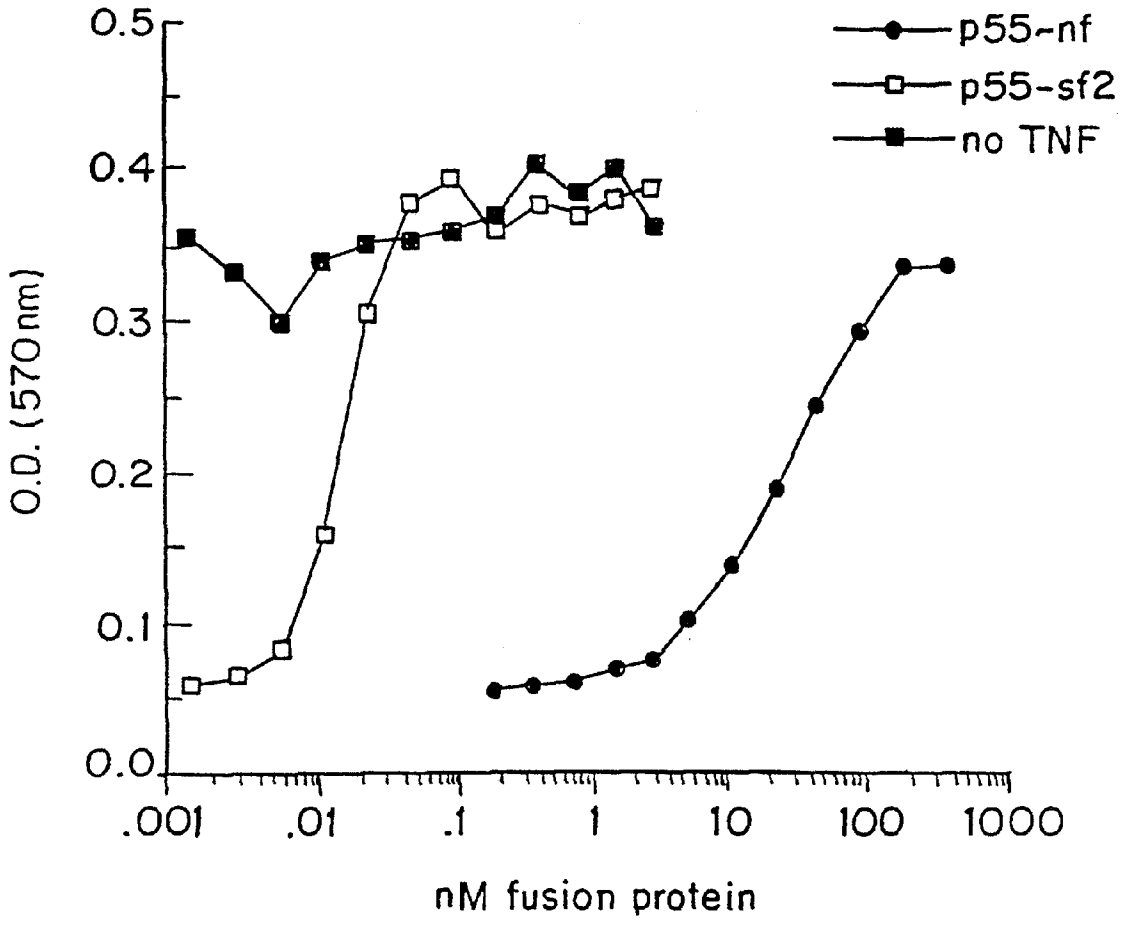

0.5
0.4
0.3
0.2
0.1
0.0
O.D. (570nm)
.001
.01
.1
1
10
100
1000
nM fusion protein
p55-nf
p55-sf2
no TNF

FIG. 31C

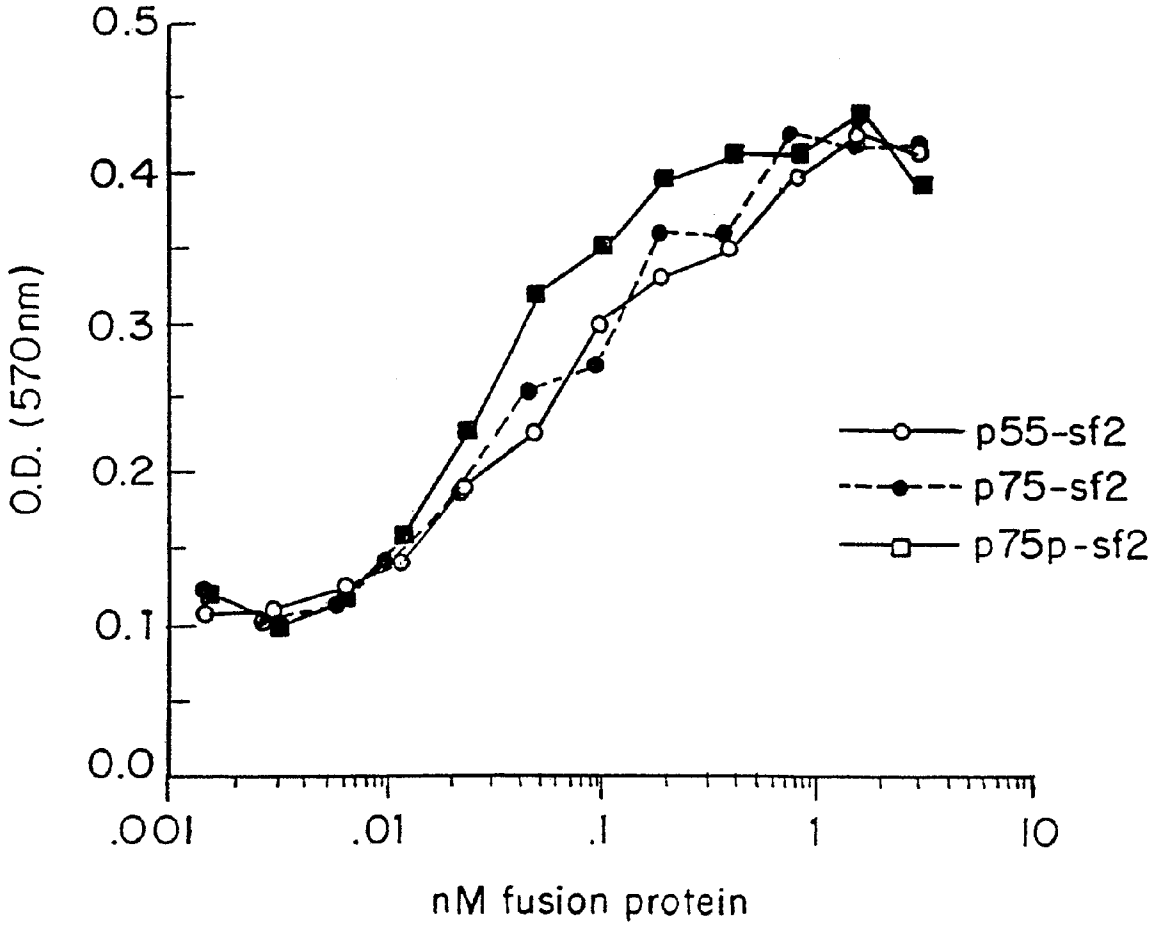

0.5
0.4
0.3
0.2
0.1
0.0
O.D. (570nm)
.001
.01
.1
1
10
nM fusion protein
p55-sf2
p75-sf2
p75p-sf2

FIG. 32

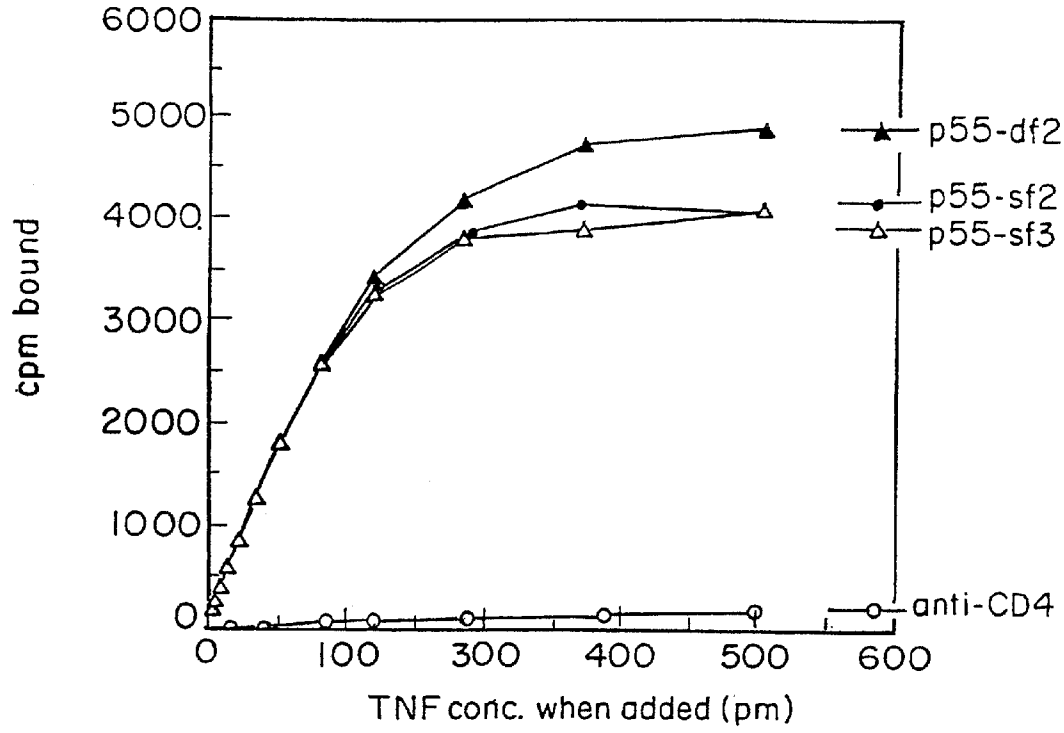


FIG. 33A

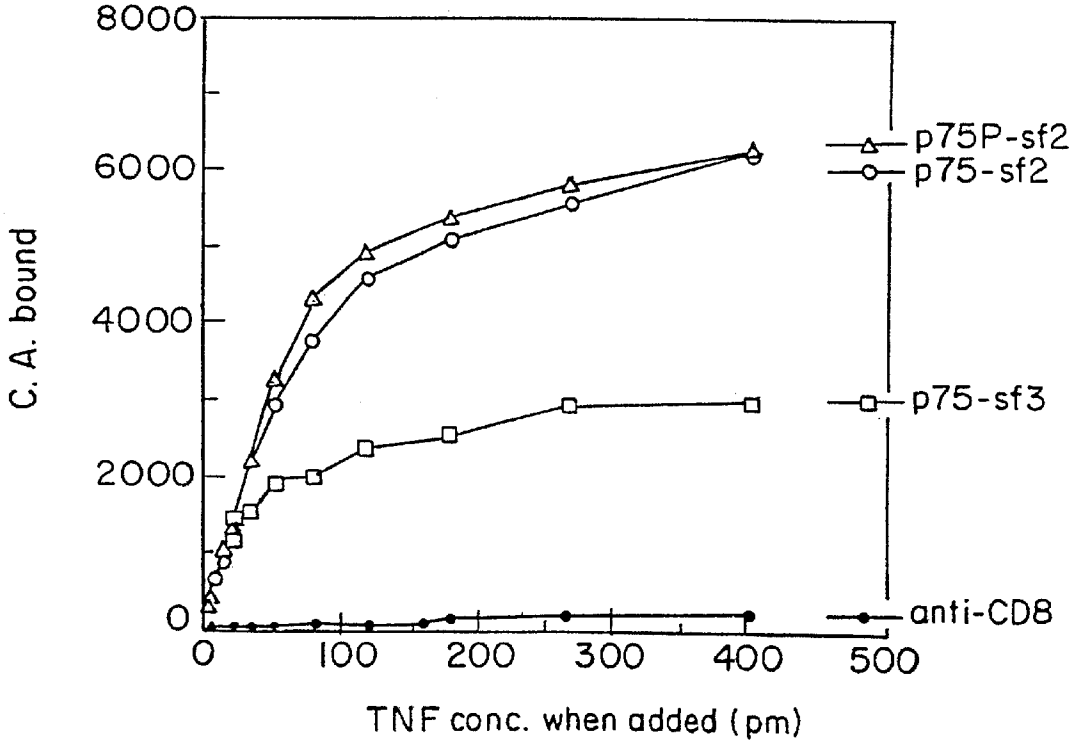


FIG. 33B

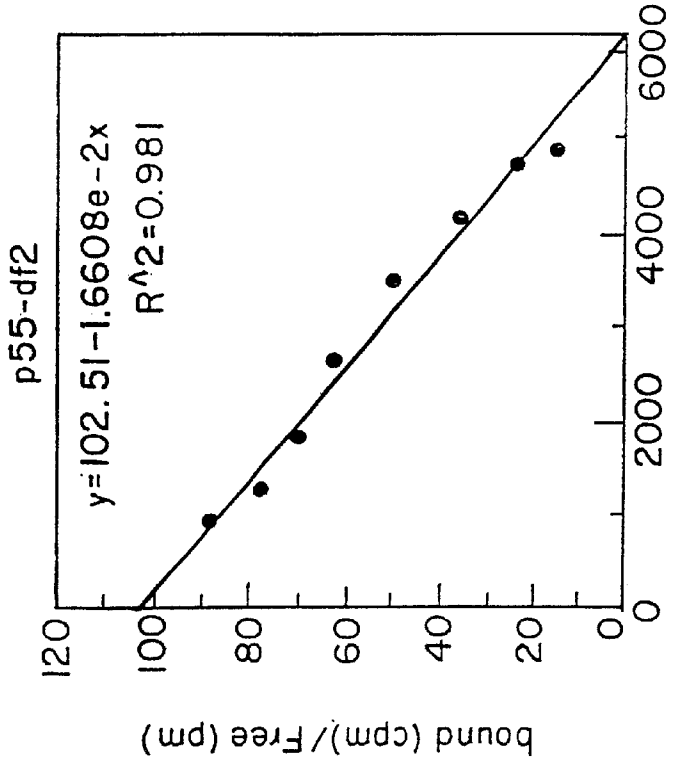


FIG. 33D

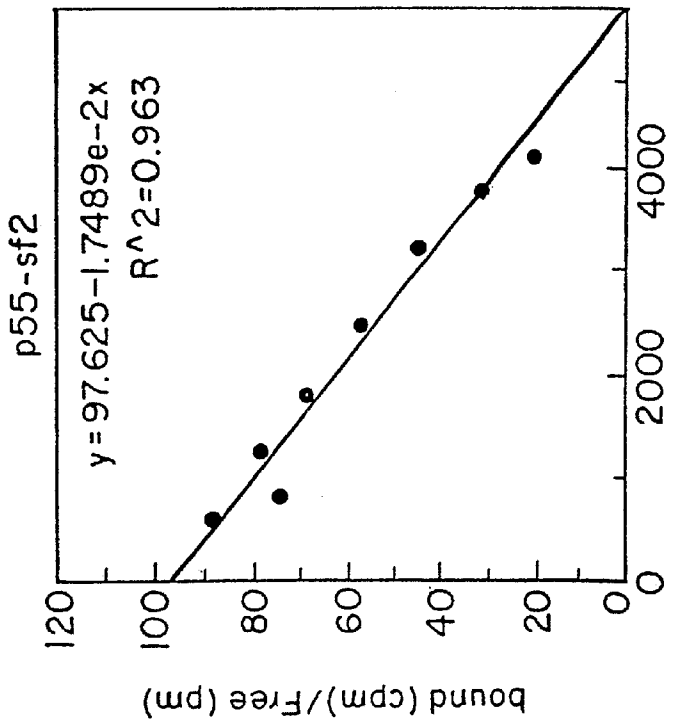


FIG. 33C

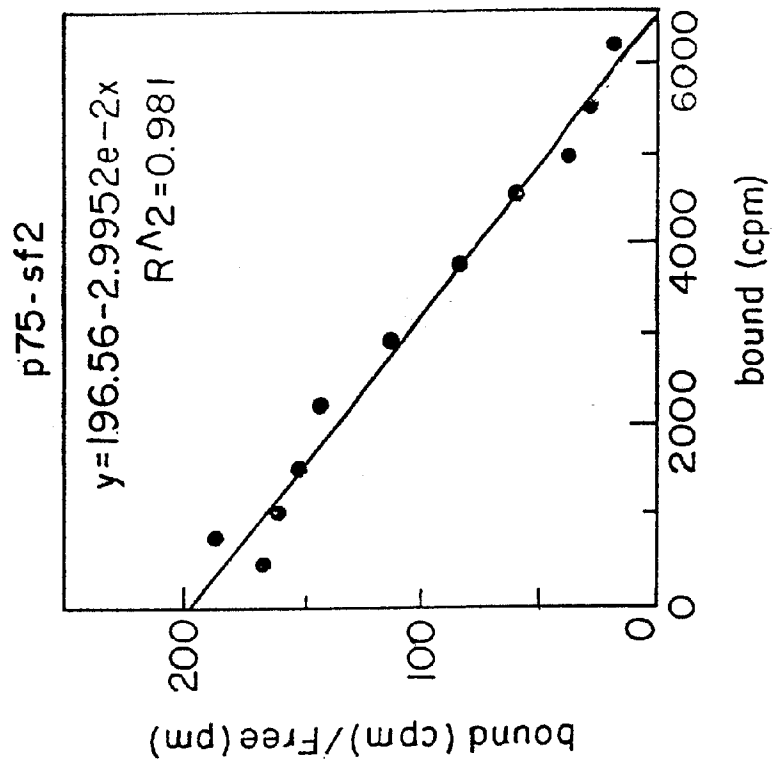


FIG. 33F

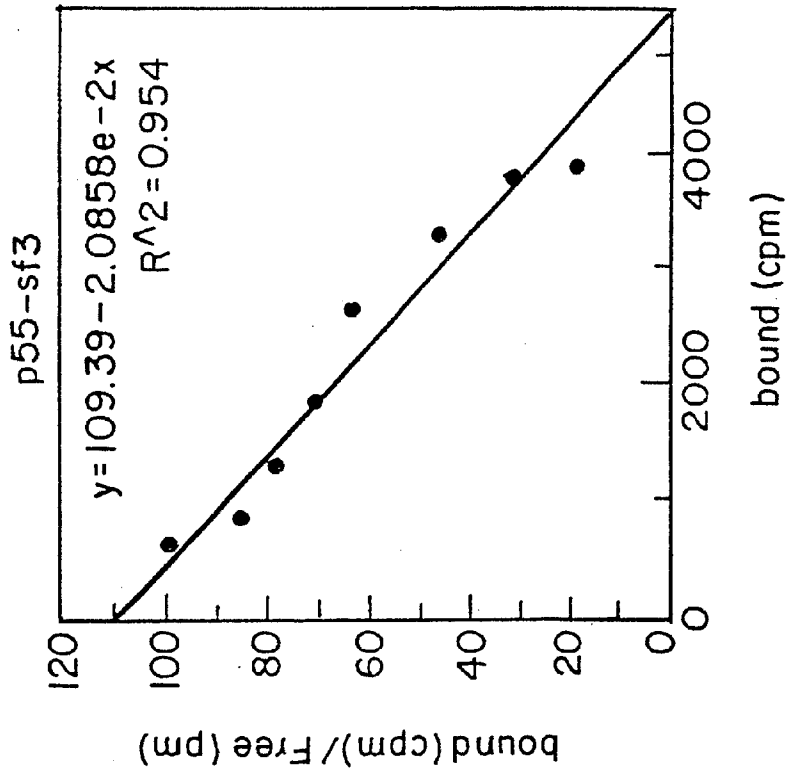


FIG. 33E

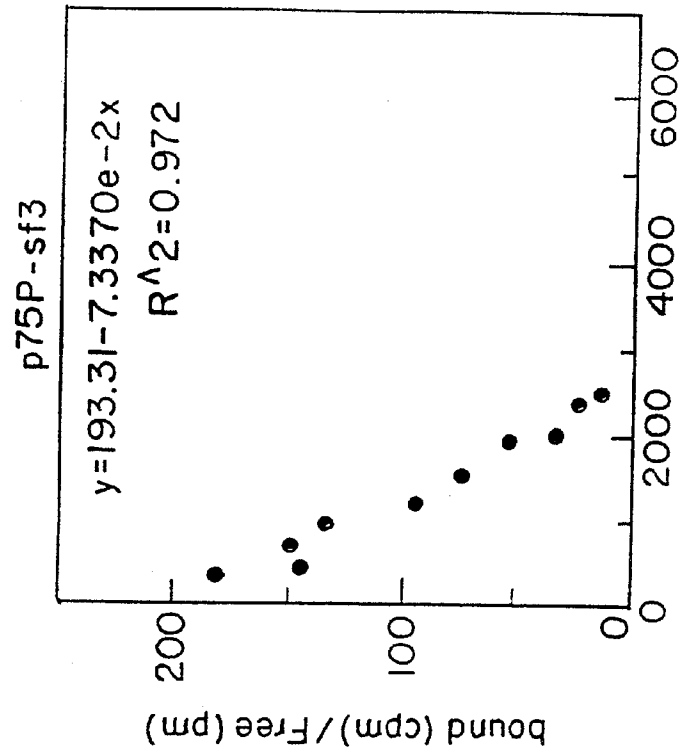


FIG. 33H

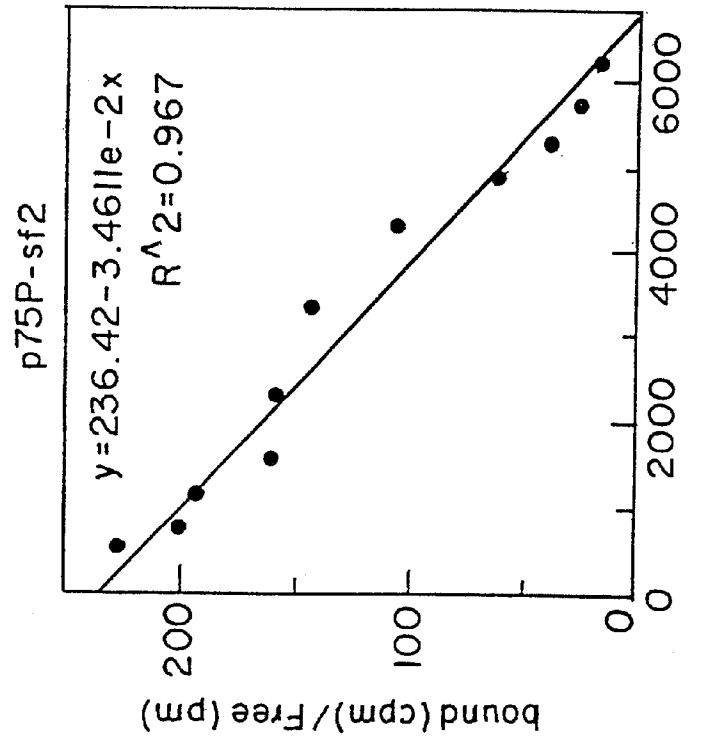


FIG. 33G

ANTI-TNFA ANTIBODIES AND ASSAYS EMPLOYING ANTI-TNFA ANTIBODIES

This application is a continuation-in-part of each of U.S. application Ser. No. 08/010,406, filed Jan. 29, 1993, now abandoned; and CIP of U.S. application Ser. No. 08/013,413, filed Feb. 2, 1993, now abandoned, which is a continuation-in-part of U.S. application Ser. No. 07/943,852, filed Sep. 11, 1992, now abandoned, which is a continuation-in-part of U.S. application Ser. No. 07/853,606, filed Mar. 18, 1992, now abandoned, which is a continuation-in-part of U.S. application Ser. No. 07/670,827, filed Mar. 18, 1991, now abandoned. Each of the above non-abandoned applications are entirely incorporated herein by reference.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention in the field of immunology and medicine relates to anti-human tumor necrosis factor-alpha (hTNFα) antibodies and peptides and nucleic acids encoding therefor, and to pharmaceutical and diagnostic compositions and production, diagnostic and therapeutic methods thereof, and to methods for treating TNF-mediated pathologies.

2. Description of the Background Art

Tumor Necrosis Factor: Monocytes and macrophages secrete cytokines known as tumor necrosis factor-α (TNFα) and tumor necrosis factor-β (TNFβ) in response to endotoxin or other stimuli. TNFα is a soluble homotrimer of 17 kD protein subunits (Smith, et al., *J. Biol. Chem.* 262:6951–6954 (1987)). A membrane-bound 26 kD precursor form of TNF also exists (Kriegler, et al., *Cell* 53:45–53 (1988)). For reviews of TNF, see Beutler, et al., *Nature* 320:584 (1986), *Old, Science* 230:630 (1986), and Le, et al., *Lab. Invest.* 56:234 (1987).

Cells other than monocytes or macrophages also make TNFα. For example, human non-monocytic tumor cell lines produce TNF (Rubin, et al., *J. Exp. Med.* 164:1350 (1986); Spriggs, et al., *Proc. Natl. Acad. Sci. USA* 84:6563 (1987)). $CD4^+$ and $CD8^+$ peripheral blood T lymphocytes and some cultured T and B cell lines (Cuturi, et al., *J. Exp. Med.* 165:1581 (1987); Sung, et al., *J. Exp. Med.* 168:1539 (1988)) also produce TNFα.

TNF causes pro-inflammatory actions which result in tissue injury, such as inducing procoagulant activity on vascular endothelial cells (Pober, et al., *J. Immunol.* 136:1680 (1986)), increasing the adherence of neutrophils and lymphocytes (Pober, et al., *J. Immunol.* 138:3319 (1987)), and stimulating the release of platelet activating factor from macrophages, neutrophils and vascular endothelial cells (Camussi, et al., *J. Exp. Med.* 166:1390 (1987)).

Recent evidence associates TNF with infections (Cerami, et al., *Immunol. Today* 9:28 (1988)), immune disorders, neoplastic pathologies (Oliff, et al., *Cell* 50:555 (1987)), autoimmune pathologies and graft-versus host pathologies (Piguet, et al., *J. Exp. Med.* 166:1280 (1987)). The association of TNF with cancer and infectious pathologies is often related to the host's catabolic state. Cancer patients suffer from weight loss, usually associated with anorexia.

The extensive wasting which is associated with cancer, and other diseases, is known as "cachexia" (Kern, et al. (*J. Parent. Enter. Nutr.* 12:286–298 (1988)). Cachexia includes progressive weight loss, anorexia, and persistent erosion of body mass in response to a malignant growth. The fundamental physiological derangement can relate to a decline in food intake relative to energy expenditure. The cachectic state causes most cancer morbidity and mortality. TNF can mediate cachexia in cancer, infectious pathology, and other catabolic states.

TNF also plays a central role in gram-negative sepsis and endotoxic shock (Michie, et al., *Br. J. Surg.* 76:670–671 (1989); Debets, et al., *Second Vienna Shock Forum*, p. 463–466 (1989); Simpson, et al., *Crit. Care Clin.* 5:27–47 (1989)), including fever, malaise, anorexia, and cachexia. Endotoxin strongly activates monocyte/macrophage production and secretion of TNF and other cytokines (Kornbluth, et al., *J. Immunol.* 137:2585–2591 (1986)). TNF and other monocyte-derived cytokines mediate the metabolic and neurohormonal responses to endotoxin (Michie, et al., *New. Engl. J. Med.* 318:1481–1486 (1988)). Endotoxin administration to human volunteers produces acute illness with flu-like symptoms including fever, tachycardia, increased metabolic rate and stress hormone release (Revhaug, et al., *Arch. Surg.* 123:162–170 (1988)). Circulating TNF increases in patients suffering from Gram-negative sepsis (Waage, et al., *Lancet* 1:355–357 (1987); Hammerle, et al., *Second Vienna Shock Forum* p. 715–718 (1989); Debets, et al., *Crit. Care Med.* 17:489–497 (1989); Calandra, et al., *J. Infect. Dis.* 161:982–987 (1990)).

TNF Antibodies

Polyclonal murine antibodies to TNF are disclosed by Cerami et al. (EPO Patent Publication 0212489, Mar. 4, 1987). Such antibodies were said to be useful in diagnostic immunoassays and in therapy of shock in bacterial infections.

Rubin et al. (EPO Patent Publication 0218868, Apr. 22, 1987) discloses murine monoclonal antibodies to human TNF, the hybridomas secreting such antibodies, methods of producing such murine antibodies, and the use of such murine antibodies in immunoassay of TNF.

Yone et al. (EPO Patent Publication 0288088, Oct. 26, 1988) discloses anti-TNF murine antibodies, including mAbs, and their utility in immunoassay diagnosis of pathologies, in particular Kawasaki's pathology and bacterial infection. The body fluids of patients with Kawasaki's pathology (infantile acute febrile mucocutaneous lymph node syndrome; Kawasaki, *Allergy* 16:178 (1967); Kawasaki, *Shonica* (*Pediatrics*) 26:935 (1985)) were said to contain elevated TNF levels which were related to progress of the pathology (Yone et al., infra).

Other investigators have described rodent or murine mAbs specific for recombinant human TNF which had neutralizing activity in vitro (Liang, et al. (*Biochem. Biophys. Res. Comm.* 137:847–854 (1986); Meager, et al., *Hybridoma* 6:305–311 (1987); Fendly et al., *Hybridoma* 6:359–369 (1987); Bringman, et al., *Hybridoma* 6:489–507 (1987); Hirai, et al., *J. Immunol. Meth.* 96:57–62 (1987); Moller, et al. (*Cytokine* 2:162–169 (1990)). Some of these mAbs were used to map epitopes of human TNF and develop enzyme immunoassays (Fendly et al., infra; Hirai et al., infra; Moller et al., infra) and to assist in the purification of recombinant TNF (Bringman et al., infra). However, these studies do not provide a basis for producing TNF neutralizing antibodies that can be used for in vivo diagnostic or therapeutic uses in humans, due to immunogenicity, lack of specificity and/or pharmaceutical suitability.

Neutralizing antisera or mAbs to TNF have been shown in mammals other than man to abrogate adverse physiological changes and prevent death after lethal challenge in experimental endotoxemia and bacteremia. This effect has been demonstrated, e.g., in rodent lethality assays and in primate pathology model systems (Mathison, et al., *J. Clin.*

Invest. 81:1925–1937 (1988); Beutler, et al., *Science* 229:869–871 (1985); Tracey, et al., *Nature* 330:662–664 (1987); Shimamoto, et al., *Immunol. Lett.* 17:311–318 (1988); Silva, et al., *J. Infect. Dis.* 162:421–427 (1990); Opal, et al., *J. Infect. Dis.* 161:1148–1152 (1990); Hinshaw, et al., *Circ. Shock* 30:279–292 (1990)).

Putative receptor binding loci of hTNF has been disclosed by Eck and Sprang (*J. Biol. Chem.* 264(29), 17595–17605 (1989), who identified the receptor binding loci of TNF-α as consisting of amino acids 11–13, 37–42, 49–57 and 155–157.

PCT publication WO91/02078 (1991) discloses TNF ligands which can bind to monoclonal antibodies having the following epitopes: at least one of 1–20, 56–77, and 108–127; at least two of 1–20, 56–77, 108–127 and 138–149; all of 1–18, 58–65, 115–125 and 138–149; all of 1–18, and 108–128; all of 56–79, 110–127 and 135– or 136–155; all of 1–30, 117–128 and 141–153; all of 1–26, 117–128 and 141–153; all of 22–40, 49–96 or 49–97, 110–127 and 136–153; all of 12–22, 36–45, 96–105 and 132–157; both of 1–20 and 76–90; all of 22–40, 69–97, 105–128 and 135–155; all of 22–31 and 146–157; all of 22–40 and 49–98; at least one of 22–40, 49–98 and 69–97, both of 22–40 and 70–87.

To date, experience with anti-TNF murine mAb therapy in humans has been limited. In a phase I study, fourteen patients with severe septic shock were administered a murine anti-TNF mAb in a single dose from 0.4–10 mg/kg (Exley, A. R. et al., *Lancet* 335:1275–1277 (1990)). However, seven of the fourteen patients developed a human anti-murine antibody response to the treatment, which treatment suffers from the known problems due to immuriogenicity from the use of murine heavy and light chain portions of the antibody. Such immunogenicity causes decreased effectiveness of continued administration and can render treatment ineffective, in patients undergoing diagnostic or therapeutic administration of murine anti-TNF antibodies.

Administration of murine TNF mAb to patients suffering from severe graft versus host pathology has also been reported (Herve, et al., *Lymphoma Res.* 9:591 (1990)).

TNF Receptors

The numerous biological effects of TNFα and the closely related cytokine, TNFβ (lymphotoxin), are mediated by two TNF transmembrane receptors, both of which have been cloned. The p55 receptor (also termed TNF-R55, TNF-RI, or TNFRβ) is a 55 kd glycoprotein shown to transduce signals resulting in cytotoxic, anti-viral, and proliferative activities of TNFα.

The p75 receptor (also termed TNF-R75, TNF-RII, or TNFRα) is a 75 kDa glycoprotein that has also been shown to transduce cytotoxic and proliferative signals as well as signals resulting in the secretion of GM-CSF. The extracellular domains of the two receptors have 28% homology and have in common a set of four subdomains defined by numerous conserved cysteine residues. The p75 receptor differs, however, by having a region adjacent to the transmembrane domain that is rich in proline residues and contains sites for O-linked glycosylation. Interestingly, the cytoplasmic domains of the two receptors share no apparent homology which is consistent with observations that they can transduce different signals to the interior of the cell.

TNFα inhibiting proteins have been detected in normal human urine and in serum of patients with cancer or endotoxemia. These have since been shown to be the extracellular domains of TNF receptors derived by proteolytic cleavage of the transmembrane forms. Many of the same stimuli that result in TNFα release also result in the release of the soluble receptors, suggesting that these soluble TNFα inhibitors can serve as part of a negative feedback mechanism to control TNFα activity.

Aderka, et al., *Isrl. J. Med. Sci.* 28:126–130 (1992) discloses soluble forms of TNF receptors (sTNF-Rs) which specifically bind TNF and thus can compete with cell surface TNF receptors to bind TNF (Seckinger, et al., *J. Exp. Med.* 167:1511–1516 (1988); Engelmann, et al., *J. Biol. Chem.* 264:11974–11980 (1989)).

Loetscher, et al., *Cell* 61:351–359 (Apr. 20, 1990) discloses the cloning and expression of human 55 kd TNF receptor with the partial amino acid sequence, complete cDNA sequence and predicted amino acid sequence.

Schall et al., *Cell* 61:361–370 (Apr. 20, 1990), discloses molecular cloning and expression of a receptor for human TNF wherein an isolated cDNA clone including a receptor as a 415 amino acid protein with an apparent molecular weight of 28 kDa, as well as the cDNA sequence and predicted amino acid sequence.

Nophar, et al., *EMBO J.* 9(10):3269–3278 (1990) discloses soluble forms of TNF receptor and that the cDNA for type I TNF-R encodes both the cell surface and soluble forms of the receptor. The cDNA and predicted amino acid sequences are disclosed.

Engelmann, et al., *J. Biol. Chem.* 265(3):1531–1536 (1990), discloses TNF-binding proteins, purified from human urine, both having an approximate molecular weight of 30 kDa and binding TNF-α more effectively than TNF-β. Sequence data is not disclosed. See also Engelmann, et al., *J. Biol. Chem.* 264(20):11974–11980 (1989).

European Patent publication number 0 433 900 A1, published Jun. 26, 1991, owned by YEDA Research and Develoopemtn Co., Ltd., Wallach, et al., discloses TNF binding protein I (TBP-I), derivatives and analogs thereof, produced expression of a DNA encoding the entire human type I TNF receptor, or a soluble domain thereof.

PCT publication number WO 92/13095, published Aug. 6, 1992, owned by Synergen, Carmichael et al., discloses methods for treating tumor necrosis factor mediated diseases by administration of a therapeutically effective amount of a TNF inhibitor selected from a 30 kDa TNF inhibitor and a 40 kDa TNF inhibitor selected from the full length 40 kDa TNF inhibitor or modifications thereof.

European Patent Publication number 0 526 905 A2, published Oct. 2, 1993, owned by YEDA Research one Development Company, Ltd., Wallach et al., discloses multimers of the soluble forms of TNF receptors produced by either chemical or recombinant methods which are useful for protecting mammals from the diliterious effects of TNF, which include portions of the hp55 TNF-receptor.

PCT publication WO 92/07076, published Apr. 30, 1992, owned by Charring Cross Sunley Research Center, Feldman et al., discloses modified human TNFα receptor which consists of the first three cysteine-rich subdomain but lacks the fourth Cysteine-rich subdomain of the extracellular binding domain of the 55 kDa or 75 kDa TNF receptor for human TNF α, or an amino acid sequence having a homology of 90% or more with the TNF receptor sequences.

European Patent Publication 0 412 486 A1, published Feb. 13, 1991, owned by YEDA Research and Development Co., Ltd., Wallach et al., discloses antibodies to TNF binding protein I (TBP-I), and fragments thereof, which can be used as diagnostic assays or pharmaceutical agents, either inhibiting or mimicking the effects of TNF on cells.

European Patent Publication number 0 398 327 A1, published Nov. 22, 1990, owned by YEDA Research and Development Co., Ltd., Wallach et al., discloses TNF bind-

ing protein (TBP) isolated and purified having inhibitory activity on the cytotoxic effect of TNF, as well as TNF binding protein II and salts, functional derivatives precursors and active fractions thereof, as well as polyclonal and monoclonal antibodies to TNF binding protein II.

European Patent Publication 0 308 378 A2, published Mar. 22, 1989, owned by YEDA Research and Development Co., Ltd., Wallach, et al., discloses TNF inhibitory protein isolated and substantially purified, having activity to inhibit the binding of TNF to TNF receptors and to inhibit the cytotoxicity of TNF. Additionally disclosed are TNF inhibitory protein, salts, functional derivatives and active fractions thereof, used to antagonize the diliterious effects of TNF.

Accordingly, there is a need to provide novel TNF antibodies or peptides which overcome the problems of murine antibody immunogenicity and which provide reduced immunogenicity and increased neutralization activity.

Citation of documents herein is not intended as an admission that any of the documents cited herein is pertinent prior art, or an admission that the cited documents is considered material to the patentability of any of the claims of the present application. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicant and does not constitute any admission as to the correctness of the dates or contents of these documents.

SUMMARY OF THE INVENTION

It is object of the present invention to overcome one or more deficiencies of the background art.

It is also an object of the present invention to provide methods having utility for in vitro, in situ and/or in vivo diagnosis and/or treatment of animal cells, tissues or pathologies associated with the presence of tumor necrosis factor (TNF), using anti-TNF antibodies and/or anti-TNF peptides.

Anti-TNF antibodies (Abs) are intended to include at least one of monoclonal rodent-human chimeric antibodies, rodent antibodies, human antibodies or any portions thereof, having at least one antigen binding region of an immunoglobulin variable region, which antibody binds TNF.

Anti-TNF peptides are capable of binding TNF under physiological conditions, and can include, but are not limited to, portions of a TNF receptor and/or portions or structural analogs of anti-TNF antibody antigen binding regions or variable regions. Such antibodies or peptides bind TNF with neutralizing and/or inhibiting biological activity.

Anti-TNF antibodies and/or anti-TNF peptides of the present invention can be routinely made and/or used according to methods of the present invention, such as, but not limited to synthetic methods, hybridomas, and/or recombinant cells expressing nucleic acid encoding such anti-TNF antibodies or peptides.

The present invention also provides antigenic polypeptides of hTNF, corresponding to peptides containing neutralizing epitopes or portions of TNF that, when such epitopes on TNF are bound by anti-TNF antibodies or peptides, neutralize or inhibit the biological activity of TNF in vitro, in situ or in vivo.

The present invention also provides anti-TNF antibodies and peptides in the form of pharmaceutical and/or diagnostic compounds and/or compositions, useful for the diagnostic and/or therapeutic methods of the present invention for diagnosing and/or treating TNF-related pathologies.

Anti-TNF Abs or anti-TNF peptides of the present invention are provided for use in diagnostic methods for detecting TNF in patients or animals suspected of suffering from conditions associated with abnormal TNF production, including methods wherein high affinity anti-TNF antibodies or peptides are contacted with a biological sample from a patient and an antigen-antibody reaction detected. Also included in the present invention are kits for detecting TNF in a solution using anti-TNF antibodies or peptides, preferably in detectably labeled form.

The present invention is also directed to an anti-hTNF chimeric antibody comprising two light chains and two heavy chains, each of the chains comprising at least part of a human constant region and at least part of a variable (V) region of non-human origin having specificity to human TNF, said antibody binding with high affinity to a inhibiting and/or neutralizing epitope of human TNF, such as the antibody cA2. The invention is also includes a fragments or a derivative such an antibody, such as one or more portions of the antibody chain, such as the heavy chain constant, joining, diversity or variable regions, or the light chain constant, joining or variable regions.

Methods are also provided for making and using anti-TNF antibodies and peptides for various utilities of the present invention, such as but not limited to, hybridoma, recombinant or chemical synthetic methods for producing anti-TNF antibodies or anti-TNF peptides according to the present invention; detecting TNF in a solution or cell; removing TNF from a solution or cell, inhibiting one or more biological activities of TNF in vitro, in situ or in vitro. Such removal can include treatment methods of the present invention for alleviating symptoms or pathologies involving TNF, such as, by not limited to bacterial, viral or parasitic infections, chronic inflammatory diseases, autoimmune diseases, malignancies, and/or neurodegenerative diseases.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a graph showing dose dependent binding of mouse mAb A2 to human TNFα.

FIG. **2** is a graph showing lack of recognition of heat-inactivated human TNFα by mAb A2.

FIG. **3** is a graph showing neutralization of in vitro TNF cytotoxicity by murine A2. Control: murine IgG1 anti-lipid A mAb (8A1) with natural human TNF. Average absorbance values for controls were as follows: no TNF added=1.08; natural TNF, no antibody=0.290; and recombinant TNF, no antibody=0.500.

FIG. **4** is a graph showing that mAb A2 and chimeric A2 do not inhibit or neutralize human lymphotoxin (TNFβ).

FIG. **5** is a graph showing that mAbs murine A2 and chimeric CA2 do not inhibit or neutralize murine TNFα.

FIGS. **6** and FIG. **7** are graphs showing that mAb A2 inhibits or neutralizes TNF produced by chimpanzee monocytes and rhTNFα.

FIGS. **8**A and **8**B provides schematic diagrams of the plasmids used for expression of the chimeric H (pA2HG1apgpt) and L (pA2HuKapgpt) chains of the chimeric A2 antibody.

FIGS. **9**A and **9**B is a graph showing results of a cross-blocking epitope ELISA with murine A2 (mA2) and chimeric (cA2) antibody competitors.

FIGS. **10**A and **10**B is a graph of a Scatchard analysis of ^{125}I-labelled mAb A2 (mA2) and chimeric A2 (cA2) binding to recombinant human TNFα immobilized on a microtiter plate. Each Ka value was calculated from the average of two independent determinations.

FIG. **11** is a graph showing neutralization of TNF cytotoxicity by chimeric A2. The control is a chimeric mouse/

human IgG1 anti-platelet mAb (7E3) reacting with natural human TNF. Average absorbance values for controls were: no TNF added=1.08; natural TNF, no Ab=0.290; and recombinant TNF, no Ab=0.500.

FIG. **12** is a graph showing in vitro neutralization of TNF-induced ELAM-1 expression by chimeric A2. The control is a chimeric mouse/human IgG1 anti-CD4 antibody.

FIG. **13** is an amino acid sequence of human TNF as SEQ ID NO:1.

FIGS. **14**A–B FIG. **14**A is a graphical representation of epitope mapping of chimeric mAb cA2 indicating relative binding of cA2 to human TNF peptide pins. FIG. **14**B is a graphical representation of epitope mapping of chimeric mAb cA2 indicating relative binding of cA2 to human TNF peptide pins in the presence of human TNF.

FIG. **15** is an amino acid sequence of human TNF showing sequences having portions of epitopes recognized by cA2, corresponding to portions of amino acids 59–80 and/or 87–108 of SEQ ID NO:1.

FIGS. **16**A–**16**B FIG. **16**A is a nucleic acid sequence (SEQ ID NO:2) and corresponding amino acid sequence (SEQ ID NO:3) of a cloned cA2 light chain variable region. FIG. **16**B is a nucleic acid sequence (SEQ ID NO:4) and corresponding amino acid sequence (SEQ ID NO:5) of a cloned cA2 heavy chain variable region.

FIG. **17** is a graphical representation of the early morning stiffness for the five patients in group I, and the four patients in group II is plotted as the mean percent of the baseline value versus time. Both groups showed an approximately 80 percent decrease or greater in early morning stiffness, which persisted for greater than 40 days.

FIG. **18** is a graphical representation of the assessment of pain using a visual analogue scale for the five patients in group I, and the four patients in group II, is plotted as the mean percent of the baseline value versus time. Both groups showed an approximately 60 to 80 percent decrease in pain score which persisted for greater than 40 days.

FIG. **19** is a graphical representation of the Ritchie Articular Index, (a scale scored of joint tenderness), is plotted as the mean percent of the baseline value versus time. Both groups showed an approximately 80 percent decrease in the Ritchie Articular Index, which persisted for greater than 40 days.

FIG. **20** is a graphical representation of the number of swollen joints for the five patients in group I and the four patients in Group II is plotted as the mean percent of baseline value versus time. Both groups showed an approximately 70 to 80 percent decrease in swollen joints, which persisted for 30 to 40 days.

FIG. **21** is a graphical representation of the serum C-reactive protein for four to five patients in group I, and three of the for patients in group II, is plotted as the mean percent of the baseline value versus time. Both groups showed an approximately 80 percent reduction in CRP which persisted for 30 to 40 days. The values for patient number 1 and patient number 7 were omitted from the computations on which the plots are based, since these patients did not have elevated CRP values at baseline.

FIG. **22** is a graphical representation of the erythrocyte sedimentation rate for the five patients in group I and three of the patients in group II is plotted as the mean percent of the baseline value versus time. Both groups showed an approximately 40 percent reduction in ESR which persisted for at least 40 days. The data from patient number 9 is omitted from the computations on which the plots were based, since this patient did not have an elevated ESR at baseline.

FIG. **23** is a graphical representation of the index of Disease Activity, (a composite score of several parameters of disease activity), for the five patients in group I, and the four patients in group II, is plotted as the mean percent of the baseline value versus time. Both groups showed a clinically significant reduction in IDA, which persisted for at least 40 days.

FIG. **24** is a graphical representation of swollen joint counts (maximum 28), as recorded by a single observer. Circles represent individual patients and horizontal bars show median values at each time point. The screening time point was within 4 weeks of entry to the study (week 0); data from patient 15 were not included after week 2 (dropout). Significance of the changes, relative to week 0, by Mann-Whitney test, adjusted: week 1, $p>0.05$; week 2, $p<0.02$; weeks 3–4, $p<0.002$; weeks 6–8, $p<0.001$.

FIGS. **26**A–B is a graphical representation of levels of serum C-reactive protein (CRP)-Serum CRP (normal range 0–10 mg/liter), measured by nephelometry. Circles represent individual patients and horizontal bars show median values at each time point. The screening time point was within 4 weeks of entry to the study (week 0); data from patient 15 were not included after week 2 (dropout). Significance of the changes, relative to week 0, by Mann-Whitney test, adjusted: week 1, $p<0.001$; week 2, $p<0.003$; week 3, $p<0.002$; week 4, $p<0.02$; week 6,8, $p<0.001$. FIG. **25**B is a schematic illustration of the construction of the vectors used to express the heavy chain of the immunoreceptors.

FIGS. **26**A–B is a schematic illustration of the genes encoding TNF receptor/IgG fusion proteins and the gene encoding the truncated light chain. The gene encoding Ig heavy chain (IgH) fusion proteins had the same basic structure as the naturally occurring, rearranged Ig genes except that the Ig variable region coding sequence was replaced with TNF receptor coding sequence. Except for the TNF receptor coding sequences and a partial human K sequence derived by modifying the murine J region coding sequence in the cM-T412 IgH gene by PCT mutagenesis, the entire genomic fragment shown originated from the cM-T412 chimeric mouse/human IgH gene. Looney et al., *Hum. Antibody Hybrid.* 3:191–200 (1992). The region deleted in the genes encoding p55-sf3 and p75P-sf3 is marked in the figure. The JC_K gene, encoding a truncated Ig Kappa light chain, was constructed by deleting the variable region coding sequence from the cM-T412 chimeric mouse/human Ig Kappa gene (Looney, infra) and using PCR mutagenesis to change the murine J sequence to a partial human J sequence. THe p55-light chain fusion in p55-df2 was made by inserting the p55 coding sequence into the EcoRV site in the JC_K gene. Tracey et al., *Nature* 330:662–666 (1987). FIG. **26**B is a schematic illustration of several immunoreceptor molecules of the present invention. The blackened ovals each represent a domain of the IgG1 constant region. THe circles represent the truncated light chain. Small circles adjacent to a p55 or p75 subunit mark the positions of human J sequence. The incomplete circles in p75-sf2 and -sf3 are to illustrate that the C-terminal 53 amino acids of the p75 extracellular domain were deleted. Lines between subunits represent disulfide bonds.

FIG. **27** is a schematic illustration of the construction of a cM-T412 heavy chain so that it has a unique cloning site for insertion of foreign genes such as p55 and p75.

FIG. **28** is a schematic illustration of the construction of the vectors used to express the heavy chain of the immunoreceptors.

FIG. **29** is a schematic illustration of the construction of a cM-T412 light chain so that it has a unique cloning site for insertion of foreign genes such as p55 and p75.

FIG. **30** is a schematic illustration of the construction of the vectors used to express the light chain of the immunoreceptors.

FIGS. **31**A–C shows graphical representations of fusion proteins protected WEHI 164 cells from TNFβ with actinomycin D and then incubated in 2 ng/ml TNFα with varying concentrations of TNFf overnight at 37° C. Cell viability was determined by measuring their uptake of MTT dye. FIG. **31**A shows p55 fusion proteins. FIG. **31**B shows p75 fusion proteins. FIG. **31**C shows comparison of the protective ability of the non-fusion form of p55 (p55-nf) to p55-sf2.

FIG. **32** is a graphical represation of data showing fusion proteins also effectively protect WEHI 164 cells from TNFβ cytotoxicity.

FIGS. **33**A–H are graphical representations of analyses of binding between the various fusion proteins and TNFα by saturation binding (FIGS. **33**A–B) and Scatchard analysis (FIGS. **33**C–H). A microtiter plate was coated with excess goat anti-Fc polyclonal antibody and incubated with 10 ng/ml of fusion protein in TBST buffer (10 mM Tris-HCl, pH 7.8, 150 mM NaCl, 0.05% Tween-20) for 1 hour. Varying amounts of ^{125}I labeled TNFα (specific activity—34.8 μCi/μg) was then incubated with the captured fusion protein in PBS (10 mM Na Phosphate, pH 7.0, 150 mM NaCl) with 1% bovine serum albumin for 2 hours. Unbound TNFα was washed away with four washes in PBS and the cpm bound was quantitated using a y-counter. All samples were analyzed in triplicate. The slope of the lines in (FIGS. **33**C–H) represent the affinity constant, K_a. The dissociation constant (K_d) values (see Table I) were derived using the equation $K_d=1/K_a$.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Tumor necrosis factor (TNF) has been discovered to mediate or be involved in many pathologies, such as, but not limited to bacterial, viral or parasitic infections, chronic inflammatory diseases, autoimmune diseases, malignancies, and/or neurodegenerative diseases. Accordingly, anti-TNF compounds and compositions of the present invention which have neutralizing and/or inhibiting activity against TNF are discovered to provide methods for treating and/or diagnosing such pathologies.

The present invention thus provides anti-TNF compounds and compositions comprising anti-TNF antibodies (Abs) and/or anti-TNF peptides which inhibit and/or neutralize TNF biological activity in vitro, in situ and/or in vivo, as specific for association with neutralizing epitopes of human tumor necrosis factor-alpha (hTNFα) and/or human tumor necrosis factor β (hTNFβ). Such anti-TNF Abs or peptides have utilities for use in research, diagnostic and/or therapeutic methods of the present invention for diagnosing and/or treating animals or humans having pathologies or conditions associated with the presence of a substance reactive with an anti-TNF antibody, such as TNF or metabolic products thereof. Such pathologies can include the generalized or local presence of TNF or related compounds, in amounts and/or concentrations exceeding, or less than, those present in a normal healthy subject, or as related to a pathological condition.

Anti-TNF Antibodies and Methods

The term "antibody" is meant to include polyclonal antibodies, monoclonal antibodies (mAbs), chimeric antibodies, anti-idiotypic (anti-Id) antibodies to antibodies that can be labeled in soluble or bound form, as well as fragments, regions or derivatives thereof, provided by any known technique, such as, but not limited to enzymatic cleavage, peptide synthesis or recombinant techniques. Such anti-TNF antibodies of the present invention are capable of binding portions of TNF that inhibit the binding of TNF to TNF receptors.

Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen. A monoclonal antibody contains a substantially homogeneous population of antibodies specific to antigens, which population contains substantially similar epitope binding sites. MAbs may be obtained by methods known to those skilled in the art. See, for example Kohler and Milstein, *Nature* 256:495–497 (1975); U.S. Pat. No. 4,376,110; Ausubel et al, eds., *CURRENT PROTOCOLS IN MOLECULAR BIOLOGY*, Greene Publishing Assoc. and Wiley Interscience, N.Y., (1987, 1992); and Harlow and Lane *ANTIBODIES: A LABORATORY MANUAL* Cold Spring Harbor Laboratory (1988); Colligan et al., eds., *Current Protocols in Immunology*, Greene Publishing Assoc. and Wiley Interscience, N.Y., (1992, 1993), the contents of which references are incoporated entirely herein by reference. Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, GILD and any subclass thereof. A hybridoma producing a mAb of the present invention may be cultivated in vitro, in situ or in vivo. Production of high titers of mAbs in vivo or in situ makes this the presently preferred method of production.

Chimeric antibodies are molecules different portions of which are derived from different animal species, such as those having variable region derived from a murine mAb and a human immunoglobulin constant region, which are primarily used to reduce immunogenicity in application and to increase yields in production, for example, where murine mAbs have higher yields from hybridomas but higher immunogenicity in humans, such that human/murine chimeric mAbs are used. Chimeric antibodies and methods for their production are known in the art (Cabilly et al, *Proc. Natl. Acad. Sci. USA* 81:3273–3277 (1984); Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851–6855 (1984); Boulianne et al., *Nature* 312:643–646 (1984); Cabilly et al., European Patent Application 125023 (published Nov. 14, 1984); Neuberger et al., *Nature* 314:268–270 (1985); Taniguchi et al., European Patent Application 171496 (published Feb. 19, 1985); Morrison et al., European Patent Application 173494 (published Mar. 5, 1986); Neuberger et al., PCT Application WO 86/01533, (published Mar. 13, 1986); Kudo et al., European Patent Application 184187 (published Jun. 11, 1986); Morrison et al., European Patent Application 173494 (published Mar. 5, 1986); Sahagan et al., J. Immunol. 137:1066–1074 (1986); Robinson et al., International Patent Publication #PCT/US86/02269 (published May 7, 1987); Liu et al., Proc. Natl. Acad. Sci. USA 84:3439–3443 (1987); Sun et al., Proc. Natl. Acad. Sci. USA 84:214–218 (1987); Better et al., Science 240:1041–1043 (1988); and Harlow and Lane ANTIBODIES: A LABORATORY MANUAL Cold Spring Harbor Laboratory (1988)). These references are entirely incorporated herein by reference.

An anti-idiotypic (anti-Id) antibody is an antibody which recognizes unique determinants generally associated with the antigen-binding site of an antibody. An Id antibody can be prepared by immunizing an animal of the same species and genetic type (e.g., mouse strain) as the source of the mAb with the mAb to which an anti-Id is being prepared. The immunized animal will recognize and respond to the idiotypic determinants of the immunizing antibody by producing an antibody to these idiotypic determinants (the anti-Id antibody). See, for example, U.S. Pat. No. 4,699,880, which is herein entirely incorporated by reference.

The anti-Id antibody may also be used as an "immunogen" to induce an immune response in yet another animal, producing a so-called anti-anti-Id antibody. The anti-anti-Id may be epitopically identical to the original mAb which induced the anti-Id. Thus, by using antibodies to the idiotypic determinants of a mAb, it is possible to identify other clones expressing antibodies of identical specificity.

Anti-TNF antibodies of the present invention can include at least one of a heavy chain constant region (H_c), a heavy chain variable region (H_v), a light chain variable region (L_v) and a light chain constant regions (L_c), wherein a polyclonal Ab, monoclonal Ab, fragment and/or regions thereof include at least one heavy chain variable region (H_v) or light chain variable region (L_v) which binds a portion of a TNF and inhibits and/or neutralizes at least one TNF biological activity.

Preferred antibodies of the present invention are high affinity human-murine chimeric anti-TNF antibodies, and fragments or regions thereof, that have potent inhibiting and/or neutralizing activity in vivo against human TNFα. Such antibodies and chimeric antibodies can include those generated by immunization using purified recombinant hTNFα (SEQ ID NO:1) or peptide fragments thereof. Such fragments can include epitopes of at least 5 amino acids of residues 87–107, or a combination of both of 59–80 and 87–108 of hTNFα (as these corresponding amino acids of SEQ ID NO:1). Additionally, preferred antibodies, fragments and regions of anti-TNF antibodies of the present invention do not recognize amino acids from at least one of amino acids 11–13, 37–42, 49–57 or 155–157 of hTNFα (of SEQ ID NO:1).

Preferred anti-TNF mAbs are also those which will competitively inhibit in vivo the binding to human TNFα of anti-TNFα murine mAb A2, chimeric mAb cA2, or an antibody having substantially the same specific binding characteristics, as well as fragments and regions thereof. Preferred antibodies of the present invention are those that bind epitopes recognized by A2 and cA2, which are included in amino acids 59–80 and/or 87–108 of hTNFα (as these corresponding amino acids of SEQ ID NO:1), such that the epitopes consist of at least 5 amino acids which comprise at least one amino acid from the above portions of human TNFα.

Preferred methods for determining mAb specificity and affinity by competitive inhibition can be found in Harlow, et al., *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1988), Colligan et al., eds., *Current Protocols in Immunology*, Greene Publishing Assoc. and Wiley Interscience, N.Y., (1992, 1993), and Muller, *Meth. Enzymol.* 92:589–601 (1983), which references are entirely incorporated herein by reference.

The techniques to raise antibodies of the present invention to small peptide sequences that recognize and bind to those sequences in the free or conjugated form or when presented as a native sequence in the context of a large protein are well known in the art. Such antibodies include murine, murine human and human-human antibodies produced by hybridoma or recombinant techniques known in the art.

As used herein, the term "antigen binding region" refers to that portion of an antibody molecule which contains the amino acid residues that interact with an antigen and confer on the antibody its specificity and affinity for the antigen. The antibody region includes the "framework" amino acid residues necessary to maintain the proper conformation of the antigen-binding residues.

Preferably, the antigen binding region will be of murine origin. In other embodiments, the antigen binding region can be derived from other animal species, in particular rodents such as rabbit, rat or hamster.

The antigen binding region of the chimeric antibody of the present invention is preferably derived from a non-human antibody specific for human TNF. Preferred sources for the DNA encoding such a non-human antibody include cell lines which produce antibody, preferably hybrid cell lines commonly known as hybridomas. A preferred hybridoma is the A2 hybridoma cell line.

An "antigen" is a molecule or a portion of a molecule capable of being bound by an antibody which is additionally capable of inducing an animal to produce antibody capable of binding to an epitope of that antigen. An antigen can have one or more than one epitope. The specific reaction referred to above is meant to indicate that the antigen will react, in a highly selective manner, with its corresponding antibody and not with the multitude of other antibodies which can be evoked by other antigens. Preferred antigens that bind antibodies, fragments and regions of anti-TNF antibodies of the present invention include at least 5 amino acids comprising at least one of amino acids residues 87–108 or both residues 59–80 and 87–108 of hTNFα (of SEQ ID NO:1). Preferred antigens that bind antibodies, fragments and regions of anti-TNF antibodies of the present invention do not include amino acids of amino acids 11–13, 37–42, 49–57 or 155–157 of hTNFα (SEQ ID NO:1)

Particular peptides which can be used to generate antibodies of the present invention can include combinations of amino acids selected from at least residues 87–108 or both residues 59–80 and 87–108, which are combined to provide an epitope of TNF that is bound by anti-TNF antibodies, fragments and regions thereof, and which binding provided anti-TNF biological activity. Such epitopes include at least 1–5 amino acids and less than 22 amino acids from residues 87–108 or each of residues 59–80 and 87–108, which in combination with other amino acids of TNF provide epitopes of at least 5 amino acids in length.

TNF residues 87–108 or both residues 59–80 and 87–108 of TNF (of SEQ ID NO:1), fragments or combinations of peptides containing therein are useful as immunogens to raise antibodies that will recognize peptide sequences presented in the context of the native TNF molecule.

The term "epitope" is meant to refer to that portion of any molecule capable of being recognized by and bound by an antibody at one or more of the Ab's antigen binding region. Epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and have specific three dimensional structural characteristics as well as specific charge characteristics. By "inhibiting and/or neutralizing epitope" is intended an epitope, which, when bound by an antibody, results in loss of biological activity of the molecule or organism containing the epitope, in vivo, in vitro or in situ, more preferably in vivo, including binding of TNF to a TNF receptor.

Epitopes recognized by antibodies, and fragments and regions thereof, of the present invention can include 5 or more amino acids comprising at least one amino acid of each or both of the following amino acid sequences of TNF, which provide a topographical or three dimensional epitope of TNF which is recognized by, and/or binds with anti-TNF activity, an antibody, and fragments, and variable regions thereof, of the present invention:

59–80: Tyr-Ser-Gln-Val-Leu-Phe-Lys-Gly-Gln-Gly-Cys-Pro-Ser-Thr-His-Val-Leu-Leu-Thr-His-Thr-Ile (AA 59–80 of SEQ ID NO:1); and

87–108: Tyr-Gln-Thr-Lys-Val-Asn-Leu-Leu-Ser-Ala-Ile-Lys-Ser-Pro-Cys-Gln-Arg-Glu-Thr-Pro-Gl u-Gly (AA 87–108 of SEQ ID NO:1).

Preferred antibodies, fragments and regions of anti-TNF antibodies of the present invention recognize epitopes including 5 amino acids comprising at least one amino acid from amino acids residues 87–108 or both residues 59–80 and 87–108 of hTNFα (of SEQ ID NO:1). Preferred antibodies, fragments and regions of anti-TNF antibodies of the present invention do not recognize epitopes from at least one of amino acids 11–13, 37–42, 49–57 or 155–157 of hTNFα (of SEQ ID NO:1). In a preferred embodiment, the epitope comprises at least 2 amino acids from residues 87–108 or both residues 59–80 and 87–108 of hTNFα (of SEQ ID NO:1). In another preferred embodiment, the epitope comprises at least 3 amino acids from residues 59–80 and 87–108 of hTNFα (of SEQ ID NO:1). In another preferred embodiment, the epitope comprises at least 4 amino acids from residues 87–108 or both residues 59–80 and 87–108 of hTNFα (of SEQ ID NO:1). In another preferred embodiment, the epitope comprises at least 5 amino acids from residues 87–108 or both residues 59–80 and 87–108 of hTNFα (of SEQ ID NO:1). In another preferred embodiment, the epitope comprises at least 6 amino acids from residues 87–108 or both residues 59–80 and 87–108 of hTNFα (of SEQ ID NO:1). In another preferred embodiment, the epitope comprises at least 7 amino acids from residues 87–108 or both residues 59–80 and 87–108 of hTNFα (of SEQ ID NO:1).

As used herein, the term "chimeric antibody" includes monovalent, divalent or polyvalent immunoglobulins. A monovalent chimeric antibody is a dimer (HL)) formed by a chimeric H chain associated through disulfide bridges with a chimeric L chain. A divalent chimeric antibody is tetramer (H_2L_2) formed by two HL dimers associated through at least one disulfide bridge. A polyvalent chimeric antibody can also be produced, for example, by employing a CH region that aggregates (e.g., from an IgM H chain, or μ chain).

Murine and chimeric antibodies, fragments and regions of the present invention comprise individual heavy (H) and/or light (L) immunoglobulin chains. A chimeric H chain comprises an antigen binding region derived from the H chain of a non-human antibody specific for TNF, which is linked to at least a portion of a human H chain C region (C_H), such as CH_1 or CH_2.

A chimeric L chain according to the present invention, comprises an antigen binding region derived from the L chain of a non-human antibody specific for TNF, linked to at least a portion of a human L chain C region (C_L).

Antibodies, fragments or derivatives having chimeric H chains and L chains of the same or different variable region binding specificity, can also be prepared by appropriate association of the individual polypeptide chains, according to known method steps, e.g., according to Ausubel infra, Harlow infra, and Colligan infra, the contents of which references are incoporated entirely herein by reference.

With this approach, hosts expressing chimeric H chains (or their derivatives) are separately cultured from hosts expressing chimeric L chains (or their derivatives), and the immunoglobulin chains are separately recovered and then associated. Alternatively, the hosts can be co-cultured and the chains allowed to associate spontaneously in the culture medium, followed by recovery of the assembled immunoglobulin, fragment or derivative.

The hybrid cells are formed by the fusion of a non-human anti-hTNFα antibody-producing cell, typically a spleen cell of an animal immunized against either natural or recombinant human TNF, or a peptide fragment of the human TNFα protein sequence. Alternatively, the non-human anti-TNFα antibody-producing cell can be a B lymphocyte obtained from the blood, spleen, lymph nodes or other tissue of an animal immunized with TNF.

The second fusion partner, which provides the immortalizing function, can be lymphoblastoid cell or a plasmacytoma or myeloma cell, which is not itself an antibody producing cell, but is malignant. Preferred fusion partner cells include the hybridoma SP2/0-Ag14, abbreviated as SP2/0 (ATCC CRL1581) and the myeloma P3X63Ag8 (ATCC TIB9), or its derivatives. See, e.g, Ausubel infra, Harlow infra, and Colligan infra, the contents of which references are incoporated entirely herein by reference.

Murine hybridomas which produce mAb specific for human TNFα or TNFβ are formed by the fusion of a mouse fusion partner cell, such as SP2/0, and spleen cells from mice immunized against purified hTNFα, recombinant hTNFα, natural or synthetic TNF peptides, including peptides including 5 or more amino acids selected from residues 59–80, and 87–108 of TNF (of SEQ ID NO:1) or other biological preparations containing TNF. To immunize the mice, a variety of different conventional protocols can be followed. For example, mice can receive primary and boosting immunizations of TNF.

The antibody-producing cell contributing the nucleotide sequences encoding the antigen-binding region of the chimeric antibody of the present invention can also be produced by transformation of a non-human, such as a primate, or a human cell. For example, a B lymphocyte which produces anti-TNF antibody can be infected and transformed with a virus such as Epstein-Barr virus to yield an immortal anti-TNF producing cell (Kozbor et al. *Immunol. Today* 4:72–79 (1983)). Alternatively, the B lymphocyte can be transformed by providing a transforming gene or transforming gene product, as is well-known in the art. See, e.g, Ausubel infra, Harlow infra, and Colligan infra, the contents of which references are incoporated entirely herein by reference.

Antibody Production Using Hybridomas

The cell fusions are accomplished by standard procedures well known to those skilled in the field of immunology. Fusion partner cell lines and methods for fusing and selecting hybridomas and screening for mAbs are well known in the art. See, e.g, Ausubel infra, Harlow infra, and Colligan infra, the contents of which references are incoporated entirely herein by reference.

The hTNFα-specific murine or chimeric mAb of the present invention can be produced in large quantities by injecting hybridoma or transfectoma cells secreting the antibody into the peritoneal cavity of mice and, after appropriate time, harvesting the ascites fluid which contains a high titer of the mAb, and isolating the mAb therefrom. For such in vivo production of the mAb with a non-murine hybridoma (e.g., rat or human), hybridoma cells are preferably grown in irradiated or athymic nude mice. Alternatively, the antibodies can be produced by culturing hybridoma or transfectoma cells in vitro and isolating secreted mAb from the cell culture medium or recombinantly, in eukaryotic or prokaryotic cells.

In a preferred embodiment, the antibody is a MAb which binds amino acids of an epitope of TNF, which antibody is designated A2, rA2 or cA2, which is produced by a hybridoma or by a recombinant host. In another preferred embodiment, the antibody is a chimeric antibody which recognizes an epitope recognized by A2. In a more preferred embodiment, the antibody is a chimeric antibody designated as chimeric A2 (cA2).

As examples of antibodies according to the present invention, murine mAb A2 of the present invention is produced by a cell line designated c134A. Chimeric anti-

body cA2 is produced by a cell line designated c168A. Cell line c134A is deposited as a research cell bank in the Centocor Cell Biology Services Depository, and cell line c168A(RCB) is deposited as a research cell bank in the Centocor Corporate Cell Culture Research and Development Depository, both at Centocor, 200 Great Valley Parkway, Malvern, Pa., 19355. The c168A cell line is also deposited at Centocor BV, Leiden, The Netherlands.

The invention also provides for "derivatives" of the murine or chimeric antibodies, fragments, regions or derivatives thereof, which term includes those proteins encoded by truncated or modified genes to yield molecular species functionally resembling the immunoglobulin fragments. The modifications include, but are not limited to, addition of genetic sequences coding for cytotoxic proteins such as plant and bacterial toxins. The fragments and derivatives can be produced from any of the hosts of this invention. Alternatively, anti-TNF antibodies, fragments and regions can be bound to cytotoxic proteins or compounds in vitro, to provide cytotoxic anti-TNF antibodies which would selectively kill cells having TNF receptors.

Fragments include, for example, Fab, Fab', $F(ab')_2$ and Fv. These fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and can have less non-specific tissue binding than an intact antibody (Wahl et al., *J. Nucl. Med.* 24:316–325 (1983)). These fragments are produced from intact antibodies using methods well known in the art, for example by proteolytic cleavage with enzymes such as papain (to produce Fab fragments) or pepsin (to produce $F(ab')_2$ fragments).

The identification of these antigen binding regions and/or epitopes recognized by mabs of the present invention provides the information necessary to generate additional monoclonal antibodies with similar binding characteristics and therapeutic or diagnostic utility that parallel the embodiments of this application.

In a preferred embodiment, the amino acids of the epitope are not of at least one of amino acids 11–13, 37–42, 49–57 and 155–157 of hTNFα (of SEQ ID NO:1).

Unexpectedly, anti-TNF antibodies or peptides of the present invention can block the action of TNF-α without binding to the putative receptor binding locus such as is presented by Eck and Sprang (J. Biol. Chem. 264(29), 17595–17605 (1989), as amino acids 11–13, 37–42, 49–57 and 155–157 of hTNFα (of SEQ ID NO:1).

Recombinant Expression of Anti-TNF Antibodies

Recombinant murine or chimeric murine-human or human-human antibodies that inhibit TNF and bind an epitope included in the amino acid sequences residues 87–108 or both residues 59–80 and 87–108 of hTNFα (of SEQ ID NO:1), can be provided according to the present invention using known techniques based on the teaching provided herein. See, e.g., Ausubel et al., eds. *Current Protocols in Molecular Biology*, Wiley Interscience, N.Y. (1987, 1992, 1993); and Sambrook et al. *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press (1989), the entire contents of which are incorporated herein by reference.

The DNA encoding an anti-TNF antibody of the present invention can be genomic DNA or cDNA which encodes at least one of the heavy chain constant region (H_c), the heavy chain variable region (H_v), the light chain variable region (L_v) and the light chain constant regions (L_c). A convenient alternative to the use of chromosomal gene fragments as the source of DNA encoding the murine V region antigen-binding segment is the use of cDNA for the construction of chimeric immunoglobulin genes, e.g., as reported by Liu et al. (*Proc. Natl. Acad. Sci., USA* 84:3439 (1987) and *J. Immunology* 139:3521 (1987), which references are hereby entirely incorporated herein by reference. The use of cDNA requires that gene expression elements appropriate for the host cell be combined with the gene in order to achieve synthesis of the desired protein. The use of cDNA sequences is advantageous over genomic sequences (which contain introns), in that cDNA sequences can be expressed in bacteria or other hosts which lack appropriate RNA splicing systems.

For example, a cDNA encoding a murine V region antigen-binding segment having anti-TNF activity can be provided using known methods based on the use of the DNA sequence presented in FIG. **16**A (SEQ ID NO:2). Alternatively, a cDNA encoding a murine C region antigen-binding segment having anti-TNF activity can be provided using known methods based on the use of the DNA sequence presented in FIG. **16**B (SEQ ID NO:3). Probes that bind a portion of the DNA sequence presented in FIG. **16**A or **16**B can be used to isolate DNA from hybridomas expressing TNF antibodies, fragments or regions, as presented herein, according to the present invention, by known methods.

Oligonucleotides representing a portion of the variable region presented in FIG. **16**A or **16**B sequence are useful for screening for the presence of homologous genes and for the cloning of such genes encoding variable or constant regions of an anti-TNF antibody. Such probes preferably bind to portions of sequences according to FIG. **17**A or **17**B which encode light chain or heavy chain variable regions which bind an activity inhibiting epitope of TNF, especially an epitope of at least 5 amino acids of residues 87–108 or a combination of residues 59–80 and 87–108 (of SEQ ID NO:1).

Such techniques for synthesizing such oligonucleotides are well known and disclosed by, for example, Wu, et al., *Prog. Nucl. Acid. Res. Molec. Biol.* 21:101–141 (1978)), and Ausubel et al, eds. *Current Protocols in Molecular Biology*, Wiley Interscience (1987, 1993), the entire contents of which are herein incorporated by reference.

Because the genetic code is degenerate, more than one codon can be used to encode a particular amino acid (Watson, et al., infra). Using the genetic code, one or more different oligonucleotides can be identified, each of which would be capable of encoding the amino acid. The probability that a particular oligonucleotide will, in fact, constitute the actual XXX-encoding sequence can be estimated by considering abnormal base pairing relationships and the frequency with which a particular codon is actually used (to encode a particular amino acid) in eukaryotic or prokaryotic cells expressing an anti-TNF antibody or fragment. Such "codon usage rules" are disclosed by Lathe, et al., *J. Molec. Biol.* 183:1–12 (1985). Using the "codon usage rules" of Lathe, a single oligonucleotide, or a set of oligonucleotides, that contains a theoretical "most probable" nucleotide sequence capable of encoding anti-TNF variable or constant region sequences is identified.

Although occasionally an amino acid sequence can be encoded by only a single oligonucleotide, frequently the amino acid sequence can be encoded by any of a set of similar oligonucleotides. Importantly, whereas all of the members of this set contain oligonucleotides which are capable of encoding the peptide fragment and, thus, potentially contain the same oligonucleotide sequence as the gene which encodes the peptide fragment, only one member of the set contains the nucleotide sequence that is identical to the nucleotide sequence of the gene. Because this member is present within the set, and is capable of hybridizing to DNA

even in the presence of the other members of the set, it is possible to employ the unfractionated set of oligonucleotides in the same manner in which one would employ a single oligonucleotide to clone the gene that encodes the protein.

The oligonucleotide, or set of oligonucleotides, containing the theoretical "most probable" sequence capable of encoding an anti-TNF antibody or fragment including a variable or constant region is used to identify the sequence of a complementary oligonucleotide or set of oligonucleotides which is capable of hybridizing to the "most probable" sequence, or set of sequences. An oligonucleotide containing such a complementary sequence can be employed as a probe to identify and isolate the variable or constant region anti-TNF gene (Sambrook et al., infra).

A suitable oligonucleotide, or set of oligonucleotides, which is capable of encoding a fragment of the variable or constant anti-TNF region (or which is complementary to such an oligonucleotide, or set of oligonucleotides) is identified (using the above-described procedure), synthesized, and hybridized by means well known in the art, against a DNA or, more preferably, a cDNA preparation derived from cells which are capable of expressing anti-TNF antibodies or variable or constant regions thereof. Single stranded oligonucleotide molecules complementary to the "most probable" variable or constant anti-TNF region peptide coding sequences can be synthesized using procedures which are well known to those of ordinary skill in the art (Belagaje, et al., *J. Biol. Chem.* 254:5765–5780 (1979); Maniatis, et al., *In: Molecular Mechanisms in the Control of Gene Expression*, Nierlich, et al., Eds., Acad. Press, NY (1976); Wu, et al., *Prog. Nucl. Acid Res. Molec. Biol.* 21:101–141 (1978); Khorana, *Science* 203:614–625 (1979)). Additionally, DNA synthesis can be achieved through the use of automated synthesizers. Techniques of nucleic acid hybridization are disclosed by Sambrook et al. (infra), and by Haymes, et al. (In: *Nucleic Acid Hybridization, A Practical Approach*, IRL Press, Washington, D.C. (1985)), which references are herein incorporated by reference. Techniques such as, or similar to, those described above have successfully enabled the cloning of genes for human aldehyde dehydrogenases (Hsu, et al., *Proc. Natl. Acad. Sci. USA* 82:3771–3775 (1985)), fibronectin (Suzuki, et al., *Eur. Mol. Biol. Organ. J.* 4:2519–2524 (1985)), the human estrogen receptor gene (Walter, et al., *Proc. Natl. Acad. Sci. USA* 82:7889–7893 (1985)), tissue-type plasminogen activator (Pennica, et al., *Nature* 301:214–221 (1983)) and human term placental alkaline phosphatase complementary DNA (Kam, et al., *Proc. Natl. Acad. Sci. USA* 82:8715–8719 (1985)).

In an alternative way of cloning a polynucleotide encoding an anti-TNF variable or constant region, a library of expression vectors is prepared by cloning DNA or, more preferably, cDNA (from a cell capable of expressing an anti-TNF antibody or variable or constant region) into an expression vector. The library is then screened for members capable of expressing a protein which competitively inhibits the binding of an anti-TNF antibody, such as A2 or cA2, and which has a nucleotide sequence that is capable of encoding polypeptides that have the same amino acid sequence as anti-TNF antibodies or fragments thereof. In this embodiment, DNA, or more preferably cDNA, is extracted and purified from a cell which is capable of expressing an anti-TNF antibody or fragment. The purified cDNA is fragmentized (by shearing, endonuclease digestion, etc.) to produce a pool of DNA or cDNA fragments. DNA or cDNA fragments from this pool are then cloned into an expression vector in order to produce a genomic library of expression vectors whose members each contain a unique cloned DNA or cDNA fragment such as in a lambda phage library, expression in prokaryotic cell (e.g., bacteria) or eukaryotic cells, (e.g., mammalian, yeast, insect or fungus). See, e.g., Ausubel, infra, Harlow, infra, Colligan, infra; Nyyssonen et al. *Bio/Technology* 11:591–595 (Can 1993); Marks et al., *Bio/Technology* 11:1145–1149 (October 1993). Once nucleic acid encoding such variable or constant anti-TNF regions is isolated, the nucleic acid can be appropriately expressed in a host cell, along with other constant or variable heavy or light chain encoding nucleic acid, in order to provide recombinant MAbs that bind TNF with inhibitory activity. Such antibodies preferably include a murine or human anti-TNF variable region which contains a framework residue having complimentarily determining residues which are responsible for antigen binding. In a preferred embodiment, an anti-TNF variable light or heavy chain encoded by a nucleic acid as described above binds an epitope of at least 5 amino acids including residues 87–108 or a combination of residues 59–80 and 87–108 of hTNF (of SEQ ID NO:1).

Human genes which encode the constant (C) regions of the murine and chimeric antibodies, fragments and regions of the present invention can be derived from a human fetal liver library, by known methods. Human C regions genes can be derived from any human cell including those which express and produce human inmuunoglobulins. The human C_H region can be derived from any of the known classes or isotypes of human H chains, including gamma, μ, α, δ or ϵ, and subtypes thereof, such as G1, G2, G3 and G4. Since the H chain isotype is responsible for the various effector functions of an antibody, the choice of C_H region will be guided by the desired effector functions, such as complement fixation, or activity in antibody-dependent cellular cytotoxicity (ADCC). Preferably, the C_H region is derived from gamma 1 (IgG1), gamma 3 (IgG3), gamma 4 (IgG4), or μ (IgM).

The human C_L region can be derived from either human L chain isotype, kappa or lambda.

Genes encoding human immunoglobulin C regions are obtained from human cells by standard cloning techniques (Sambrook, et al. (*Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989) and Ausubel et al, eds. *Current Protocols in Molecular Biology* (1987–1993)). Human C region genes are readily available from known clones containing genes representing the two classes of L chains, the five classes of H chains and subclasses thereof. Chimeric antibody fragments, such as $F(ab')_2$ and Fab, can be prepared by designing a chimeric H chain gene which is appropriately truncated. For example, a chimeric gene encoding an H chain portion of an $F(ab')_2$ fragment would include DNA sequences encoding the CH_1 domain and hinge region of the H chain, followed by a translational stop codon to yield the truncated molecule.

Generally, the murine, human or murine and chimeric antibodies, fragments and regions of the present invention are produced by cloning DNA segments encoding the H and L chain antigen-binding regions of a TNF-specific antibody, and joining these DNA segments to DNA segments encoding C_H and C_L regions, respectively, to produce murine, human or chimeric immunoglobulin-encoding genes.

Thus, in a preferred embodiment, a fused chimeric gene is created which comprises a first DNA segment that encodes at least the antigen-binding region of non-human origin, such as a functionally rearranged V region with joining (J) segment, linked to a second DNA segment encoding at least a part of a human C region.

Therefore, cDNA encoding the antibody V and C regions, the method of producing the chimeric antibody according to the present invention involves several steps, outlined below:

1. isolation of messenger RNA (mRNA) from the cell line producing an anti-TNF antibody and from optional additional antibodies supplying heavy and light constant regions; cloning and cDNA production therefrom;
2. preparation of a full length cDNA library from purified mRNA from which the appropriate V and/or C region gene segments of the L and H chain genes can be: (i) identified with appropriate probes, (ii) sequenced, and (iii) made compatible with a C or V gene segment from another antibody for a chimeric antibody;
3. Construction of complete H or L chain coding sequences by linkage of the cloned specific V region gene segments to cloned C region gene, as described above;
4. Expression and production of L and H chains in selected hosts, including prokaryotic and eukaryotic cells to provide murine-murine, human-murine, human-human or human murine antibodies.

One common feature of all immunoglobulin H and L chain genes and their encoded mRNAs is the J region. H and L chain J regions have different sequences, but a high degree of sequence homology exists (greater than 80%) among each group, especially near the C region. This homology is exploited in this method and consensus sequences of H and L chain J regions can be used to design oligonucleotides for use as primers for introducing useful restriction sites into the J region for subsequent linkage of V region segments to human C region segments.

C region cDNA vectors prepared from human cells can be modified by site-directed mutagenesis to place a restriction site at the analogous position in the human sequence. For example, one can clone the complete human kappa chain C (C_k) region and the complete human gamma-1 C region ($C_{gamma\text{-}1}$). In this case, the alternative method based upon genomic C region clones as the source for C region vectors would not allow these genes to be expressed in bacterial systems where enzymes needed to remove intervening sequences are absent. Cloned V region segments are excised and ligated to L or H chain C region vectors. Alternatively, the human $C_{gamma\text{-}1}$ region can be modified by introducing a termination codon thereby generating a gene sequence which encodes the H chain portion of an Fab molecule. The coding sequences with linked V and C regions are then transferred into appropriate expression vehicles for expression in appropriate hosts, prokaryotic or eukaryotic.

Two coding DNA sequences are said to be "operably linked" if the linkage results in a continuously translatable sequence without alteration or interruption of the triplet reading frame. A DNA coding sequence is operably linked to a gene expression element if the linkage results in the proper function of that gene expression element to result in expression of the coding sequence.

Expression vehicles include plasmids or other vectors. Preferred among these are vehicles carrying a functionally complete human C_H or C_L chain sequence having appropriate restriction sites engineered so that any V_H or V_L chain sequence with appropriate cohesive ends can be easily inserted therein. Human C_H or C_L chain sequence-containing vehicles thus serve as intermediates for the expression of any desired complete H or L chain in any appropriate host.

A chimeric antibody, such as a mouse-human or human-human, will typically be synthesized from genes driven by the chromosomal gene promoters native to the mouse H and L chain V regions used in the constructs; splicing usually occurs between the splice donor site in the mouse J region and the splice acceptor site preceding the human C region and also at the splice regions that occur within the human C_H region; polyadenylation and transcription termination occur at native chromosomal sites downstream of the human coding regions.

Non-Limiting Exemplary Chimeric A2 (cA2) Anti-TNF Antibody of the Present Invention

Murine MAbs are undesirable for human therapeutic use, due to a short free circulating serum half-life and the stimulation of a human anti-murine antibody (HAMA) response. A murine-human chimeric anti-human TNFα MAb was developed in the present invention with high affinity, epitope specificity and the ability to neutralize the cytotoxic effects of human TNF. Chimeric A2 anti-TNF consists of the antigen binding variable region of the high-affinity neutralizing mouse anti-human TNF IgG1 antibody, designated A2, and the constant regions of a human IgG1, kappa immunoglobulin. The human IgG1 Fc region is expected to: improve allogeneic antibody effector function; increase the circulating serum half-life; and decrease the immunogenicity of the antibody. A similar murine-human chimeric antibody (chimeric 17-1A) has been shown in clinical studies to have a 6-fold longer in vivo circulation time and to be significantly less immunogenic than its corresponding murine MAb counterpart (LoBuglio et al., *Proc Natl Acad Sci USA* 86: 4220–4224, 1988).

The avidity and epitope specificity of the chimeric A2 is derived from the variable region of the murine A2. In a solid phase ELISA, cross-competition for TNF was observed between chimeric and murine A2, indicating an identical epitope specificity of cA2 and murine A2. The specificity of cA2 for TNF-α was confirmed by its inability to neutralize the cytotoxic effects of lymphotoxin(TNF-β). Chimeric A2 neutralizes the cytotoxic effect of both natural and recombinant human TNF in a dose dependent manner. From binding assays of cA2 and recombinant human TNF, the affinity constant of cA2 was calculated to be $1.8\times10^{9}M^{-1}$.

ANTI-TNF Immunoreceptor Peptides

Immunoreceptor peptides of this invention can bind to TNFα and/or TNFβ. The immunoreceptor comprises covalently attached to at least a portion of the TNF receptor at least one immunoglobulin heavy or light chain. In certain preferred embodiments, the heavy chain constant region comprises at least a portion of CH_1. Specifically, where a light chain is included with an immunoreceptor peptide, the heavy chain must include the area of CH_1 responsible for binding a light chain constant region.

An immunoreceptor peptide of the present invention can preferably comprise at least one heavy chain constant region and, in certain embodiments, at least one light chain constant region, with a receptor molecule covalently attached to at least one of the immunoglobulin chains. Light chain or heavy chain variable regions are included in certain embodiments. Since the receptor molecule can be linked within the interior of an immunoglobulin chain, a single chain can have a variable region and a fusion to a receptor molecule.

The portion of the TNF receptor linked to the immunoglobulin molecule is capable of binding TNFα and/or TNFβ. Since the extracellular region of the TNF receptor binds TNF, the portion attached to the immunoglobulin molecule of the immunoreceptor consists of at least a portion of the extracellular region of the TNF receptor. In certain preferred embodiments, the entire extracellular region of p55 is included. In other preferred embodiments, the entire extracellular region of p75 is included. In further preferred

embodiments, the extracellular region of p75 is truncated to delete at least a portion of a region of O-linked glycosylation and/or a proline-rich region while leaving intact the intramolecular disulfide bridges. Such immunoreceptors comprise at least a portion of a hinge region wherein at least one heavy chain is covalently linked to a truncated p75 extracellular region capable of binding to TNFα or TNFβ or both. Such a truncated molecule includes, for example, sequences 1–178, 1–182 or at least 5 amino acid portions thereof, such as 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, . . . 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290 or any value thereon.

Certain embodiments can also include, for example, the C-terminal half of the hinge region to provide a disulfide bridge between heavy chains where both CH_2 and CH_3 chains are present and CH_1 is absent. Alternatively, for example, the N-terminal half of the hinge region can be included to provide a disulfide bridge with a light chain where only the CH_1 region is present.

In certain preferred embodiments of this invention, the non-immunoglobulin molecule is covalently linked to the N-terminus of at least one CH_1 region. In other preferred embodiments, the non-immunoglobulin molecule is covalently linked to an interior section of at least one heavy and/or light chain region. Thus, a portion of the TNF receptor can be, for example, at the end of the immunoglobulin chain or in the middle of the chain.

Where the TNF receptor is attached to the middle of the immunoglobulin, the immunoglobulin chain can be truncated, for example, to compensate for the presence of foreign amino acids, thus resulting in a fusion molecule of approximately the same length as a natural immunoglobulin chain. Alternatively, for example, the immunoglobulin chain can be present substantially in its entirety, thus resulting in a chain that is longer than the corresponding natural immunoglobulin chain. Additionally, the immunoglobulin molecule can be truncated to result in a length intermediate between the size of the entire chain linked to the receptor molecule and the size of the immunoglobulin chain alone.

In certain preferred embodiments, the heavy chain is an IgG class heavy chain. In other preferred embodiments, the heavy chain is an IgM class heavy chain.

In certain preferred embodiments, the heavy chain further comprises at least about 8 amino acids of a J region.

In certain preferred embodiments, at least a portion of the hinge region is attached to the CH_1 region. For example, where CH_1 and CH_2 are present in the molecule, the entire hinge region is also present to provide the disulfide bridges between the two heavy chain molecules and between the heavy and light chains. Where only CH_1 is present, for example, the molecule need only contain the portion of the hinge region corresponding to the disulfide bridge between the light and heavy chains, such as the first 7 amino acids of the hinge.

It will be understood by one skilled in the art, once armed with the present disclosure, that the immunoreceptor peptides of the invention can be, for example, monomeric or dimeric. For example, the molecules can have only one light chain and one heavy chain or two light chains and two heavy chains.

At least one of the non-immunoglobulin molecules linked to an immunoglobin molecule comprises at least a portion of p55 or at least a portion of p75. The portion of the receptor that is included encompasses the TNF binding site.

In certain preferred embodiments, the non-immunoglobulin molecule comprises at least 5 amino acid segments of sequences 2–159 of p55. In other preferred embodiments, the non-immunoglobulin molecule comprises at least 5 amino acid portions of sequences 1–235 of p75. In further preferred embodiments, the non-immunoglobulin molecule comprises at least 5 amino acid portions of sequences 1–182 of p75. The above 5 amino acid portions can be selected from 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, . . . 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290.

In certain preferred embodiments, each of the two heavy chains and each of the two light chains is linked to a portion of the TNF receptor, thus forming a tetravalent molecule. Such a tetravalent molecule can have, for example, four p55 receptor molecules; two on the two heavy chains and two on the two light chains. Alternatively, a tetravalent molecule can have, for example, a p55 receptor molecule attached to each of the two heavy chains and a p75 receptor molecule attached to each of the two light chains. A tetravalent molecule can also have, for example, p55 receptor attached to the light chains and p75 receptor attached to the heavy chains. Additionally, a tetravalent molecule can have one heavy chain attached to p55, one heavy chain attached to p75, one light chain attached to p75, and one light chain attached to p55. See, for example, the molecules depicted in FIG. **26**A. Further, the molecules can have six receptors attached, for example; two within the heavy chains and four at the ends of the heavy and light chains. Other potential multimers and combinations would also be within the scope of one skilled in the art, once armed with the present disclosure.

In further preferred embodiments, at least one of the heavy chains has a variable region capable of binding to a second target molecule. Such molecules include, for example, CD3, so that one half of a fusion molecule is a monomeric anti-CD3 antibody.

Additionally, in other embodiments of the present invention, the immunoreceptor peptides further include an irrelevant variable region on the light chain and/or heavy chain. Preferably, however, such a region is absent due to the lowered affinity for TNF which can be present due to stearic hindrance.

In certain preferred embodiments, the heavy chain is linked to a non-immunoglobulin molecule capable of binding to a second target molecule, such as a cytotoxic protein, thus creating a part immunoreceptor, part immunotoxin that is capable of killing those cells expressing TNF. Such cytotoxic proteins, include, but are not limited to, Ricin-A, Pseudomonas toxin, Diphtheria toxin and TNF. Toxins conjugated to ligands are known in the art (see, for example, Olsnes, S. et al., *Immunol. Today* 1989, 10, 291–295). Plant and bacterial toxins typically kill cells by disruption the protein synthetic machinery.

The Immunoreceptors of this invention can be conjugated to additional types of therapeutic moieties including, but not limited to, radionuclides, cytotoxic agents and drugs. Examples of radionuclides include ^{212}Bi, ^{131}I, ^{186}Re, and ^{90}Y, which list is not intended to be exhaustive. The radionuclides exert their cytotoxic effect by locally irradiating the cells, leading to various intracellular lesions, as is known in the art of radiotherapy.

Cytotoxic drugs which can be conjugated to the immunoreceptors and subsequently used for in vivo therapy include, but are not limited to, daunorubicin, doxorubicin, methotrexate, and Mitomycin C. Cytotoxic drugs interfere with critical cellular processes including DNA, RNA, and

protein synthesis. For a fuller exposition of these classes of drugs which are known in the art, and their mechanisms of action, see Goodman, A. G., et al., *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, 8th Ed., Macmillan Publishing Col, 1990. Katzung, ed., Basic and Clinical Pharmacology, Fifth Edition, p 768–769, 808–809, 896, Appleton and Lange, Norwalk, Conn.

In preferred embodiments, immunoreceptor molecules of the invention are capable of binding with high affinity to a neutralizing epitope of human TNFα or TNFβ in vivo. Preferably, the binding affinity is at least about 1.6×10^{10} M-1. See, for example, Table 1 below. Additionally, in preferred embodiments, immunoreceptor molecules of the invention are capable of neutralizing TNF at an efficiency of about a concentration of less than 130 pM to neutralize 39.2 pM human TNFα. See, for example, Table 1.

TABLE 1

Summary of affinities of different fusion proteins for TNFα

protein	IC_{50}*	Molar ration fp: TNFα at IC_{50}	K_D (pM)
p55-sf2	70	1.8	57
p55-df2	55	1.4	60
p55-sf3	100	2.6	48
p55-nf	36,000	900	n.d.
p75-sf2	130	3.3	33
p75P-sf2	70	1.8	29
p75P-sf3	130	3.3	15

*IC_{50} = concentration of fusion protein required to inhibit 2 ng/ml (39.2 pM) TNFα by 50% .62

Once armed with the present disclosure, one skilled in the art would be able to create fragments of the immunoreceptor peptides of the invention. Such fragments are intended to be within the scope of this invention. For example, once the molecules are isolated, they can be cleaved with protease to generate fragments that remain capable of binding TNF.

Once armed with the present disclosure, one skilled in the art would also be able to create derivatives of the immunoreceptor peptides of the invention. Such derivatives are intended to be within the scope of this invention. For example, amino acids in the immunoreceptor that constitute a protease recognition site can be modified to avoid protease cleavage and thus confer greater stability, such as KEX2 sites.

One skilled in the art, once armed with the present disclosure, would be able to synthesize the molecules of the invention. The immunoreceptor peptides can be constructed, for example, by vector-mediated synthesis, as described in Example XXIV. In general, two expression vectors are preferably used; one for the heavy chain, one for the light chain. A vector for expression an immunoglobulin preferably consists of a promoter linked to the signal sequence, followed by the constant region. The vector additionally preferably contains a gene providing for the selection of transfected cells expressing the construct. In certain preferred embodiments, sequences derived from the J region are also included.

The immunoglobulin gene can be from any vertebrate source, such as murine, but preferably, it encodes an immunoglobulin having a substantial number of sequences that are of the same origin as the eventual recipient of the immunoreceptor peptide. For example, if a human is treated with a molecule of the invention, preferably the immunoglobulin is of human origin.

TNF receptor constructs for linking to the heavy chain can be synthesized, for example, using DNA encoding amino acids present in the cellular domain of the receptor. Putative receptor binding loci of hTNF have been presented by Eck and Sprange, *J. Biol. Chem.* 1989, 264(29), 17595–17605, who identified the receptor binding loci of TNF-α as consisting of amino acids 11–13, 37–42, 49–57 and 155–157. PCT application WO91/02078 (priority date of Aug. 7, 1989) discloses TNF ligands which can bind to monoclonal antibodies having the following epitopes of at least one of 1–20, 56–77, and 108–127; at least two of 1–20, 56–77, 108–127 and 138–149; all of 1–18, 58–65, 115–125 and 138–149; all of 1–18, and 108–128; all of 56–79, 110–127 and 135– or 136–155; all of 1–30, 117–128 and 141–153; all of 1–26, 117–128 and 141–153; all of 22–40, 49–96 or –97, 110–127 and 136–153; all of 12–22, 36–45, 96–105 and 132–157; all of both of 1–20 and 76–90; all of 22–40, 69–97, 105–128 and 135–155; all of 22–31 and 146–157; all of 22–40 and 49–98; at least one of 22–40, 49–98 and 69–97, both of 22–40 and 70–87. Thus, one skilled in the art, once armed with the present disclosure, would be able to create TNF receptor fusion proteins using portions of the receptor that are known to bind TNF.

Advantages of using an immunoglobulin fusion protein (immunoreceptor peptide) of the present invention include one or more of (1) possible increased avidity for multivalent ligands due to the resulting bivalency of dimeric fusion proteins, (2) longer serum half-life, (3) the ability to activate effector cells via the Fc domain, (4) ease of purification (for example, by protein A chromatography), (5) affinity for TNFα and TNFβ and (6) the ability to block TNFα or TNFβ cytotoxicity.

TNF receptor/IgG fusion proteins have shown greater affinity for TNF α in vitro than their monovalent, non-fusion counterparts. These types of fusion proteins, which also bind murine TNF with high affinity, have also been shown to protect mice from lipopolysaccharide-induced endotoxemia. Lesslauer et al., *Eur. J. Immunol.* 1991, 21, 2883–2886; and Ashkenazi et al., *Proc. Natl. Acad. Sci. USA* 1991, 88, 10535–10539. Unlike the molecules of the present invention, the TNF receptor/IgG fusion proteins reported to date have had the receptor sequence fused directly to the hinge domain of IgGs such that the first constant domain (CH_1) of the heavy chain was omitted. Lesslauer et al., *Eur. J. Immunol.* 1991, 21, 2883–2886; Ashkenzi et al., *Proc. Natl. Acad. Sci. USA* 1991, 88, 10535–10539; and Peppel et al., *J. Exp. Med.* 1991, 174, 1483–1489.

While this generally permits secretion of the fusion protein in the absence of an Ig light chain, a major embodiment of the present invention provides for the inclusion of the CH_1 domain, which can confer advantages such as (1) increased distance and/or flexibility between two receptor molecules resulting in greater affinity for TNF, (2) the ability to create a heavy chain fusion protein and a light chain fusion protein that would assemble with each other and dimerize to form a tetravalent (double fusion) receptor molecule, and (3) a tetravalent fusion protein can have increased affinity and/or neutralizing capability for TNF compared to a bivalent (single fusion) molecule.

Unlike other TNF receptor/IgG fusion proteins that have been reported, the fusion proteins of a major embodiment of the present invention include the first constant domain (CH_1) of the heavy chain. The CH_1 domain is largely responsible for interactions with light chains. The light chain, in turn, provides a vehicle for attaching a second set of TNF receptor molecules to the immunoreceptor peptide.

It was discovered using the molecules of the present invention that the p55/light chain fusion proteins and p55/heavy chain fusion proteins would assemble with each other

and dimerize to form an antibody-like molecule that is tetravalent with respect to p55. The resulting tetravalent p55 molecules can confer more protection against, and have greater affinity for, TNFα or TNFβ than the bivalent p55 molecules. Despite the presumed close proximity of the two light chain p55 domains to the heavy chain p55 domains, they do not appear to stereocilia hinder or reduce the affinity for TNF.

Inclusion of the CH_1 domain also meant that secretion of the fusion protein was likely to be inefficient in the absence of light chain. This has been shown to be due to a ubiquitous immunoglobulin binding protein (BiP) that binds to the C_H1 domain of heavy chains that are not assembled with a light chain and sequesters them in the endoplasmic reticulum. Karlsson et al., *J. Immunol. Methods* 1991, 145, 229–240.

In initial experiments, an irrelevant light chain was co-transfected with the p55-heavy chain construct and subsequent analyses showed that the two chains did assemble and that the resulting fusion protein protected WEHI cells from TNFα. However, it was considered likely that the variable region of the irrelevant light chain would stereocilia hinder interactions between the p55 subunits and TNFα. For this reason, a mouse-human chimeric antibody light chain gene was engineered by (1) deleting the variable region coding sequence, and (2) replacing the murine J coding sequence with human J coding sequence. Use of this truncated light chain, which was shown to assemble and disulfide bond with heavy chains, increased the efficiency of TNF inhibition by approximately 30-fold compared to use of a complete irrelevant light chain.

Comparison of the abilities of p75-sf2 and p75P-sf2 to inhibit TNF cytotoxicity indicated that the C-terminal 53 amino acids of the extracellular domain of p75, which defines a region that is rich in proline residues and contains the only sites of O-linked glycosylation, are not necessary for high-affinity binding to TNFα or TNFβ. In fact, the p75P-sf2 construct repeatedly showed higher affinity binding to TNFβ than p75-sf2. Surprisingly, there was no difference observed between the two constructs in their affinity for TNFα.

It is possible that a cell-surface version of p75-P would also bind TNFβ with higher affinity than the complete p75 extracellular domain. A similar region is found adjacent to the transmembrane domain in the low affinity nerve growth factor receptor whose extracellular domain shows the same degree of similarity to p75 as p55 does. Mallett et al., *Immunol. Today* 1991, 12, 220–223.

Two groups have reported that in cell cytotoxicity assays, their p55 fusion protein could be present at a 3-fold (Lesslauer et al., *Eur. J. Immunol.* 1991, 21, 2883–2886) or 6–8 fold (Ashkenazi et al., *Proc. Natl. Acad. Sci. USA* 1991, 88, 10535–10539) lower concentration than their monovalent p55 and still get the same degree of protection, while another group (Peppel et al., *J. Exp. Med.* 1991, 174, 1483–1489) showed that their p55 fusion protein could be present at a 1000-fold lower concentration than monomeric p55. Thus, the prior art has shown unpredictability in the great variability in the efficiency of different fusion proteins.

The molecules of the present invention have demonstrated the same degree of protection against TNF in a 5000-fold lower molar concentration than monomeric p55. (See Table 1.) It is believed that the presence of the CH_1 chain in the molecules of a major embodiment of the present invention can confer greater flexibility to the molecule and avoid stearic hindrance with the binding of the TNF receptor.

Recombinant Expression of Anti-TNF Antibodies and Anti-TNF Peptides.

A nucleic acid sequence encoding at least one anti-TNF peptide or Ab fragment of the present invention may be recombined with vector DNA in accordance with conventional techniques, including blunt-ended or staggered-ended termini for ligation, restriction enzyme digestion to provide appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and ligation with appropriate ligases. Techniques for such manipulations are disclosed, e.g., by Ausubel, infra, Sambrook, infra, entirely incorporated herein by reference, and are well known in the art.

A nucleic acid molecule, such as DNA, is said to be "capable of expressing" a polypeptide if it contains nucleotide sequences which contain transcriptional and translational regulatory information and such sequences are "operably linked" to nucleotide sequences which encode the polypeptide. An operable linkage is a linkage in which the regulatory DNA sequences and the DNA sequence sought to be expressed are connected in such a way as to permit gene expression as anti-TNF peptides or Ab fragments in recoverable amounts. The precise nature of the regulatory regions needed for gene expression may vary from organism to organism, as is well known in the analogous art. See, e.g., Sambrook, supra and Ausubel supra.

The present invention accordingly encompasses the expression of an anti-TNF peptide or Ab fragment, in either prokaryotic or eukaryotic cells, although eukaryotic expression is preferred.

Preferred hosts are bacterial or eukaryotic hosts including bacteria, yeast, insects, fungi, bird and mammalian cells either in vivo, or in situ, or host cells of mammalian, insect, bird or yeast origin. It is preferred that the mammalian cell or tissue is of human, primate, hamster, rabbit, rodent, cow, pig, sheep, horse, goat, dog or cat origin, but any other mammalian cell may be used.

Further, by use of, for example, the yeast ubiquitin hydrolase system, in vivo synthesis of ubiquitin-transmembrane polypeptide fusion proteins may be accomplished. The fusion proteins so produced may be processed in vivo or purified and processed in vitro, allowing synthesis of an anti-TNF peptide or Ab fragment of the present invention with a specified amino terminus sequence. Moreover, problems associated with retention of initiation codon-derived methionine residues in direct yeast (or bacterial) expression may be avoided. Sabin et al., *Bio/Technol.* 7(7): 705–709 (1989); Miller et al., *Bio/Technol.* 7(7): 698–704 (1989).

Any of a series of yeast gene expression systems incorporating promoter and termination elements from the actively expressed genes coding for glycolytic enzymes produced in large quantities when yeast are grown in mediums rich in glucose can be utilized to obtain anti-TNF peptides or Ab fragments of the present invention. Known glycolytic genes can also provide very efficient transcriptional control signals. For example, the promoter and terminator signals of the phosphoglycerate kinase gene can be utilized.

Production of anti-TNF peptides or Ab fragments or functional derivatives thereof in insects can be achieved, for example, by infecting the insect host with a baculovirus engineered to express transmembrane polypeptide by methods known to those of skill. See Ausubel et al, eds. *Current Protocols in Molecular Biology*, Wiley Interscience, §§16.8–16.11 (1987, 1993).

In a preferred embodiment, the introduced nucleotide sequence will be incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. Any

of a wide variety of vectors may be employed for this purpose. See, e.g., Ausubel et al, infra, §§ 1.5, 1.10, 7.1, 7.3, 8.1, 9.6, 9.7, 13.4, 16.2, 16.6, and 16.8–16.11. Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species.

Preferred prokaryotic vectors known in the art include plasmids such as those capable of replication in *E. coli* (such as, for example, pBR322, ColE1, pSC101, pACYC 184, πVX). Such plasmids are, for example, disclosed by Maniatis, T., et al. (*Molecular Cloning, A Laboratory Manual*, Second Edition, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989); Ausubel, infra. Bacillus plasmids include pC194, pC221, pT127, etc. Such plasmids are disclosed by Gryczan, T. (In: *The Molecular Biology of the Bacilli*, Academic Press, NY (1982), pp. 307–329). Suitable Streptomyces plasmids include pIJ101 (Kendall, K. J., et al., *J. Bacteriol.* 169:4177–4183 (1987)), and streptomyces bacteriophages such as φC31 (Chater, K. F., et al., In: *Sixth International Symposium on Actinomycetales Biology*, Akademiai Kaido, Budapest, Hungary (1986), pp. 45–54). Pseudomonas plasmids are reviewed by John, J. F., et al. (*Rev. Infect. Dis.* 8:693–704 (1986)), and Izaki, K. (*Jpn. J. Bacteriol.* 33:729–742 (1978); and Ausubel et al, supra).

Alternatively, gene expression elements useful for the expression of cDNA encoding anti-TNF antibodies or peptides include, but are not limited to (a) viral transcription promoters and their enhancer elements, such as the SV40 early promoter (Okayama, et al., *Mol. Cell. Biol.* 3:280 (1983)), Rous sarcoma virus LTR (Gorman, et al., *Proc. Natl. Acad. Sci., USA* 79:6777 (1982)), and Moloney murine leukemia virus LTR (Grosschedl, et al., *Cell* 41:885 (1985)); (b) splice regions and polyadenylation sites such as those derived from the SV40 late region (Okayama et al., infra); and (c) polyadenylation sites such as in SV40 (Okayama et al., infra).

Immunoglobulin cDNA genes can be expressed as described by Liu et al., infra, and Weidle et al., *Gene* 51:21 (1987), using as expression elements the SV40 early promoter and its enhancer, the mouse immunoglobulin H chain promoter enhancers, SV40 late region mRNA splicing, rabbit β-globin intervening sequence, immunoglobulin and rabbit β-globin polyadenylation sites, and SV40 polyadenylation elements. For immunoglobulin genes comprised of part cDNA, part genomic DNA (Whittle et al., *Protein Engineering* 1:499 (1987)), the transcriptional promoter is human cytomegalovirus, the promoter enhancers are cytomegalovirus and mouse/human immunoglobulin, and mRNA splicing and polyadenylation regions are from the native chromosomal immunoglobulin sequences.

In one embodiment, for expression of cDNA genes in rodent cells, the transcriptional promoter is a viral LTR sequence, the transcriptional promoter enhancers are either or both the mouse immunoglobulin heavy chain enhancer and the viral LTR enhancer, the splice region contains an intron of greater than 31 bp, and the polyadenylation and transcription termination regions are derived from the native chromosomal sequence corresponding to the immunoglobulin chain being synthesized. In other embodiments, cDNA sequences encoding other proteins are combined with the above-recited expression elements to achieve expression of the proteins in mammalian cells.

Each fused gene is assembled in, or inserted into, an expression vector. Recipient cells capable of expressing the chimeric immunoglobulin chain gene product are then transfected singly with an anti-TNF peptide or chimeric H or chimeric L chain-encoding gene, or are co-transfected with a chimeric H and a chimeric L chain gene. The transfected recipient cells are cultured under conditions that permit expression of the incorporated genes and the expressed immunoglobulin chains or intact antibodies or fragments are recovered from the culture.

In one embodiment, the fused genes encoding the anti-TNF peptide or chimeric H and L chains, or portions thereof, are assembled in separate expression vectors that are then used to co-transfect a recipient cell.

Each vector can contain two selectable genes, a first selectable gene designed for selection in a bacterial system and a second selectable gene designed for selection in a eukaryotic system, wherein each vector has a different pair of genes. This strategy results in vectors which first direct the production, and permit amplification, of the fused genes in a bacterial system. The genes so produced and amplified in a bacterial host are subsequently used to co-transfect a eukaryotic cell, and allow selection of a co-transfected cell carrying the desired transfected genes.

Examples of selectable genes for use in a bacterial system are the gene that confers resistance to ampicillin and the gene that confers resistance to chloramphenicol. Preferred selectable genes for use in eukaryotic transfectants include the xanthine guanine phosphoribosyl transferase gene (designated gpt) and the phosphotransferase gene from Tn5 (designated neo).

Selection of cells expressing gpt is based on the fact that the enzyme encoded by this gene utilizes xanthine as a substrate for purine nucleotide synthesis, whereas the analogous endogenous enzyme cannot. In a medium containing (1) mycophenolic acid, which blocks the conversion of inosine monophosphate to xanthine monophosphate, and (2) xanthine, only cells expressing the gpt gene can survive. The product of the neo blocks the inhibition of protein synthesis by the antibiotic G418 and other antibiotics of the neomycin class.

The two selection procedures can be used simultaneously or sequentially to select for the expression of immunoglobulin chain genes introduced on two different DNA vectors into a eukaryotic cell. It is not necessary to include different selectable markers for eukaryotic cells; an H and an L chain vector, each containing the same selectable marker can be co-transfected. After selection of the appropriately resistant cells, the majority of the clones will contain integrated copies of both H and L chain vectors and/or anti-TNF peptides.

Alternatively, the fused genes encoding the chimeric H and L chains can be assembled on the same expression vector.

For transfection of the expression vectors and production of the chimeric antibody, the preferred recipient cell line is a myeloma cell. Myeloma cells can synthesize, assemble and secrete immunoglobulins encoded by transfected immunoglobulin genes and possess the mechanism for glycosylation of the immunoglobulin. A particularly preferred recipient cell is the recombinant Ig-producing myeloma cell SP2/0 (ATCC #CRL 8287). SP2/0 cells produce only immunoglobulin encoded by the transfected genes. Myeloma cells can be grown in culture or in the peritoneal cavity of a mouse, where secreted immunoglobulin can be obtained from ascites fluid. Other suitable recipient cells include lymphoid cells such as B lymphocytes of human or non-human origin, hybridoma cells of human or non-human origin, or interspecies heterohybridoma cells.

The expression vector carrying a chimeric antibody construct or anti-TNF peptide of the present invention can be introduced into an appropriate host cell by any of a variety of suitable means, including such biochemical means as transformation, transfection, conjugation, protoplast fusion, calcium phosphate-precipitation, and application with polycations such as diethylaminoethyl (DEAE) dextran, and such mechanical means as electroporation, direct microinjection, and microprojectile bombardment (Johnston et al., *Science* 240:1538 (1988)). A preferred way of introducing DNA into lymphoid cells is by electroporation (Potter et al., *Proc. Natl. Acad. Sci. USA* 81:7161 (1984); Yoshikawa, et al.,*Jpn. J. Cancer Res.* 77:1122–1133). In this procedure, recipient cells are subjected to an electric pulse in the presence of the DNA to be incorporated. Typically, after transfection, cells are allowed to recover in complete medium for about 24 hours, and are then seeded in 96-well culture plates in the presence of the selective medium. G418 selection is performed using about 0.4 to 0.8 mg/ml G418. Mycophenolic acid selection utilizes about 6 μg/ml plus about 0.25 mg/ml xanthine. The electroporation technique is expected to yield transfection frequencies of about 10^{-5} to about 10^{-4} for Sp2/0 cells. In the protoplast fusion method, lysozyme is used to strip cell walls from catarrhal harboring the recombinant plasmid containing the chimeric antibody gene. The resulting spheroplasts are fused with myeloma cells with polyethylene glycol.

The immunoglobulin genes of the present invention can also be expressed in nonlymphoid mammalian cells or in other eukaryotic cells, such as yeast, or in prokaryotic cells, in particular bacteria.

Yeast provides substantial advantages over bacteria for the production of immunoglobulin H and L chains. Yeasts carry out post-translational peptide modifications including glycosylation. A number of recombinant DNA strategies now exist which utilize strong promoter sequences and high copy number plasmids which can be used for production of the desired proteins in yeast. Yeast recognizes leader sequences of cloned maamalian gene products and secretes peptides bearing leader sequences (i.e., pre-peptides) (Hitzman, et al., 11*th International Conference on Yeast, Genetics and Molecular Biology*, Montpelier, France, Sep. 13–17, 1982).

Yeast gene expression systems can be routinely evaluated for the levels of production, secretion and the stability of anti-TNF peptides, antibody and assembled murine and chimeric antibodies, fragments and regions thereof. Any of a series of yeast gene expression systems incorporating promoter and termination elements from the actively expressed genes coding for glycolytic enzymes produced in large quantities when yeasts are grown in media rich in glucose can be utilized. Known glycolytic genes can also provide very efficient transcription control signals. For example, the promoter and terminator signals of the phosphoglycerate kinase (PGK) gene can be utilized. A number of approaches can be taken for evaluating optimal expression plasmids for the expression of cloned immunoglobulin cDNAs in yeast (see Glover, ed., *DNA Cloning*, Vol. II, pp45–66, IRL Press, 1985).

Bacterial strains can also be utilized as hosts for the production of antibody molecules or peptides described by this invention, *E. coli* K12 strains such as *E. coli* W3110 (ATCC 27325), and other enterobacteria such as *Salmonella typhimurium* or *Serratia marcescens*, and various Pseudomonas species can be used.

Plasmid vectors containing replicon and control sequences which are derived from species compatible with a host cell are used in connection with these bacterial hosts. The vector carries a replication site, as well as specific genes which are capable of providing phenotypic selection in transformed cells. A number of approaches can be taken for evaluating the expression plasmids for the production of murine and chimeric antibodies, fragments and regions or antibody chains encoded by the cloned immunoglobulin cDNAs in bacteria (see Glover, ed., *DNA Cloning*, Vol. I, IRL Press, 1985, Ausubel, infra, Sambrook, infra, Colligan, infra).

Preferred hosts are mammalian cells, grown in vitro or in vivo. Mammalian cells provide post-translational modifications to immunoglobulin protein molecules including leader peptide removal, folding and assembly of H and L chains, glycosylation of the antibody molecules, and secretion of functional antibody protein.

Mammalian cells which can be useful as hosts for the production of antibody proteins, in addition to the cells of lymphoid origin described above, include cells of fibroblast origin, such as Vero (ATCC CRL 81) or CHO-K1 (ATCC CRL 61).

Many vector systems are available for the expression of cloned anti TNF peptides H and L chain genes in mammalian cells (see Glover, ed., *DNA Cloning*, Vol. II, pp143–238, IRL Press, 1985). Different approaches can be followed to obtain complete H_2L_2 antibodies. As discussed above, it is possible to co-express H and L chains in the same cells to achieve intracellular association and linkage of H and L chains into complete tetrameric H_2L_2 antibodies and/or anti-TNF peptides. The co-expression can occur by using either the same or different plasmids in the same host. Genes for both H and L chains and/or anti-TNF peptides can be placed into the same plasmid, which is then transfected into cells, thereby selecting directly for cells that express both chains. Alternatively, cells can be transfected first with a plasmid encoding one chain, for example the L chain, followed by transfection of the resulting cell line with an H chain plasmid containing a second selectable marker. Cell lines producing anti-TNF peptides and/or H_2L_2 molecules via either route could be transfected with plasmids encoding additional copies of peptides, H, L, or H plus L chains in conjunction with additional selectable markers to generate cell lines with enhanced properties, such as higher production of assembled H_2L_2 antibody molecules or enhanced stability of the transfected cell lines.

Anti-idiotype Abs. In addition to monoclonal or chimeric anti-TNF antibodies, the present invention is also directed to an anti-idiotypic (anti-Id) antibody specific for the anti-TNF antibody of the invention. An anti-Id antibody is an antibody which recognizes unique determinants generally associated with the antigen-binding region of another antibody. The antibody specific for TNF is termed the idiotypic or Id antibody. The anti-Id can be prepared by immunizing an animal of the same species and genetic type (e.g. mouse strain) as the source of the Id antibody with the Id antibody or the antigen-binding region thereof. The immunized animal will recognize and respond to the idiotypic determinants of the immunizing antibody and produce an anti-Id antibody. The anti-Id antibody can also be used as an "immunogen" to induce an immune response in yet another animal, producing a so-called anti-anti-Id antibody. The anti-anti-Id can be epitopically identical to the original antibody which induced the anti-Id. Thus, by using antibodies to the idiotypic determinants of a mAb, it is possible to identify other clones expressing antibodies of identical specificity.

Accordingly, mAbs generated against TNF according to the present invention can be used to induce anti-Id antibod-

ies in suitable animals, such as BALB/c mice. Spleen cells from such immunized mice can be used to produce anti-Id hybridomas secreting anti-Id mAbs. Further, the anti-Id mAbs can be coupled to a carrier such as keyhole limpet hemocyanin (KLH) and used to immunize additional BALB/c mice. Sera from these mice will contain anti-anti-Id antibodies that have the binding properties of the original mAb specific for a TNF epitope.

Screening Methods for determining tissue necrosis factor neutralizing and/or inhibiting activity are also provided in the present invention. In the context of the present invention, TNF neutralizing activity or TNF inhibiting activity refers to the ability of a TNF neutralizing compound to block at least one biological activity of TNF, such as preventing TNF from binding to a TNF receptor, blocking production of TNF by intracellular processing, such as transcription, translation or post-translational modification, expression on the cell surface, secretion or assembly of the bioactive trimer of TNF. Additionally, TNF neutralizing compounds can act by inducing regulation of metabolic pathways such as those involving the up or down regulation of TNF production. Alternatively TNF neutralizing compounds can modulate cellular sensitivity to TNF by decreasing such sensitivity. TNF neutralizing compounds can be selected from the group consisting of antibodies, or fragments or portions thereof, peptides, peptido mimetic compounds or organo mimetic compounds that neutralizes TNF activity in vitro, in situ or in vivo is considered a TNF neutralizing compound if used according to the present invention. Screening methods which can be used to determine TNF neutralizing activity of a TNF neutralizing compound can include in vitro or in vivo assays. Such in vitro assays can include a TNF cytotoxicity assay, such as a radioimmuno assay which determine a decrease in cell death by contact with TNF, such as chimpanzee or human TNF in isolated or recombinant form, wherein the concurrent presence of a TNF neutralizing compound reduces the degree or rate of cell death. The cell death can be determined using ID50 values which represent the concentration of a TNF neutralizing compound which decreases the cell death rate by 50%. For example, MAb's A2 and cA2 are found to have ID50 about 17 mg/ml +/−3 mg/ml, such as 14–20 mg/ml, or any range or value therein. Such a TNF cytotoxicity assay is presented in example II.

Alternatively or additionally, another in vitro assay which can be used to determine neutralizing activity of a TNF neutralizing compound is an assay which measures the neutralization of TNF induced procoagulant activity, such as presented in example XI.

Alternatively or additionally, TNF neutralizing activity of a TNF neutralizing compound can be measured by an assay for the neutralization of TNF induced IL-6 secretion, such as using cultured human umbilical vein endothelial cells (HUVEC), for example. Also presented in example 11.

Alternatively or additionally, in vivo testing of TNF neutralizing activity of TNF neutralizing compounds can be tested using survival of mouse given lethal doses of Rh TNF with controlled and varied concentrations of a TNF neutralizing compound, such as TNF antibodies. Preferably galactosamine sensitive mice are used. For example, using a chimeric human anti-TNF antibody as a TNF neutralizing compound, a concentration of 0.4 milligrams per kilogram TNF antibody resulted in a 70–100% increase in survival and a 2.0 mg/kg dose of TNF antibody resulted in a 90–100% increase in survival rate using such an assay, for example, as presented in example 12.

Additionally, after TNF neutralizing compounds are tested for safety in animal models such as chimpanzees, for example as presented in Example XVII, TNF neutralizing compounds can be used to treat various TNF related pathologies, as described herein, and as presented in Examples XVIII–XXII.

Accordingly, any suitable TNF neutralizing compound can be used in methods according to the present invention. Examples of such TNF neutralizing compound can be selected from the group consisting of antibodies or portions thereof specific to neutralizing epitopes of TNF, p55 receptors, p75 receptors, or complexes thereof, portions of TNF receptors which bind TNF, peptides which bind TNF, any peptido mimetic drugs which bind TNF and any organo mimetic drugs that block TNF.

Such TNF neutralizing compounds can be determined by routine experimentation based on the teachings and guidance presented herein, by those skilled in the relevant arts.

Structural Analogs of Anti-TNF Antibodies and Anti-TNF Peptides

Structural analogs of anti-TNF Abs and peptides of the present invention are provided by known method steps based on the teaching and guidance presented herein.

Knowledge of the three-dimensional structures of proteins is crucial in understanding how they function. The three-dimensional structures of more than 400 proteins are currently available in protein structure databases (in contrast to around 15,000 known protein sequences in sequence databases). Analysis of these structures shows that they fall into recognizable classes of motifs. It is thus possible to model a three-dimensional structure of a protein based on the proteins homology to a related protein of known structure. Many examples are known where two proteins that have relatively low sequence homology, can have very similar three dimensional structures or motifs.

In recent years it has become possible to determine the three dimensional structures of proteins of up to about 15 kDa by nuclear magnetic resonance (NMR). The technique only requires a concentrated solution of pure protein. No crystals or isomorphous derivatives are needed. The structures of a number proteins have been determined by this method. The details of NMR structure determination are well-known in the art (See, e.g., Wuthrich, *NMR of Proteins and Nucleic Acids*, Wiley, New York, 1986; Wuthrich, K. *Science* 243:45–50 (1989); Clore et al., *Crit. Rev. Bioch. Molec. Biol.* 24:479–564 (1989); Cooke et al. *Bioassays* 8:52–56 (1988), which references are hereby incorporated herein by reference).

In applying this approach, a variety of ^{1}H NMR 2D data sets are collected for anti-TNF Abs and/or anti-TNF peptides of the present invention. These are of two main types. One type, COSY (Correlated Spectroscopy) identifies proton resonances that are linked by chemical bonds. These spectra provide information on protons that are linked by three or less covalent bonds. NOESY (nuclear Overhauser enhancement spectroscopy) identifies protons which are close in space (less than 0.5 nm). Following assignment of the complete spin system, the secondary structure is defined by NOESY. Cross peaks (nuclear Overhauser effects or NOE's) are found between residues that are adjacent in the primary sequence of the peptide and can be seen for protons less than 0.5 nm apart. The data gathered from sequential NOE's combined with amide proton coupling constants and NOE's from non-adjacent amino acids, that are adjacent to the secondary structure, are used to characterize the secondary structure of the polypeptides. Aside from predicting secondary structure, NOE's indicate the distance that protons are in space in both the primary amino acid sequence and the secondary structures. Tertiary structure predictions are determined, after all the data are considered, by a "best fit" extrapolation.

Types of amino acid are first identified using through-bond connectivities. The second step is to assign specific amino acids using through-space connectivities to neighboring residues, together with the known amino acid sequence. Structural information is then tabulated and is of three main kinds: The NOE identifies pairs of protons which are close in space, coupling constants give information on dihedral angles and slowly exchanging amide protons give information on the position of hydrogen bonds. The restraints are used to compute the structure using a distance geometry type of calculation followed by refinement using restrained molecular dynamics. The output of these computer programs is a family of structures which are compatible with the experimental data (i.e. the set of pairwise <0.5 nm distance restraints). The better that the structure is defined by the data, the better the family of structures can be superimposed, (i.e., the better the resolution of the structure). In the better defined structures using NMR, the position of much of backbone (i.e. the amide, Cα and carbonyl atoms) and the side chains of those amino acids that lie buried in the core of the molecule can be defined as clearly as in structures obtained by crystallography. The side chains of amino acid residues exposed on the surface are frequently less well defined, however. This probably reflects the fact that these surface residues are more mobile and can have no fixed position. (In a crystal structure this might be seen as diffuse electron density).

Thus, according to the present invention, use of NMR spectroscopic data is combined with computer modeling to arrive structural analogs of at least portions of anti-TNF Abs and peptides based on a structural understanding of the topography. Using this information, one of ordinary skill in the art will know how to achieve structural analogs of anti-TNF Abs and/or peptides, such as by rationally-based amino acid substitutions allowing the production of peptides in which the TNF binding affinity is modulated in accordance with the requirements of the expected therapeutic or diagnostic use of the molecule, preferably, the achievement of greater specificity for TNF binding.

Alternatively, compounds having the structural and chemical features suitable as anti-TNF therapeutics and diagnostics provide structural analogs with selective TNF affinity. Molecular modeling studies of TNF binding compounds, such as TNF receptors, anti-TNF antibodies, or other TNF binding molecules, using a program such as MACROMODEL®, INSIGHT®, and DISCOVER® provide such spatial requirements and orientation of the anti-TNF Abs and/or peptides according to the present invention. Such structural analogs of the present invention thus provide selective qualitative and quantitative anti-TNF activity in vitro, in situ and/or in vivo.

Therapeutic Methods for Treating TNF-Related Pathologies The anti-TNF peptides, antibodies, fragments and/or derivatives of the present invention are useful for treating a subject having a pathology or condition associated with abnormal levels of a substance reactive with an anti-TNF antibody, in particular TNF, such as TNFα or TNFβ, in excess of, or less than, levels present in a normal healthy subject, where such excess or diminished levels occur in a systemic, localized or particular tissue type or location in the body. Such tissue types can include, but are not limited to, blood, lymph, CNS, liver, kidney, spleen, heart muscle or blood vessels, brain or spinal cord white matter or grey matter, cartilage, ligaments, tendons, lung, pancreas, ovary, testes, prostrate. Increased or decreased TNF concentrations relative to normal levels can also be localized to specific regions or cells in the body, such as joints, nerve blood vessel junctions, bones, specific tendons or ligaments, or sites of infection, such as bacterial or viral infections.

TNF related pathologies include, but are not limited to, the following:

(A) acute and chronic immune and autoimmune pathologies, such as systemic lupus erythematosus (SLE) rheumatoid arthritis, thyroidosis, graft versus host disease, scleroderma, diabetes mellitus, Graves' disease, and the like;

(B) infections, including, but not limited to, sepsis syndrome, cachexia, circulatory collapse and shock resulting from acute or chronic bacterial infection, acute and chronic parasitic and/or infectious diseases, bacterial, viral or fungal, such as a HIV, AIDS (including symptoms of cachexia, autoimmune disorders, AIDS dementia complex and infections);

(C) inflammatory diseases, such as chronic inflammatory pathologies and vacsular inflammatory pathologies, including chronic inflammatory pathologies such as sarcoidosis, chronic inflammatory bowel disease, ulcerative colitis, and Crohn's pathology and vascular inflammatory pathologies, such as, but not limited to, disseminated intravascular coagulation, atherosclerosis, and Kawasaki's pathology:

(D) neurodegenerative diseases, including, but are not limited to,

demyelinating diseases, such as multiple sclerosis and acute transverse myelitis;

extrapyramidal and cerebellar disorders' such as lesions of the corticospinal system;

disorders of the basal ganglia or cerebellar disorders;

hyperkinetic movement disorders such as Huntington's Chorea and senile chorea;

drug-induced movement disorders, such as those induced by drugs which block CNS dopamine receptors;

hypokinetic movement disorders, such as Parkinson's disease;

Progressive supranucleo palsy;

Cerebellar and Spinocerebellar Disorders, such as astructural lesions of the cerebellum;

spinocerebellar degenerations (spinal ataxia, Friedreich's ataxia, cerebellar cortical degenerations, multiple systems degenerations (Mencel, Dejerine-Thomas, Shi-Drager, and Machado-Joseph); and systemic disorders (Refsum's disease, abetalipoprotemia, ataxia, telangiectasia, and mitochondrial multi.system disorder);

demyelinating core disorders, such as multiple sclerosis, acute transverse myelitis;

disorders of the motor unit, such as neurogenic muscular atrophies (anterior horn cell degeneration, such as amyotrophic lateral sclerosis, infantile spinal muscular atrophy and juvenile spinal muscular atrophy); Alzheimer's disease; Down's Syndrome in middle age; Diffuse Lewy body disease; Senile Dementia of Lewy body type; Wernicke-Korsakoff syndrome; chronic alcoholism; Creutzfeldt-Jakob disease; Subacute sclerosing panencephalitis, Hallerrorden-Spatz disease; and Dementia pugilistica, or any subset thereof;

(E) malignant pathologies involving TNF-secreting tumors or other malignancies involving TNF, such as, but not limited to leukemias (acute, chronic myelocytic, chronic lymphocytic and/or myelodyspastic syndrome); lymphomas (Hodgkin's and non-

Hodgkin's lymphomas, such as malignant lymphomas (Burkitt's lymphoma or Mycosis fungoides)); and

(F) alcohol-induced hepatitis.

See, e.g., Berkow et al, eds., The Merck Manual, 16th edition, chapter 11, pp 1380–1529, Merck and Co., Rahway, N.J., 1992, which reference, and references cited therein, are entirely incorporated herein by reference.

Such treatment comprises parenterally administering a single or multiple doses of the antibody, fragment or derivative. Preferred for human pharmaceutical use are high affinity potent hTNFα-inhibiting and/or neutralizing murine and chimeric antibodies, fragments and regions of this invention.

Anti-TNF peptides or MAbs of the present invention can be administered by any means that enables the active agent to reach the agent's site of action in the body of a mammal. In the case of the antibodies of this invention, the primary focus is the ability to reach and bind with TNF released by monocytes and macrophages or other TNF producing cells. Because proteins are subject to being digested when administered orally, parenteral administration, i.e., intravenous, subcutaneous, intramuscular, would ordinarily be used to optimize absorption.

Therapeutic Administration

Anti-TNF peptides and/or MAbs of the present invention can be administered either as individual therapeutic agents or in combination with other therapeutic agents. They can be administered alone, but are generally administered with a pharmaceutical carrier selected on the basis of the chosen route of administration and standard pharmaceutical practice.

The dosage administered will, of course, vary depending upon known factors such as the pharmacodynamic characteristics of the particular agent, and its mode and route of administration; age, health, and weight of the recipient; nature and extent of symptoms, kind of concurrent treatment, frequency of treatment, and the effect desired. Usually a daily dosage of active ingredient can be about 0.01 to 100 milligrams per kilogram of body weight. Ordinarily 1.0 to 5, and preferably 1 to 10 milligrams per kilogram per day given in divided doses 1 to 6 times a day or in sustained release form is effective to obtain desired results.

As a non-limiting example, treatment of TNF-related pathologies humans or animals can be provided as a daily dosage of anti-TNF peptides, monoclonal chimeric and/or murine antibodies of the present invention 0.1 to 100 mg/kg, such as 0.5, 0.9, 1.0, 1.1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 40, 45, 50, 60, 70, 80, 90 or 100 mg/kg, per day, on at least one of day 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40, or alternatively, at least one of week 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20, or any combination thereof, using single or divided doses of every 24, 12, 8, 6, 4, or 2 hours, or any combination thereof.

Since circulating concentrations of TNF tend to be extremely low, in the range of about 10 pg/ml in non-septic individuals, and reaching about 50 pg/ml in septic patients and above 100 pg/ml in the sepsis syndrome (Hammerle, A. F. et al., 1989, infra) or can be only be detectable at sites of TNF-mediated pathology, it is preferred to use high affinity and/or potent in vivo TNF-inhibiting and/or neutralizing antibodies, fragments or regions thereof, for both TNF immunoassays and therapy of TNF-mediated pathology. Such antibodies, fragments, or regions, will preferably have an affinity for hTNFα, expressed as Ka, of at least 10^8 M^{-1}, more preferably, at least 10^9 M^{-1}, such as 10^8–10^{10} M^{-1}, 5×10^8 M^{-1}, 8×10^8 M^{-1}, 2×10^9 M^{-1}, 4×10^9 M^{-1}, 6×10^9 M^{-1}, 8×10^9 M^{-1}, or any range or value therein.

Preferred for human therapeutic use are high affinity murine and chimeric antibodies, and fragments, regions and derivatives having potent in vivo TNFα-inhibiting and/or neutralizing activity, according to the present invention, that block TNF-induced IL-6 secretion. Also preferred for human therapeutic uses are such high affinity murine and chimeric anti-TNFα antibodies, and fragments, regions and derivatives thereof, that block TNF-induced procoagulant activity, including blocking of TNF-induced expression of cell adhesion molecules such as ELAM-1 and ICAM-1 and blocking of TNF mitogenic activity, in vivo, in situ, and in vitro.

Dosage forms (composition) suitable for internal administration generally contain from about 0.1 milligram to about 500 milligrams of active ingredient per unit. In these pharmaceutical compositions the active ingredient will ordinarily be present in an amount of about 0.5–95% by weight based on the total weight of the composition.

For parenteral administration, anti-TNF peptides or antibodies can be formulated as a solution, suspension, emulsion or lyophilized powder in association with a pharmaceutically acceptable parenteral vehicle. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Liposomes and nonaqueous vehicles such as fixed oils can also be used. The vehicle or lyophilized powder can contain additives that maintain isotonicity (e.g., sodium chloride, mannitol) and chemical stability (e.g., buffers and preservatives). The formulation is sterilized by commonly used techniques.

Suitable pharmaceutical carriers are described in the most recent edition of Remington's Pharmaceutical Sciences, A. Osol, a standard reference text in this field of art.

For example, a parenteral composition suitable for administration by injection is prepared by dissolving 1.5% by weight of active ingredient in 0.9% sodium chloride solution.

Anti-TNF peptides and/or antibodies of this invention can be adapted for therapeutic efficacy by virtue of their ability to mediate antibody-dependent cellular cytotoxicity (ADCC) and/or complement-dependent cytotoxicity (CDC) against cells having TNF associated with their surface. For these activities, either an endogenous source or an exogenous source of effector cells (for ADCC) or complement components (for CDC) can be utilized. The murine and chimeric antibodies, fragments and regions of this invention, their fragments, and derivatives can be used therapeutically as immunoconjugates (see for review: Dillman, R. O., Ann. Int. Med. 111:592–603 (1989)). Such peptides or Abs can be coupled to cytotoxic proteins, including, but not limited to ricin-A, Pseudomonas toxin and Diphtheria toxin. Toxins conjugated to antibodies or other ligands or peptides are well known in the art (see, for example, Olsnes, S. et al., *Immunol. Today* 10:291–295 (1989)). Plant and bacterial toxins typically kill cells by disrupting the protein synthetic machinery.

Anti-TNF peptides and/or antibodies of this invention can be conjugated to additional types of therapeutic moieties including, but not limited to, radionuclides, therapeutic agents, cytotoxic agents and drugs. Examples of radionuclides which can be coupled to antibodies and delivered in vivo to sites of antigen include ^{212}Bi, ^{131}I, ^{186}Re, and ^{90}Y, which list is not intended to be exhaustive. The radionuclides exert their cytotoxic effect by locally irradiating the cells, leading to various intracellular lesions, as is known in the art of radiotherapy.

Cytotoxic drugs which can be conjugated to anti-TNF peptides and/or antibodies and subsequently used for in vivo therapy include, but are not limited to, daunorubicin, doxorubicin, methotrexate, and Mitomycin C. Cytotoxic drugs interfere with critical cellular processes including DNA, RNA, and protein synthesis. For a description of these classes of drugs which are well known in the art, and their mechanisms of action, see Goodman, et al., *Goodman and Gilman's THE PHARMACOLOGICAL BASIS OF THERAPEUTICS*, 8th Ed., Macmillan Publishing Co., 1990.

Anti-TNF peptides and/or antibodies of this invention can be advantageously utilized in combination with other monoclonal or murine and chimeric antibodies, fragments and regions, or with lymphokines or hemopoietic growth factors, etc., which serve to increase the number or activity of effector cells which interact with the antibodies.

Anti-TNF peptides and/or antibodies, fragments or derivatives of this invention can also be used in combination with TNF therapy to block undesired side effects of TNF. Recent approaches to cancer therapy have included direct administration of TNF to cancer patients or immunotherapy of cancer patients with lymphokine activated killer (LAK) cells (Rosenberg et al., *New Eng. J. Med.* 313:1485–1492 (1985)) or tumor infiltrating lymphocytes (TIL) (Kurnick et al. (*Clin. Immunol. Immunopath.* 38:367–380 (1986); Kradin et al., *Cancer Immunol. Immunother.* 24:76–85 (1987); Kradin et al., *Transplant. Proc.* 20:336–338 (1988)). Trials are currently underway using modified LAK cells or TIL which have been transfected with the TNF gene to produce large amounts of TNF. Such therapeutic approaches are likely to be associated with a number of undesired side effects caused by the pleiotropic actions of TNF as described herein and known in the related arts. According to the present invention, these side effects can be reduced by concurrent treatment of a subject receiving TNF or cells producing large amounts of TIL with the antibodies, fragments or derivatives of the present invention. Effective doses are as described above. The dose level will require adjustment according to the dose of TNF or TNF-producing cells administered, in order to block side effects without blocking the main anti-tumor effect of TNF. One of ordinary skill in the art will know how to determine such doses without undue experimentation.

Treatment of Arthritis. In rheumatoid arthritis, the main presenting symptoms are pain, stiffness, swelling, and loss of function (Bennett J C. The etiology of rheumatoid arthritis. In Textbook of Rheumatology (Kelley W N, Harris E D, Ruddy S, Sledge C B, eds.) W B Saunders, Philadelphia pp 879–886, 1985). The multitude of drugs used in controlling such symptoms seems largely to reflect the fact that none is ideal. Although there have been many years of intense research into the biochemical, genetic, microbiological, and immunological aspects of rheumatoid arthritis, its pathogenesis is not completely understood, and none of the treatments clearly stop progression of joint destruction (Harris E D. Rheumatoid Arthritis: The clinical spectrum. In Textbook of Rheumatology (Kelley, et al., eds.) W B Saunders, Philadelphia pp 915–990, 1985).

TNFα is of major importance in the pathogenesis of rheumatoid arthritis. TNFα is present in rheumatoid arthritis joint tissues and synovial fluid at the protein and mRNA level (Buchan G, Barrett K, Turner M, Chantry D, Maini R N, and Feldmann M. Interleukin-1 and tumour necrosis factor mRNA expression in rheumatoid arthritis: prolonged production of IL-1_{α}. *Clin. Exp. Immunol* 73: 449–455, 1988), indicating local synthesis. However detecting TNFα in rheumatoid arthritis joints even in quantities sufficient for bioactivation does not necessarily indicate that it is important in the pathogenesis of rheumatoid arthritis, nor that it is a good candidate therapeutic target. In order to address these questions, the effects of anti-TNF antibody and peptides (rabbit or monoclonal) on rheumatoid joint cell cultures, and for comparison, osteoarthritic cell cultures, have been studied. IL-1 production was abolished, showing TNFα as a suitable therapeutic target for the therapy of rheumatoid arthritis, since anti-TNFα blocks both TNF and IL-1, the two cytokines known to be involved in cartilage and bone destruction (Brennan et al., *Lancet* 11: 244–247, 1989).

Subsequent studies in rheumatoid arthritis tissues have supported this hypothesis. Anti-TNF Abs abrogated the production of another proinflammatory cytokine, GM-CSF (Haworth et al., *Eur. J. Immunol.* 21:2575–2579, 1991). This observation has been independently confirmed (Alvaro-Gracia et al., 1991). It has also been demonstrated that anti-TNF diminishes cell adhesion and HLA class II expression in rheumatoid arthritis joint cell cultures.

Diagnostic Methods

The present invention also provides the above anti-TNF peptides and antibodies, detectably labeled, as described below, for use in diagnostic methods for detecting TNFα in patients known to be or suspected of having a TNFα-mediated condition.

Anti-TNF peptides and/or antibodies of the present invention are useful for immunoassays which detect or quantitate TNF, or anti-TNF antibodies, in a sample. An immunoassay for TNF typically comprises incubating a biological sample in the presence of a detectably labeled high affinity anti-TNF peptide and/or antibody of the present invention capable of selectively binding to TNF, and detecting the labeled peptide or antibody which is bound in a sample. Various clinical assay procedures are well known in the art, e.g., as described in *Immunoassays for the 80's*, A. Voller et al., eds., University Park, 1981.

Thus, an anti-TNF peptide or antibody, can be added to nitrocellulose, or other solid support which is capable of immobilizing cells, cell particles or soluble proteins. The support can then be washed with suitable buffers followed by treatment with the detectably labeled TNF-specific peptide or antibody. The solid phase support can then be washed with the buffer a second time to remove unbound peptide or antibody. The amount of bound label on the solid support can then be detected by known method steps.

By "solid phase support" or "carrier" is intended any support capable of binding peptide, antigen or antibody. Well-known supports or carriers, include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material can have virtually any possible structural configuration so long as the coupled molecule is capable of binding to TNF or an anti-TNF antibody. Thus, the support configuration can be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface can be flat such as a sheet, culture dish, test strip, etc. Preferred supports include polystyrene beads. Those skilled in the art will know many other suitable carriers for binding antibody, peptide or antigen, or can ascertain the same by routine experimentation.

Well known method steps can determine binding activity of a given lot of anti-TNF peptide and/or antibody. Those skilled in the art can determine operative and optimal assay conditions by routine experimentation.

Detectably labeling a TNF-specific peptide and/or antibody can be accomplished by linking to an enzyme for use in an enzyme immunoassay (EIA), or enzyme-linked immunosorbent assay (ELISA). The linked enzyme reacts with the exposed substrate to generate a chemical moiety which can be detected, for example, by spectrophotometric, fluorometric or by visual means. Enzymes which can be used to detectably label the TNF-specific antibodies of the present invention include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase.

By radioactively labeling the TNF-specific anti-bodies, it is possible to detect TNF through the use of a radioimmunoassay (RIA) (see, for example, Work, et al., *Laboratory Techniques and Biochemistry in Molecular Biology*, North Holland Publishing Company, N.Y. (1978). The radio-active isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography. Isotopes which are particularly useful for the purpose of the present invention are: ^{3}H, ^{125}I, ^{131}I, ^{35}S, ^{14}C, and, preferably, ^{125}I.

It is also possible to label the TNF-specific antibodies with a fluorescent compound. When the fluorescent labeled antibody is exposed to light of the proper wave length, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labelling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

The TNF-specific antibodies can also be detectably labeled using fluorescence-emitting metals such as ^{152}Eu, or others of the lanthanide series. These metals can be attached to the TNF-specific antibody using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) or ethylenediamine-tetraacetic acid (EDTA).

The TNF-specific antibodies also can be detectably labeled by coupling to a chemiluminescent compound. The presence of the chemiluminescently labeled antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound can be used to label the TNF-specific antibody, fragment or derivative of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

Detection of the TNF-specific antibody, fragment or derivative can be accomplished by a scintillation counter, for example, if the detectable label is a radioactive gamma emitter, or by a fluorometer, for example, if the label is a fluorescent material. In the case of an enzyme label, the detection can be accomplished by colorometric methods which employ a substrate for the enzyme. Detection can also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

For the purposes of the present invention, the TNF which is detected by the above assays can be present in a biological sample. Any sample containing TNF can be used. Preferably, the sample is a biological fluid such as, for example, blood, serum, lymph, urine, inflammatory exudate, cerebrospinal fluid, amniotic fluid, a tissue extract or homogenate, and the like. However, the invention is not limited to assays using only these samples, it being possible for one of ordinary skill in the art to determine suitable conditions which allow the use of other samples.

In situ detection can be accomplished by removing a histological specimen from a patient, and providing the combination of labeled antibodies of the present invention to such a specimen. The antibody (or fragment) is preferably provided by applying or by overlaying the labeled antibody (or fragment) to a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of TNF but also the distribution of TNF in the examined tissue. Using the present invention, those of ordinary skill will readily perceive that any of a wide variety of histological methods (such as staining procedures) can be modified in order to achieve such in situ detection.

The antibody, fragment or derivative of the present invention can be adapted for utilization in an immunometric assay, also known as a "two-site" or "sandwich" assay. In a typical immunometric assay, a quantity of unlabeled antibody (or fragment of antibody) is bound to a solid support that is insoluble in the fluid being tested and a quantity of detectably labeled soluble antibody is added to permit detection and/or quantitation of the ternary complex formed between solid-phase antibody, antigen, and labeled antibody.

Typical, and preferred, immunometric assays include "forward" assays in which the antibody bound to the solid phase is first contacted with the sample being tested to extract the TNF from the sample by formation of a binary solid phase antibody-TNF complex. After a suitable incubation period, the solid support is washed to remove the residue of the fluid sample, including unreacted TNF, if any, and then contacted with the solution containing a known quantity of labeled antibody (which functions as a "reporter molecule"). After a second incubation period to permit the labeled antibody to complex with the TNF bound to the solid support through the unlabeled antibody, the solid support is washed a second time to remove the unreacted labeled antibody. This type of forward sandwich assay can be a simple "yes/no" assay to determine whether TNF is present or can be made quantitative by comparing the measure of labeled antibody with that obtained for a standard sample containing known quantities of TNF. Such "two-site" or "sandwich" assays are described by Wide (*Radioimmune Assay Method*, Kirkham, ed., Livingstone, Edinburgh, 1970, pp. 199–206).

Other type of "sandwich" assays, which can also be useful with TNF, are the so-called "simultaneous" and "reverse" assays. A simultaneous assay involves a single incubation step wherein the antibody bound to the solid support and labeled antibody are both added to the sample being tested at the same time. After the incubation is completed, the solid support is washed to remove the residue of fluid sample and uncomplexed labeled antibody. The presence of labeled antibody associated with the solid support is then determined as it would be in a conventional "forward" sandwich assay.

In the "reverse" assay, stepwise addition first of a solution of labeled antibody to the fluid sample followed by the addition of unlabeled antibody bound to a solid support after a suitable incubation period, is utilized. After a second

incubation, the solid phase is washed in conventional fashion to free it of the residue of the sample being tested and the solution of unreacted labeled antibody. The determination of labeled antibody associated with a solid support is then determined as in the "simultaneous" and "forward" assays. In one embodiment, a combination of antibodies of the present invention specific for separate epitopes can be used to construct a sensitive three-site immunoradiometric assay.

TNF REMOVAL FROM SOLUTIONS

The murine and chimeric antibodies, fragments and regions, fragments, or derivatives of this invention, attached to a solid support, can be used to remove TNF from fluids or tissue or cell extracts. In a preferred embodiment, they are used to remove TNF from blood or blood plasma products. In another preferred embodiment, the murine and chimeric antibodies, fragments and regions are advantageously used in extracorporeal immunoadsorbent devices, which are known in the art (see, for example, *Seminars in Hematology*, 26 (2 Suppl. 1) (1989)). Patient blood or other body fluid is exposed to the attached antibody, resulting in partial or complete removal of circulating TNF (free or in immune complexes), following which the fluid is returned to the body. This immunoadsorption can be implemented in a continuous flow arrangement, with or without interposing a cell centrifugation step. See, for example, Terman, et al., *J. Immunol.* 117:1971–1975 (1976).

Having now generally described the invention, the same will be further understood by reference to certain specific examples which are included herein for purposes of illustration only and are not intended to be limiting unless otherwise specified.

EXAMPLE I

Production a Mouse Anti-Human TNF mAb

To facilitate clinical study of TNF mAb a high-affinity potent inhibiting and/or neutralizing mouse anti-human TNF IgG1 mAb designated A2 was produced.

Female BALB/c mice, 10 weeks old, were obtained from the Jackson Laboratory (Bar Harbor, Me.). Forty μg of purified *E. coli*-derived recombinant human TNF (rhTNF) emulsified with an equal volume of complete Freund's adjuvant (obtained from Difco Laboratories) in 0.4 ml was injected subcutaneously and intraperitoneally (i.p.) into a mouse. One week later, an injection of 5 μg of rhTNF in incomplete Freund's adjuvant was given i.p. followed by four consecutive i.p. injections of 10 μg of TNF without adjuvant. Eight weeks after the last injection, the mouse was boosted i.p. with 10 μg of TNF.

Four days later, the mouse was sacrificed, the spleen was obtained and a spleen cell suspension was prepared. Spleen cells were fused with cells of the nonsecreting hybridoma, Sp2/0 (ATCC CRL1581), at a 4:1 ratio of spleen cells to Sp2/0 cells, in the presence of 0.3 ml of 30% polyethylene glycol, PEG 1450. After incubation at 37° C. for 6 hours, the fused cells were distributed in 0.2 ml aliquots into 96-well plates at concentrations of 2×10^4 SP2/0 cells per well. Feeder cells, in the form of 5×10^4 normal BALB/c spleen cells, were added to each well.

The growth medium used consisted of RPMl-1640 medium, 10% heat-inactivated fetal bovine serum (FBS) (HYCLONE), 0.1 mM minimum essential medium (MEM) nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (GIBCO Laboratories) and, for selection, hypoxanthine-aminopterin-thymidine (HAT) (Boehringer Mannheim). A solid-phase radioimmunoassay (RIA) was employed for screening supernatants for the presence of mAbs specific for rhTNFα. This assay is described in Example II, below. The background binding in this assay was about 500 cpm. A supernatant was considered positive if it yielded binding of 2000 cpm or higher.

Of 322 supernatants screened, 25 were positive by RIA. Of these 25, the one with the highest binding (4800 cpm) was designated A2. Positive wells were subcloned at limiting dilution on mouse feeder cells. Upon further analysis of the supernatants in neutralization assays, A2 was found to be the only positive clone showing potent inhibiting and/or neutralizing activity. Thus, the hybridoma line A2 was selected. This line was maintained in RPMl-1640 medium with 10% FBS (GIBCO), 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

Alternatively, anti-TNF antibodies which inhibit TNF biological activity can be screened by binding to peptide including at least 5 amino acids of residues 87–108 or both residues 59–80 and 87–108 of TNF (of SEQ ID NO:1) or combinations of peptides contained therein, which are used in place of the rTNF protein, as described above.

EXAMPLE II

Characterization of an Anti-TNF Antibody of the Present Invention

Radioimmunoassays

E. coli-derived rhTNF was diluted to 1 μg/ml in BCB buffer, pH 9.6, and 0.1 ml of the solution was added to each assay well. After incubation at 4° C. overnight, the wells were washed briefly with BCB, then sealed with 1% bovine incubated with 40 pg/ml of natural (GENZYME, Boston, Mass.) or recombinant (SUNTORY, Osaka, Japan) human TNFα with varying concentrations of mAb A2 in the presence of 20 μg/ml cycloheximide at 39° C. overnight. Controls included medium alone or medium +TNF in each well. Cell death was measured by staining with naphthol blue-black, and the results read spectrophotometrically at 630 nm. Absorbance at this wave length correlates with the number of live cells present.

It was found that A2 inhibited or neutralized the cytotoxic effect of both natural and rhTNF in a dose-dependent manner (FIG. **3**).

In another experiment, the specificity of this inhibiting and/or neutralizing activity was tested. A673/6 cells were seeded at 3×10^4 cells/well 20 hr before the TNF bioassay. Two-fold serial dilutions of rhTNF, *E. coli*-derived recombinant human lymphotoxin (TNFβ), and *E. coli*-derived recombinant murine TNF were prepared. The A2 hybridoma supernatant was added to an equal volume of the diluted TNF preparations, and the mixtures were incubated at room temperature for 30 min. Aliquots of 0.1 ml were transferred to the wells containing A673/6 cells, 20 μg/ml of cycloheximide was added, and the cells were incubated at 39° C. overnight. The cells were then fixed and stained for evaluation of cytotoxicity. The results indicate that mAb A2 specifically inhibited or neutralized the cytotoxicity of rhTNFα, whereas it had no effect on human lymphotoxin (TNFβ) (FIG. **4**) or murine TNF (FIG. **5**).

Experiments were next performed to analyze the cross-reactivity of mAb A2 with TNF derived from non-human primates. Monocytes isolated from B514 (baboon), J91 (cynomolgus) and RH383 (rhesus) blood by Ficoll gradient centrifugation and adherence, were incubated at 1×10^5 cells/well in RPMl 1640 medium with 5% FBS and 2 μg/ml of *E.*

coli LPS for 3 or 16 hr at 37° C. to induce TNF production. Supernatants from duplicate wells were pooled and stored at 4° C. for less than 20 hr until the TNF bioassay was performed, as described above, using A673/6 cells. Two-fold dilutions of the culture supernatants were mixed with either medium or purified mAb A2 at a final concentration of 1 μg/ml, incubated at room temperature for 30 min and aliquots transferred to the indicator cells. The results showed that mAb A2 failed to significantly inhibit or neutralize the cytotoxic activity of TNF produced by baboon, cynomolgus and rhesus monkey monocytes.

A further experiment was conducted with chimpanzee TNF. Monocytes isolated from CH563 (chimpanzee) blood were incubated as described above to generate TNF-containing supernatants. The ability of 10 μg/ml of mAb A2 to inhibit or neutralize the bioactivity of these supernatants was assayed as above. Human TNF was used as a positive control. Results, shown in FIG. **6**, indicate that mAb A2 had potent inhibiting and/or neutralizing activity for chimpanzee TNF, similar to that for human TNF (FIG. **7**).

The inhibiting and/or neutralizing activity of mAb A2 was compared with three other murine mAbs specific for human TNF, termed TNF-1, TNF-2 and TNF-3, and a control mAb. Two-fold serial dilutions of purified mAbs were mixed with rhTNF (40 pg/ml), incubated at room temperature for 30 min, and aliquots tested for TNF bioactivity as above. It was found that mAbs TNF-1, TNF-2 and TNF-3 each had a similar moderate degree of inhibiting and/or neutralizing activity. In contrast, mAb A2 had much more potent inhibiting and/or neutralizing activity.

EXAMPLE III

General Strategy for Cloning Antibody V and C Genes

The strategy for cloning the V regions for the H and L chain genes from the hybridoma A2, which secretes the anti-TNF antibody described above, was based upon the linkage in the genome between the V region and the corresponding J (joining) region for functionally rearranged (and expressed) Ig genes. J region DNA probes can be used to screen genomic libraries to isolate DNA linked to the J regions. Although DNA in the germline configuration (i.e., unrearranged) would also hybridize to J probes, this DNA would not be linked to a Ig V region sequence and can be identified by restriction enzyme analysis of the isolated clones.

The cloning utilized herein was to isolate V regions from rearranged H and L chain genes using J_H and J_k probes. These clones were tested to see if their sequences were expressed in the A2 hybridoma by Northern analysis. Those clones that contained expressed sequence were cloned into expression vectors containing human C regions and transfected into mouse myeloma cells to determine if an antibody was produced. The antibody from producing cells was then tested for binding specificity and functionally compared to the A2 murine antibody.

EXAMPLE IV

Construction of a L Chain Genomic Library

To isolate the L chain V region gene from the A2 hybridoma, a size-selected genomic library was constructed using the phage lambda vector charon 27. High molecular weight DNA was isolated from A2 hybridoma cells and digested to completion with restriction endonuclease HindIII. The DNA was then fractionated on a 0.8% agarose gel and the DNA fragments of three different size ranges of approximately 3 kb, 4 kb and 6 kb were isolated from the gel by electroelution. The size ranges for library construction were chosen based upon the size of Hind III fragments that hybridized on a southern blot with the J_k probe. After phenol/chloroform extraction and ethanol precipitation, the DNA fragments from each size class were ligated with lambda charon 27 arms and packaged into phage particles in vitro using Gigapack Gold from Stratagene (LaJolla, Calif.).

These libraries were screened directly at a density of approximately 20,000 plaques per 150 mm petri dish using a ^{32}P-labeled J_k probe. The mouse L chain J_k probe was a 2.7 kb HindIII fragment containing all five J_k segments. The probe was labeled with ^{32}P by random priming using a kit obtained from Boehringer Mannheim. Free nucleotides were removed by centrifugation through a Sephadex G-50 column. The specific activities of the probe was approximately 10^9 cpm/μg.

Plaque hybridizations were carried out in 5×SSC, 50% formamide, 2×Denhardt's reagent, and 200 μg/ml denatured salmon sperm DNA at 42° C. for 18–20 hours. Final washes were in 0.5×SSC, 0.1% SDS at 65° C. Positive clones were identified after autoradiography.

EXAMPLE V

Construction of H Chain Genomic Library

To isolate the V region gene for the A2 H chain, a genomic library was constructed in the lambda gt10 vector system. High molecular weight DNA was digested to completion with restriction endonuclease EcoRI and fragments of approximately 7.5 kb were isolated after agarose gel electrophoresis. These fragments were ligated with lambda gt10 arms and packaged into phage particles in vitro using Gigapack Gold.

This library was screened at a density of 20,000 plaques per 150 mm plate using a J_H probe. The J_H probe was a 2 kb BamHI/EcoRI fragment containing both J3 and J4 segments. The probe was labeled as in Example III and had a similar specific radioactivity. Hybridization and wash conditions were identical to those used in Example III.

EXAMPLE VI

Cloning of the TNF-Specific V Gene Regions

Several positive clones were isolated from the H and L chain libraries after screening approximately 10^6 plaques from each library using the J_H and J_k probes, respectively. Following plaque purification, bacteriophage DNA was isolated for each positive clone, digested with either EcoRI (H chain clones) or HindIII (L chain clones) and fractionated on 1% agarose gels. The DNA was transferred to nitrocellulose and the blots were hybridized with the J_H or the J_K probe.

Several H chain clones were obtained that contained 7.5 kb EcoRI DNA encoding fragments of MAbs to the J_H probe. For the light chain libraries, several clones from each of the three size-selected libraries were isolated that contained HindIII fragments that hybridize to the J_k probe. For the L chain, several independently derived HindIII fragments of 2.9 kb from the 2 kb library hybridized with a 1250 bp mRNA from A2, but not with SP2/0 mRNA (see Example VII). In addition, several HindIII fragments derived from the 4 kb library hybridized both to the A2 mRNA and the fusion partner mRNA. A 5.7 kb HindIII fragment from the 6 kb library did not hybridize to either RNA.

The observed lengths of hybridizing A2 mRNA were the correct sizes for H and L chain mRNA, respectively. Because the RNA expression was restricted to the A2 hybridoma, it was assumed that the 7.5 kb H chain fragments and the 2.9 kb L chain fragments contained the correct V region sequences from A2. One example of each type was chosen for further study. The important functional test is the demonstration that these V regions sequences, when combined with appropriate C region sequences, are capable of directing the synthesis of an antibody with a specificity and affinity similar to that of the murine A2 antibody.

The 7.5 kb H chain fragment and the 2.9 kb L chain fragment were subcloned into plasmid vectors that allow expression of the chimeric mouse/human proteins in murine myeloma cells (see Examples VIII and IX). These plasmids were co-transfected into SP2/0 cells to ascertain if intact antibody molecules were secreted, and if so, if they were of the correct specificity and affinity. Control transfections were also performed pairing the putative anti-TNF H chain with an irrelevant, but expressed, L chain; the putative anti-TNF L chain was also paired with an irrelevant, but expressed, H chain. The results indicated that the 7.5 kb H chain fragment could be expressed, whereas the 2.9 kb L chain fragment could not. This was confirmed by DNA sequence analysis that suggested portions of the coding region were not in the proper amino acid reading frame when compared to other known L chain amino acid sequences.

Because the 2.9 kb HindIII fragment appeared not to contain a functional V gene, the 4.0 kb and 5.7 kb HindIII fragments isolated from L chain libraries were cloned into expression vectors and tested for expression of chimeric antibody after co-transfection with the 7.5 kb H chain. The 5.7 kb HindIII fragment was incapable of supporting antibody expression, whereas the 4.0 kb HindIII fragment did support antibody expression. The antibody resulting from the co-transfection of the 7.5 kb putative H chain V region and the 4.0 kb L chain V region was purified, tested in solid phase TNF binding assay, and found to be inactive. It was concluded that the V region contained on the 4.0 kb HindIII fragment was not the correct anti-TNF V regions, but was contributed to the hybridoma by the fusion partner. This was subsequently confirmed by sequence analysis of cDNA derived from the A2 hybridoma and from the fusion partner.

Other independently derived L chain clones containing 2.9 kb HindIII fragments that hybridized with A2 mRNA were characterized in more detail. Although the restriction maps were similar, the clones fell into two classes with respect to the presence or absence of an AccI enzyme site. The original (non-functional) 2.9 kb fragment (designated clone 8.3) was missing an AccI site present in some other clones (represented by clone 4.3). The DNA sequence of clone 4.3 was extremely similar to clone 8.3, but contained a single amino acid reading frame with close homology to known L chains, unlike clone 8.3. The 2.9 kb HindIII fragment from clone 4.3 was subcloned into the L chain expression vector and co-transfected with the putative anti-TNF H chain into SP2/0 cells. An antibody was synthesized, purified and tested in the solid phase TNF binding assay. This antibody bound to TNF, and therefore, the clone 4.3 L chain V region was assumed to be the correct one.

The A2 murine hybridoma has been shown to contain at least four rearranged L chain V region genes. At least two of these are expressed as proteins: clone 4.3 (the correct anti-TNF L chain gene) and the gene contained in the 4.0 kb HindIII fragment (contributed by the fusion partner). The expression of two L chains implies that the resulting antibody secreted from the murine hybridoma is actually a mixture of antibodies, some using the correct L chain, some using the incorrect L chain, and some using one of each. The presence of two different L chains in the murine A2 antibody has been confirmed by SDS gel and N-terminal protein sequence analysis of the purified antibody. Because construction of the chimeric A2 antibody involves cloning the individual H and L chain genes and expressing them in a non-producing cell line, the resulting antibody will have only the correct L chain and therefore should be a more potent antibody (see Examples X, XI and XII).

EXAMPLE VII

Northern Analysis of Cloned DNA

Cloned DNA corresponding to the authentic H and L chain V regions from the A2 hybridoma would be expected to hybridize to A2 mRNA. Non-functional DNA rearrangements at either the H or L chain genetic loci should not be expressed.

Ten μg total cellular RNA was subjected to electrophoresis on 1% agarose/formaldehyde gels (Sambrook et al, infra) and transferred to nitrocellulose. Blots were hybridized with random primed DNA probes in 50% formamide, 2×Denhardt's solution, 5×SSC, and 200 μg/ml denatured salmon sperm DNA at 42° C. for 10 hours. Final wash conditions were 0.5×SSC, 0.1% SDS at 65° C.

The subdoned DNA fragment s were labeled with ^{32}P by random priming and hybridized to Northern blots containing total RNA derived from A2 cells or from cells of SP2/0, the fusion partner parent of A2. The 7.5 kb EcoRI H chain fragment hybridized with a 2 kb mRNA from A2, but not with SP2/0 mRNA. Similarly, the 2.9 kb L chain HindIII fragment (clone 4.3) hybridized with a 1250 bp mRNA from A2, but not with SP2/0 mRNA. The observed lengths of A2 mRNA hybridizing were the correct sizes for H and L chain mRNA, respectively, confirming that the V region sequences on these DNA fragments are expressed in A2 hybridoma cells.

EXAMPLE VIII

Construction of Expression Vectors

The putative L (clone 4.3) and H chain V genes described above were joined to human kappa and gaimal constant region genes in expression vectcrs. The 7.5 kb EcoRI fragment corresponding to the putative V_H region gene from A2 was cloned into an expression vector containing the human C_{gamma1} gene and the Ecogpt gene to yield the plasmid designated pA2HG1apgpt (see FIG. **8**).

The 2.9 kb putative V_L fragment from clone 4.3 was cloned into a vector containing the human kappa C_k gene and the Ecogpt gene to allow selection in mammalian cells. The resulting plasmid was designated pA2HuKapgpt (See FIG. **8**).

EXAMPLE IX

Expression of Chimeric Antibody Genes

To express the chimeric H and L chain genes, the expression plasmids were transfected into cells of the non-producing mouse myeloma cell line, SP2/0. Plasmid DNA to be transfected was purified by centrifuging to equilibrium in ethidium bromide/cesium chloride gradients twice. Plasmid DNA (10–50 μg) was added to 10^7 SP2/0 cells in medium containing Hank's salts, and the mixture was placed in a

BIORAD electroporation apparatus. Electroporation was performed at 20 volts, following which the cells were plated in 96 well microtiter plates.

Mycophenolic acid selection was applied after 24 hours and drug resistant colonies were identified after 1–2 weeks. Resistant colonies were expanded to stable cell lines and tissue culture supernatant from these cell lines was tested for antibody using an ELISA assay with goat anti-human IgG Fc antibody and goat anti-human H+L conjugated with alkaline phosphatase (obtained from Jackson Laboratories).

The chimeric A2 antibody was purified from tissue culture supernatant by Protein A-Sepharose chromatography. The supernatant was adjusted to 0.1M Tris, 0.002M EDTA, pH 8.0 and loaded on a Protein A-Sepharose column equilibrated in the same buffer. The IgG was eluted with 0.1M citrate, pH 3.5, inhibited or neutralized with 1M Tris, and dialyzed into phosphate buffered saline (PBS).

The purified chimeric antibody was evaluated for its binding and inhibiting and/or neutralizing activity.

EXAMPLE X

Specificity of an Anti-TNF Chimeric Antibody

Since the antigen binding domain of cA2 was derived from murine A2, these mAbs would be expected to compete for the same binding site on TNF. Fixed concentrations of chimeric A2 and murine mAb A2 were incubated with increasing concentrations of murine and chimeric A2 competitor, respectively, in a 96-well microtiter plate coated with rhTNF (Dainippon, Osaka, Japan). Alkaline-phosphatase conjugated anti-human immunoglobulin and anti-mouse immunoglobulin second antibodies were used to detect the level of binding of chimeric and murine A2, respectively. Cross-competition for TNF antigen was observed in this solid-phase ELISA format (FIG. **9**). This finding is consistent with the expected identical epitope specificity of cA2 and murine A2.

The affinity constant for binding of mouse mAb A2 and cA2 to rhTNFα was determined by Scatchard analysis (see, for example, Scatchard, *Ann. N.Y. Acad. Sci.* 51:660 (1949)). The results are shown in FIG. **10**. This analysis involved measuring the direct binding of ^{125}I labelled cA2 to immobilized rhTNFα in a 96-well plate. The antibodies were each labelled to a specific activity of about 9.7 μCi/μg by the iodogen method. An affinity constant (Ka) of 0.5×10^9 liters/mole was calculated for the mouse mAb A2. Unexpectedly, the chimeric A2 antibody had a higher affinity, with a Ka of 1.8×10^9 liters/mole. Thus, the chimeric anti-TNFα antibody of the present invention was shown to exhibit a significantly higher affinity of binding to human TNFα than did the parental murine A2 mAb. This finding was surprising, since murine and chimeric antibodies, fragments and regions would be expected to have affinities that are equal to or less than that of the parent mAb.

Such high affinity anti-TNF antibodies, having affinities of binding to TNFα of at least 1×10^8 M^{-1}, more preferably at least 1×10^9 M^{-1} (expressed as Ka) are preferred for immunoassays which detect very low levels of TNF in biological fluids. In addition, anti-TNF antibodies having such high affinities are preferred for therapy of TNF-α-mediated conditions or pathology states.

The specificity of cA2 for TNF was confirmed by testing for cross-neutralization of human lymphotoxin (TNF-β). Lymphotoxin shares some sequence homology and certain biological activities, for example, tumor cell cytotoxicity, with TNF (Pennica, et al., *Nature* 312:724–729 (1984)). Cultured human A673 cells were incubated with increasing concentrations of human lymphotoxin (GENENTECH, San Francisco, Calif.) with or without 4 μg/ml chimeric A2 in the presence of 20 μg/ml cycloheximide at 39° C. overnight. Cell death was measured by vital staining with naphthol blue-black, as above. The results indicated that cA2 was ineffective at inhibiting and/or neutralizing human lymphotoxin, confirming the TNFα-specificity of the chimeric antibody.

The ability of A2 or cA2 to react with TNF from different animal species was also evaluated. As mentioned earlier, there are multiple epitopes on human TNF to which inhibiting and/or neutralizing mAbs will bind (Moller, et al., infra). Human TNF has bioactivity in a wide range of host animal species. However, certain inhibiting and/or neutralizing epitopes on human TNF are conserved amongst different animal species and others appear to be restricted to humans and chimpanzees.

Neutralization experiments utilized endotoxin-activated cell supernatants from freshly isolated human, chimpanzee, rhesus and cynomolgus monkey, baboon, pig, dog, rabbit, or rat monocytes as the TNF source. As discussed above, murine mAb A2 inhibited or neutralized activity of only human and chimpanzee TNF, and had no effect on TNF derived from other primates and lower animals. A2 also did not inhibit or neutralize the cytotoxic effect of recombinant mouse TNF.

Thus, the epitope recognized by A2 is one shared by human and chimpanzee TNFα. Chimeric A2 was also tested in this manner for cross-reactivity with monocyte-derived TNF from rat, rabbit, dog and pig, as well as with purified recombinant mouse TNFα, and natural and recombinant human TNFα. Chimeric A2 only inhibited or neutralized natural and recombinant human TNFα. Therefore, cA2 appears to share species specificity with murine A2.

EXAMPLE XI

In Vitro Activity and Neutralization Efficacy of a Chimeric Anti-TNF Antibody

Both the murine and chimeric anti-TNFα antibodies, A2 and cA2 were determined to have potent TNF-inhibiting and/or neutralizing activity. In the TNF cytotoxicity assay described above, murine A2, at a concentration of about 125 ng/ml completely inhibited or neutralized the biological activity of a 40 pg/ml challenge of rhTNFα. Two separate determinations of inhibiting and/or neutralizing potency, expressed as the 50% Inhibitory Dose (ID50) were determined to be 15.9±1.01 and 17.9±1.6 ng/ml (Mean±Std error). Thus the mAb A2 has an ID50 of about 17 ng/ml.

In this same experimental system, three other murine anti-TNFα antibodies (termed TNF-1, TNF-2 and TNF-3) of comparable binding affinity to TNF were found to have ID50 values of 1–2 orders of magnitude greater, and thus were significantly less potent in neutralization than A2.

The ability of cA2 to inhibit or neutralize human TNFα bioactivity in vitro was tested using the bioassay system described above. Cultured A673 cells were incubated with 40 pg/ml natural (Genzyme, Boston, Mass.) or recombinant (Suntory, Osaka, Japan) human TNF with or without antibody overnight as above, and cell death was measured by vital staining. As expected based upon the above results with the A2 mouse mAb, cA2 also inhibited or neutralized both natural and rhTNF in a dose-dependent manner in the cytotoxicity assay (FIG. **11**). In this assay format, levels of cA2 as low as 125 ng/ml completely abolished the toxic

activity of TNF. Upon repeated analysis, the cA2 exhibited greater TNF-inhibiting and/or neutralizing activity than did the parent murine A2 mAb. Such inhibiting and/or neutralizing potency, at antibody levels below 1 μg/ml, can easily be attained in the blood of a subject to whom the antibody is administered. Accordingly, such highly potent inhibiting and/or neutralizing anti-TNF antibodies, in particular the chimeric antibody, are preferred for therapeutic use in TNFα-mediated pathologies or conditions.

As mentioned above, TNF induces cellular secretion of IL-6. Furthermore, there is evidence that IL-6 is involved in the pathophysiology of sepsis, although the precise role of IL-6 in that syndrome is unclear (Fong, et al., *J Exp Med* 170:1627–1633 (1989); Starnes Jr., et al., *J Immunol* 145:4185–4191 (1990)). The ability of cA2 to inhibit or neutralize TNF-induced IL-6 secretion was evaluated using cultured human diploid FS-4 fibroblasts. The results in Table 2 show that cA2 was effective in blocking IL-6 secretion in cells that had been incubated overnight with TNF. TNF-induced IL-6 secretion was not inhibited in the absence of a mAb or in the presence of a control mAb specific for an irrelevant antigen.

TABLE 2

IN VITRO NEUTRALIZATION OF TNF-INDUCED IL-6 SECRETRION

	TNF Concentration (ng/ml)			
Antibody	0	0.3	1.5	7.5
None	<0.20	1.36	2.00	2.56
Control mAb	<0.20	1.60	1.96	2.16
cA2	<0.20	<9.20	<0.20	0.30

Values represent mean concentrations of IL-6 of duplicate wells, in ng/ml. RhTNF (Suntory, Osaka, Japan), with or without 4 μg/ml antibody, was added to cultures of FS-4 fibroblasts and after 18 h, the supernatant was assayed for IL-6 using the QUANTIKINE Human IL-6 Immunoassay (from R&D Systems, Minneapolis, MN). Control mAb = chimeric mouse/human IgGl anti-platelet mAb (7E3).

The ability of TNF to activate procoagulant and adhesion molecule activities of endothelial cells (EC) is thought to be an important component of pathology pathophysiology. In particular, this can be associated with the vascular damage, disseminated intravascular coagulation, and severe hypotension that is associated with the sepsis syndrome. Therefore, the ability of cA2 to block TNF-induced activation of cultured human umbilical vein endothelial cells (HUVEC) was evaluated. TNF stimulation of procoagulant activity was determined by exposing intact cultured HUVEC cells to TNF (with or without antibody) for 4 hours and analyzing a cell lysate in a human plasma clotting assay. The results in Table 3 show the expected upregulation by TNF of HUVEC procoagulant activity (reflected by a decreased clotting time). Chimeric antibody cA2 effectively inhibited or neutralized this TNF activity in a dose-dependent manner.

TABLE 3

IN VITRO NEUTRALIZATION OF TNF-INDUCED PROCOAGULANT ACTIVITY

		TNF Concentration (ng/ml)		
Antibody	μg/ml	250	25	0
None	—	64 ± 4*	63 ± 1	133 ± 13
Control Ab	10.00	74 ± 6	N.D.	178 ± 55

TABLE 3-continued

IN VITRO NEUTRALIZATION OF TNF-INDUCED PROCOAGULANT ACTIVITY

		TNF Concentration (ng/ml)		
Antibody	μg/ml	250	25	0
cA2	10.00	114 ± 5	185 ± 61	141 ± 18
cA2	3.30	113 ± 2	147 ± 3	N.D.
cA2	1.10	106 ± 1	145 ± 8	N.D.
A2	0.37	73 ± 17	153 ± 4	N.D.
cA2	0.12	64 ± 1	118 ± 13	N.D.

* Values represent mean plasma clotting time, in seconds (± S.D.). Clotting time was determined in normal human plasma after addition of the rhTNF (Dainippon, Osaka, Japan) with or without antibody-treated HUVEC lysate and Ca^{++} at 37° C. N.D. = Not done. Control Ab is a chimeric mouse/human IgGl anti-CD4 antibody.

In addition to stimulating procoagulant activity, TNF also induces surface expression of endothelial cell adhesion molecules such as ELAM-1 and ICAM-1. The ability of cA2 to inhibit or neutralize this activity of TNF was measured using an ELAM-1 specific detection radioimmunoassay. Cultured HUVEC were stimulated with 250 ng/ml rhTNF (Dainippon, Osaka, Japan) with or without antibody at 37° C. overnight in a 96-well plate format. Surface expression of ELAM-1 was determined by sequential addition of a mouse anti-human ELAM-1 mAb and ^{125}I-labelled rabbit anti-mouse immunoglobulin second antibody directly to culture plates at 4° C.

As shown in FIG. **12**, TNF induced the expression of ELAM-1 on the surface of cultured HUVEC cells, and this activity was again effectively blocked in a dose-related manner by cA2.

Finally, TNF is known to stimulate mitogenic activity in cultured fibroblasts. Chimeric A2 inhibited or neutralized TNF-induced mitogenesis of human diploid FS-4 fibroblasts cultures, confirming the potent inhibiting and/or neutralizing capability of cA2 against a broad spectrum of in vitro TNF biological activities.

EXAMPLE XII

Determination of Amino Acid Sequences (epitope) on Human TNF-α Recognized by cA2 mAb

Reagents The following reagents are readily available from commercial sources. FMOC-L-Ala-OPfp, FMOC-L-Cys(Trt)-OPfp, FMOC-L-Asp(OtBu)-OPfp, FMOC-L-Glu (OtBu)-OPfp, FMOC-L-Phe-OPfp, FMOC-Gly-OPfp, FMOC-L-His(Boc)-OPfp, FMOC-L-Ile-OPfp, FMOC-L-Lys(Boc)-OPfp, FMOC-L-Leu-OPfp, FMOC-L-Asn-OPfp, FMOC-L-Pro-OPfp, FMOC-L-Gln-OPfp, FMOC-L-Arg (Mtr)-OPfp, FMOC-L-Ser(tBu)-ODhbt, FMOC-L-Thr (tBu)-ODhbt, FMOC-L-Val-OPfp, FMOC-L-Trp-OPfp, FMOC-L-Try(tBu)-OPfp, and 1-hydroxybenotriazol (HOBT) were obtained from Cambridge Research Biochemicals. Piperidine and was obtained from Applied Biosystems, Inc. 1-Methyl-2-Pyrrolidinone (NMP) was obtained from EM Science; Methanol from J T Baker; Acetic Anhydride from Applied Biosystems, Inc., Trifluoroaccetic acid (TFA) from Applied Biosystems, Inc.; Diisopropylamne (DIEA), Triethylamine, Dithiothreitol (DTT) and Anisole from Aldrich and Hydrochloric Acid (HCl) from J T Baker.

Abbreviations: FMOC, 9-fluorenylmethoxycarbonyl; tBu t-butyl ether; OtB, t-butyl ester; Boc, t-butyloxycarbonyl; Mtr, 4-methoxy-2,3,6-trimethylbenzenesulfonyl; Trt, trityl; OPfp, pentafluorophenylester; ODhbt. oxo-benzotriazone ster;

A chimeric antibody of the present invention, designated cA2, was used to determine which portions of the TNF amino acid sequence were involved in inhibitory binding by the antibody by epitope mapping, whereby the amino acid sequences of TNF-α recognized by cA2 have been identified.

The complete primary sequence of human TNFα, according to Pennica et al, Nature 312:724–729 (1984) is shown in FIG. **13** (SEQ ID NO:1). Overlapping decapeptides beginning with every second amino acid and covering the entire amino acid sequence of human TNF-α were synthesized on polyethylene pins using the method of Gysen (Gysen et al., Peptides: Chemistry and Biological, Proceedings of the Twelfth American Peptide Symposium, p. 519–523, Ed, G. R. Marshall, Escom, Leiden, 1988). Sets of peptide pins bearing free N-terminal amino groups and acetylated N-terminal amino groups were individually prepared. Both sets of peptide pins were incubated in solutions containing the anti-TNF mAb cA2 to determine the amino acid sequences that make up the cA2 epitope on human TNF-α, as described below. FIG. **14**A shows the results of binding to the overlapping decapeptides that comprise the entire sequence of human TNFα. The O.D. (optional density) correlates directly with the increased degree of cA2 binding. FIG. **14**B shows the results of binding of cA2 to the same set of peptide pins in the presence of human TNFα. This competitive binding study delineates peptides which can show non-specific binding to cA2.

There are at least two non-contiguous peptide sequences of TNF-α recognized by cA2. Using the conventional protein numbering system wherein the N-terminal amino acid is number 1, the cA2 mAb recognizes an epitope composed at least in part of amino acids located within residues 87–108 or both residues 59–80 and 87–108 of TNF (SEQ ID NO:1). FIG. **15** presents these non-contiguous sequences within the TNF sequence.

Unexpectedly, the mAb cA2 blocks the action of TNF-α without binding to the putative receptor binding locus, which can include one or more of, e.g., 11–13, 37–42, 49–57 or 155–157 of hTNFα (of SEQ ID NO:1). Preferred anti-TNF mAbs are those that inhibit this binding of human TNF-α to its receptors by virtue of their ability to bind to one or more of these peptide sequences. These antibodies can block the activity of TNF by virtue of binding to the cA2 epitope, such binding demonstrated to inhibit TNF activity. The identification of those peptide sequences recognized by cA2 provides the information necessary to generate additional MAbs with binding characteristics and therapeutic utility that parallel the embodiments of this application.

Peptide Pin Synthesis. Using an epitope mapping kit purchased from Cambridge Research Biochemicals, Inc. (CRB), dodecapeptides corresponding to the entire sequence of human TNF-α were synthesized on polyethylene pins.

A synthesis schedule was generated using the CRB epitope mapping software. Prior to the first amino acid coupling, the pins were deprotected with a 20% piperidine in NMP solution for 30 minutes at room temperature. After deprotected, the pins were washed with NMP for five minutes at room temperature, followed by three methanol washes. Following the wash steps, the pins were allowed to air dry for at least 10 minutes.

The following procedure was performed for each coupling cycle:

1) The amino acid derivatives and the HOBT were weighted out according to the weights required in the synthesis schedule.
2) The HOBT was dissolved in the appropriate amount of NMP according to the synthesis schedule.
3) The amino acid derivatives were dissolved in the recommended amount of HOBT solution and 150 microliters were pipeted into the appropriate wells as directed by the well position sheet of the synthesis schedule.
4) The blocks containing the pins were placed into the wells, and the "sandwich" units stored in plastic bags in a 30° C. water bath for 18 hours.
5) The pins were removed from the wells and washed once (for 5 minutes) with NMP, three times (for two minutes) with methanol and air dried for 10 minutes.
6) The pins were deprotected as described above and the procedure repeated.

To acetylate the peptides on one block of pins, the peptide pins were washed, deprotected and treated with 150 microliters of a solution containing NMP; acetic anhydride:triethylamine (5:2:1) for 90 minutes at 30° C., followed by the washing procedure outlined above. The second set of peptide pins was deprotected by not acetylated to give free N-terminal amino groups.

The final deprotection of the peptides to remove the side chain protecting groups was done using a mixture of TFA:anisole:dithiothreitol, 95:2.5:2.5 (v/v/w) for four hours at ambient temperature. After deprotection, the pins were air dried for 10 minutes, followed by a 15 minute sonication in a solution of 0.1% HCl in methanol/distilled water (1:1). The pins dried over night and were then ready for testing. ELISA Assay for cA2 Binding to TNF-α Peptide PINs

Reagents: Disruption Buffer: Sodium dihydrogen phosphate (31.2 g, Sigma cat # S-0751 or equivalent) and sodium dodecylsulfate (20.0 g, Sigma cat # L-3771 or equivalent) were dissolved in 2.0 L of milliQ water. The pH was adjusted to 7.2±0.1 with 50% w/w sodium hydroxide (VWR cat # VW6730-3 or equivalent).

Blocking Buffer: Sodium dihydrogen phosphate (0.39 g, Sigma cat #S-0751 or equivalent) disodium hydrogen phosphate (1.07 g, Baker cat # 3828-1 or equivalent) and sodium chloride (8.50 g, Baker cat # 3624-5 or equivalent were dissolved in 1.0 L of milliQ water. The pH was adjusted to 7.2±0.1 with 50% w/w sodium hydroxide (VWR cat VW6730-3 or equivalent). Chicken egg albumin (10.0 g, Sigma cat #A-5503 or equivalent) and bovine serum albumin (10.0 g, Sigma, cat #A-3294 or equivalent) were dissolved at room temperature with gentle stirring. The solution was filtered, and to the solution was added Tween 20 (2.0 ml, Sigma cat #P-13.79 or equivalent). The solution was stirred gently at room temperature for 30 min, filtered and stored at 40°.

PBS/Tween 20: A 10×concentrate was prepared by dissolving sodium dihydrogen phosphate (3.90 g, Sigma cat # S-0751 or equivalent), disodium hydrogen phosphate (10.70 g, Baker cat #3828-1 or equivalent) and sodium chloride (85.0 g, Baker cat #3624-5 or equivalent) in 1.0 L of milliQ water. The pH was adjusted to 7.2±0.1 with 50% w/w sodium hydroxide (VWR cat #VW 6730 or equivalent). To the solution was added Tween 20 (5.0 mL, Sigma cat #P-1379 or equivalent), and the mixture stirred gently. Just prior to use 100 mL of this solution was diluted to 1.0 L with milliQ water.

Substrate solution: Substrate buffer was prepared by dissolving citric acid (4.20 g, Malinckrodt cat #0627 or equivalent) and disodium hydrogen phosphate (7.10 g, Baker cat #3828-1 or equivalent) in 1.0 L of milliQ water. The pH was adjusted to 5.00 with 50% w/w sodium hydroxide (VWR cat #VW6730-3 or equivalent). Immediately

prior to use an OPD substrate tablet (30 mg, Sigma cat #P-8412 or equivalent and 30% (v/v) hydrogen peroxide (40 μL, Sigma cat #P-1379 or equivalent) were added to the substrate buffer 25.0 mL). The solution was wrapped in foil and mixed thoroughly.

4 NH_2SO_4; Sulfuric acid (53 mL, EM Science cat #SX1244-5 or equivalent) was slowly added to MILLI-Q water (447 mL) and cooled to room temperature prior to use.

Equipment: Molecular Devices Model nu-max plate reader or equivalent. Scientific Products Model R4140 Oscillating table shaker and equivalent. BRANSON Model 5200 ultra-sonic bath or equivalent. FINNPIPETTE Model 4172317 multichannel pipeter or equivalent. CORNING Model 25801 96 well disposable polystyrene Elisa Plates.

Prior to use and after each subsequent use the peptide pins were cleaned using the following procedure. Disruption buffer (2.0 L) was heated to 60° and placed in an ultra-sonic bath in a fume hood. To the disruption buffer was added dithiolthreitol (2.5 g, Sigma cat #D-0632 or equivalent). The peptide pins were sonicated in this medium for 30 min, washed thoroughly with milliQ waster, suspended in a boiling ethanol bath for 2 min, and air-dried.

Blocking buffer (200 μL) was added to a 96 well disposable polystyrene Elisa plate and the peptide pins suspended in the wells. The peptide pins and plate were incubated for 2 h at room temperature on an oscillating table shaker. The plates and peptide pins were washed with PBS/Tween 20 (four times). To each well was added a 20 μg/ml concentration of cA2 antibody (diluted with blocking buffer, 175 μL/well). TNF competition was done by incubation of TNFα (40 μg/ml) and cA2 (20 μg/ml) in BSA/ovalbumin/BBS for three hours at room temperature. The peptide pins were suspended in the plate and incubated at 4° overnight. The peptide pins and plate were washed with PBS/Tween 20 (four times). To each well was added anti-human goat antibody conjugated to horseradish peroxidase (diluted with blocking buffer to 1/2000, 175 μL/well, Jackson IMMUNORESEARCH Labs). The peptide pins were suspended in the plate, and incubated for 1 h at room temperature on a oscillating table shaker. The plates and peptide pins were washed with PBS/Tween 20 (four times). To each well added freshly prepared substrate solution (150 μL/well), the peptide pins were suspended in the plate and incubated for 1 h at room temperature on an oscillating table shaker. The peptide pins were removed and to each well is added 4N H_2SO_4 (50 μL). The plates were read in a Molecular Devices plate reader (490 nm, subtracting 650 nm as a blank), and the results are shown in FIGS. **14**A and **14**B, as described above.

EXAMPLE XIII

Production Mouse Anti-Human TNF mAb Using TNF Peptide Fragments

Female BALB/c mice, as in Example I above, are injected subcutaneously and intraperitoneally (i.p.) with forty μg of purified *E. coli*-derived recombinant human TNF (rhTNF) fragments comprising anti-TNF epitopes of at least 5 amino acids located within the non-contiguous sequence 59–80, 87–108 or both residues 59–80 and 87–108 of TNF (of SEQ ID NO:1), as presented above, emulsified with an equal volume of complete Freund's adjuvant (obtained from Difco Laboratories) in 0.4 ml is into a mouse. One week later, a booster injection of 5 μg of these rhTNF fragments in incomplete Freund's adjuvant is given i.p. followed by four consecutive i.p. injections of 10 μg of TNF fragments including anti-TNF epitopes including amino acids from residues 59–80, 87–108 or both 59–80 and 87–108 of hTNFα (of SEQ ID NO:1) without adjuvant. Eight weeks after the last injection, the mouse is boosted i.p. with 10 μg of TNF.

Four days later, the mouse is sacrificed, the spleen is obtained and a spleen cell suspension is prepared. Spleen cells are fused with cells of the nonsecreting hybridoma, Sp2/0 (ATCC CRL1581), at a 4:1 ratio of spleen cells to Sp2/0 cells, in the presence of 0.3 ml of 30% polyethylene glycol, PEG 1450. After incubation at 37° C. for 6 hours, the fused cells are distributed in 0.2 ml aliquots into 96-well plates at concentrations of 2×10^4 SP2/0 cells per well. Feeder cells, in the form of 5×10^4 normal BALB/c spleen cells, are added to each well.

The growth medium used consisted of RPMl-1640 medium, 10% heat-inactivated fetal bovine serum (FBS) (Hyclone), 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (GIBCO Laboratories) and, for selection, hypoxanthine-aminopterin-thymidine (HAT) (Boehringer Mannheim). A solid-phase radioimmunoassay (RIA) is employed for screening supernatants for the presence of mabs specific for rhTNFα fragments including portions of residues 59–80, 87–108 or both 59–80 and 87–108 of hTNFα (of SEQ ID NO:1). This assay is described in Example II, above. The background binding in this assay is about 500 cpm. A supernatant is considered positive if it yielded binding of 2000 cpm or higher.

Of the supernatants screened, one or more positive supernatants are routinely identified by RIA. Of these positive supernatants, the highest binding (as shown by the higher cpm values) are subcloned at limiting dilution on mouse feeder cells. Upon further analysis of the supernatants in neutralization assays, routinely one or more antibodies are found to have potent inhibiting and/or neutralizing activity. These positive and inhibiting and/or neutralizing hybridoma-lines are then selected and maintained in RPMl-1640 medium with 10% FBS (GIBCO), 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

EXAMPLE XIV

Production of Murine and Chimeric Antibodies, Fragments and Regions from TNF Peptides

Murine and chimeric antibodies, fragments and regions are obtained by construction of chimeric expression vectors encoding the mouse variable region of antibodies obtained in Example XIII and human constant regions, as presented in Examples IV–IX above.

The resulting chimeric A2 antibody is purified from tissue culture supernatant by Protein A-Sepharose chromatography. The supernatant is adjusted to 0.1M Tris, 0.002M EDTA, pH 8.0 and loaded on a Protein A-Sepharose column equilibrated in the same buffer. The IgG is then eluted with 0.1M citrate, pH 3.5, neutralized with 1M Tris, and dialyzed into phosphate buffered saline (PBS).

The purified murine and chimeric antibodies, fragments and regions are evaluated for its binding and inhibiting and/or neutralizing activity.

EXAMPLE XV

In Vitro Activity and Neutralization Efficacy of a Chimeric Anti-TNF Antibody

Both the murine and chimeric anti-TNFα antibodies of the present invention, as obtained according to Examples

XIII and XIV, are determined to have potent TNF-inhibiting and/or neutralizing activity, as shown for example, in the TNF cytotoxicity assay described above, expressed as the 50% Inhibitory Dose (ID50).

In this same experimental system, three other murine anti-TNFα antibodies (termed TNF-1, TNF-2 and TNF-3) of comparable binding affinity to TNF are found to have ID50 values of 1–2 orders of magnitude greater, and thus have significantly less potent in neutralization, than both the murine and chimeric anti-TNFα antibodies of the present invention.

The ability of both the murine and chimeric anti-TNFα antibodies of the present invention, as obtained according to Examples XIII and XIV, to inhibit or neutralize human TNFα bioactivity in vitro is tested using the bioassay system described above. Cultured cells producing the murine or chimeric anti-TNFα antibodies of the present invention, as obtained according to Examples XIII and XIV, are incubated with 40 pg/ml natural (Genzyme, Boston, Mass.) or recombinant (Suntory, Osaka, Japan) human TNF with or without antibody overnight as above, and cell death is measured by vital staining. As expected, both the murine and chimeric anti-TNFα antibodies of the present invention, as obtained according to Examples XIII and XIV, inhibited or neutralized both natural and rhTNF in a dose-dependent manner in the cytotoxicity assay. Such inhibiting and/or neutralizing potency, at antibody levels below 1 μg/ml, can easily be attained in the blood of a subject to whom the antibody is administered. Accordingly, such highly potent inhibiting and/or neutralizing anti-TNF antibodies, in particular the chimeric antibody, are preferred for therapeutic use in TNFα-mediated pathologies or conditions.

The ability of cA2 to inhibit or neutralize TNF-induced IL-6 secretion is evaluated using cultured human diploid FS-4 fibroblasts. The results are expected to show that both murine and chimeric anti-TNFα antibodies of the present invention, as obtained according to Examples XIII and XIV, are effective in blocking IL-6 secretion in cells that had been incubated overnight with TNF. TNF-induced IL-6 secretion is not inhibited in the absence of a mAb or in the presence of a control mAb specific for an irrelevant antigen.

The ability of TNF to activate procoagulant and adhesion molecule activities of endothelial cells (EC) is thought to be an important component of pathology pathophysiology. In particular, this can be associated with the vascular damage, disseminated intravascular coagulation, and severe hypotension that is associated with the sepsis syndrome. Therefore, the ability of both the murine and chimeric anti-TNFα antibodies of the present invention, as obtained according to Examples XIII and XIV, to block TNF-induced activation of cultured human umbilical vein endothelial cells (HUVEC) is evaluated. TNF stimulation of procoagulant activity is determined by exposing intact cultured HUVEC cells to TNF (with or without antibody) for 4 hours and analyzing a cell lysate in a human plasma clotting assay. The results are expected to show the expected upregulation by TNF of HUVEC procoagulant activity (reflected by a decreased clotting time). Both the murine and chimeric anti-TNFα antibodies of the present invention, as obtained according to Examples XIII and XIV, are expected to effectively inhibit or neutralize this TNF activity in a dose-dependent manner.

In addition to stimulating procoagulant activity, TNF also induces surface expression of endothelial cell adhesion molecules such as ELAM-1 and ICAM-1. The ability of both the murine and chimeric anti-TNFα antibodies of the present invention, as obtained according to Examples XIII and XIV, are expected to inhibit or neutralize this activity of TNF is measured using an ELAM-1 specific detection radioimmunoassay. Cultured HUVEC are stimulated with 250 ng/ml rhTNF (Dainippon, Osaka, Japan) with or without antibody at 37° C. overnight in a 96-well plate format. Surface expression of ELAM-1 is determined by sequential addition of a mouse anti-human ELAM-1 mAb and ^{125}I-labelled rabbit anti-mouse immunoglobulin second antibody directly to culture plates at 4° C.

TNF is expected to induce the expression of ELAM-1 on the surface of cultured HUVEC cells, and this activity is again expected to be effectively blocked in a dose-related manner by both the murine and chimeric anti-TNFα antibodies of the present invention, as obtained according to Examples XIII and XIV.

Finally, TNF is known to stimulate mitogenic activity in cultured fibroblasts. Both the murine and chimeric anti-TNFα antibodies of the present invention, as obtained according to Examples XIII and XIV, are expected to inhibit or neutralize TNF-induced mitogenesis of human diploid FS-4 fibroblasts cultures, confirming the potent inhibiting and/or neutralizing capability of both the murine and chimeric anti-TNFα antibodies of the present invention, as obtained according to Examples XIII and XIV against a broad spectrum of in vitro TNF biological activities.

EXAMPLE XVI

In Vivo Activity and Efficacy of cA2 Antibody

Evidence that the potent in vitro inhibiting and/or neutralizing capability of cA2 is manifest in vivo was obtained. Earlier animal studies showed that administration of TNF to experimental animals mimics the pathology state obtained with either Gram-negative bacterial infection or direct endotoxin administration (Tracey, et al., 1986. infra; Tracey, et al., 1987, infra; Lehmann, et al., infra).

An in vivo model wherein lethal doses of human TNF are administered to galactosamine-sensitized mice (Lehmann, V. et al., infra) is substantially modified for testing the capability of both the murine and chimeric anti-TNFα antibodies of the present invention, as obtained according to Examples XIII and XIV above, to inhibit or neutralize TNF in vivo. An i.p. challenge with 5 μg (0.25 mg/kg) of rhTNF resulted in 80–90 percent mortality in untreated control animals and in animals treated i.v. 15–30 minutes later with either saline or 2 mg/kg control antibody (a chimeric IgG1 derived from murine 7E3 anti-platelet mAb). In contrast, treatment with both the murine and chimeric anti-TNFα antibodies of the present invention, as obtained according to Examples XIII and XIV, is expected to reduce mortality to 0–30 percent with 0.4 mg/kg of antibody, and to 0–10 percent with 20 mg/kgs. These expected results indicate that both the murine and chimeric anti-TNFα antibodies of the present invention, as obtained according to Examples XIII and XIV, are capable of inhibiting and/or neutralizing the biological activity of TNF in vivo as well as in vitro.

TABLE 4

PREVENTION OF HUMAN TNF-INDUCED LETHALITY BY CHIMERIC A2

Anitbody	Outcome (Survivors/Total) Experiment #1	Experiment #2
None	1/10	N.D.
Control Ab, 2 mg/kg	2/10	1/10
cA2 (2 mg/kg) (p = 0.0001)	9/10 (p = 0.0055)	10/10
cA2 (0.4 mg/kg) (p = 0.0001)	7/10 (p = 0.07)	10/10

Female C3H/HeN mice were administered 5 μg rhTNF (Dainippon, Osaka, Japan) + 18 mg galactosamine i.p. and antibody was administered 15–30 minutes later i.v. Deaths were recorded 48 h post-challenge. Control MAb = chimeric mouse/human IgGl anti-platelet MAb (7E3). N.D. = not done. p values refer to comparison with the control Ab.

EXAMPLE XVII

cA2 MAb Safety in Chimpanzees

The epitope specificity of A2 can be for an epitope which predominates in humans and chimpanzees. Therefore, the chimpanzee was chosen as a relevant mammalian species to determine the toxicological potential and provide safety information for cA2. Chimpanzees were dosed at levels of 15 mg/kg for four to five consecutive days and 30 mg/kg once or for three consecutive days. No adverse clinical signs, and no changes considered to be cA2 treatment related were observed in the monitored parameters including routine hematology and blood chemistry. Thus, doses of up to 30 mg/kg for three consecutive days were well tolerated in chimpanzees.

EXAMPLE XVIII

Clinical Activity and Efficacy of cA2 Antibody

Chimeric IgG1 anti-human TNF MAb cA2 was administered to healthy male human volunteers as patients. One hour after receiving 4 ng/kg of an NIH reference endotoxin, the volunteers were administered either saline, as a control, or 0.01, 0.10 or 10 mg/kg of cA2 in a pharmaceutically acceptable form. TNF levels in serum were measured over time and were found to show a dose dependent decrease in peak TNF levels with no TNF being detected in volunteers receiving a 10 mg/kg dose of cA2. Accordingly, therapy with an anti-TNF antibody of the present invention is expected to inhibit TNF-mediated effects in humans.

Patients receiving endotoxin developed pronounced leukopenia thought to be due to margination of white blood cells. As the white blood cells become activated, they can attach to endothelial receptors with resultant endothelial damage. At doses of 1.0 to 10.0 mg/kg, this leukopenia is prevented, whereas, at 0.01 and 0.1 mg/kg dosages, a drop in white cell count was observed. The drop was most pronounced among the polymorph cell line. In all patients there was a subsequent leukocytosis, which was unchanged by treatment with anti-TNF anti-body cA2. This blocking effect on white blood cell margination is expected to represent a protective effect against the endothelial damage associated with TNF. It is expected in the art that this TNF-related endothelial damage plays a significant role in the morbidity and mortality associated with sepsis, and it is therefore expected that the anti-TNF antibodies of the present invention will provide a protective effect against these damaging effects, as presented herein.

EXAMPLE XIX

Treatment of Sepsis in Humans Using a Chimeric Anti-TNF Antibody

The chimeric anti-TNF MAb cA2 has been used in two phase I/II studies. In a phase I/II study in septic patients, 20 patients with the sepsis syndrome received a single dose of either 0.1, 1.0, 5.0 or 10 milligrams of cA2 per kilogram bodyweight. Another 60 patients received 100 milligrams of HA-1A, a human anti-lipid A Mab currently under evaluation for gram negative sepsis, followed with either placebo or 1.0, 5.0, or 10 milligrams cA2 per kilogram bodyweight. The cA2 was administered as a single, intravenous infusion over a 60 minute period. Clinical assessment, vital signs, and laboratory parameters were measured before, during and periodically for 28 days after the infusion. In this study, cA2 was well tolerated. No adverse events were reported as "probably" or "definitely" related to cA2. All deaths were reported as "definitely not" related to cA2.

Accordingly, human treatment of rheumatoid arthritis in human patients was expected, and found, to provide a suitable treatment, as described herein.

EXAMPLE XX

CLINICAL TREATMENT OF RHEUMATOID ARTHRITIS BY A ANTI-TNF ANTIBODY OR PEPTIDE OF THE PRESENT INVENTION

A Phase I open label study was conducted for methods and compositions of the present invention using a chimeric anti-TNF MAb for the treatment of patients with severe refractory rheumatoid arthritis. Nine patients were enrolled in the study. The first five patients were treated with chimeric anti-TNF antibody (cA2), 10 mg/kg as a single dose infused over a period of two hours. These patients were subsequently retreated with a second infusion of 10 mg/kg on day 14 of the study. The second group of five patients received an infusion of 5 mg/kg on the first day of the study. They were then treated with additional infusions of 5 mg/kg on days 5, 9, and 13. Four of the planned five patients in this second group have been treated to date. Preparation, Administration, and Storage of Test Material The chimeric monoclonal anti-TNF antibody was supplied in single-use glass vials containing 20 mL with 100 mg of anti-TNF (5 mg/mL). The anti-TNF antibody was stored at 2–8° C. Prior to infusion, the antibody was withdrawn from the vials and filtered through a low-protein-binding 0.22 μm filter. This filtered antibody was then diluted to a final volume of 300 mL with normal saline. The 300 mL antibody preparation was then infused via an in-line filter over a period of not less than two hours.

Prior to each repeat infusion of study medication a test dose of 0.1 mL of the infusion was diluted in 10 mL of normal saline and administered by slow IV push over 5 minutes. The patient was observed for 15 minutes for signs or symptoms of an immediate hypersensitivity reaction. If no reaction was observed in this time period, the full dose was administered as described above.

Administration Protocol

Group 1 (patients 1–5): a total of 2 infusions, on day 1 and day 15 of the trial; dosage 10 mg/kg on each occasion;

Group 2 (patients 6–9): a total of 4 infusions, on days 1, 5, 9 and 13 of the trial; dosage 5 mg/kg on each occasion.

All infusions were administered iv over 2 hours in a total volume of cA2+saline of 300 ml. Infusions subsequent to the

first in any patient were preceded by a small test dose administered as an iv push. All patients had at least three years of disease activity with rheumatoid arthritis. The patients ranged in age from 23 to 63. All patients had failed therapy with at least three different DMARD (Disease Modifying Anti-Rheumatic Drug). Six of the nine patients had serum rheumatoid factors, and all nine patients had erosions present on X-rays.

Clinical Monitoring

Patients were monitored during and for 24 hours after infusions for hemodynamic change, fever or other adverse events. Clinical and laboratory monitoring for possible adverse events was undertaken on each follow-up assessment day. Clinical response parameters were performed at the time-points as specified in the flow charts present in Table 9A and Table 9B. These evaluations were performed prior to receiving any infusions.

Clinical response studies will be comprised of the following parameters:

1. Number of tender joints and assessment of pain/tenderness

The following scoring will be used:

0=No pain/tenderness

1=Mild pain. The patient says it is tender upon questioning.

2=Moderate pain. The patient says it is tender and winces.

3=Severe pain. The patient says it is tender and winces and withdraws.

2. Number of swollen joints

Both tenderness and swelling will be evaluated for each joint separately. MCP's, PIP's etc. will not be considered as one joint for the evaluation.

3. Duration of morning stiffness (in minutes)

4. Grip strength

5. Visual analog pain scale (0–10 cm)

6. Patients and blinded evaluators will be asked to assess the clinical response to the drug. Clinical response will be assessed using a subjective scoring system as follows:

5=Excellent response (best possible anticipated response)

4=Good response (less than best possible anticipated response)

3=Fair response (definite improvement but could be better)

2=No response (no effect)

1=Worsening (disease worse)

Measurement of index of disease activity is scored according to the following Table 5.

TABLE 5

Clinical characteristics of patients 1–5

Patient Number	Age/ Sex	Disease Duration (years)	Rheumat. Factor	Erosions/ Nodules	Previous Treatment (DMARDs only)	Concomitant Anti-rheumatic Theraphy
01	48/F	7	+ve	+ve/+ve	*Sal,DP, Myo,Aur, MTX, Aza, Chl.	**Pred 5 mg
02	63/F	7	−ve	+ve/−ve	Sal,Myo, DP.	Para 1–2 g
03	59/M	3	+ve	+ve/−ve	Aur,Chl, Myo,MTX, Sal.	Pred 10 mg Ind 225 mg

TABLE 5-continued

Clinical characteristics of patients 1–5

Patient Number	Age/ Sex	Disease Duration (years)	Rheumat. Factor	Erosions/ Nodules	Previous Treatment (DMARDs only)	Concomitant Anti-rheumatic Theraphy
04	56/M	10	+ve	+ve/−ve	Myo,DP, Aza,Sal.	Pred 12.5 mg Ibu 2 g, Para 1–2 g
05	28/F	3	+ve	+ve/−ve	Myo,Sal, DP,Aza.	Pred 8 mg, Para 1–2 g Cod 16 mg

*Sal = Sulphasalazine; DP = D-penicillamine; Myo = Myocrisin; Aur = auranofin; MTX = methotrexate; Aza = azathioprine; Chl = hydroxychloroquine.

**Pred = prednisolone (dosage/day); Para = paracetamol; Ind = Indomethacin; Ibu-ibuprofen; Cod = codeine phosphate.

TABLE 6

Clinical characteristics of patients 6–9

Patient Number	Age/ Sex	Disease Duration (years)	Rheumat. Factor	Erosions/ Nodules	Previous Treatment (DMARDs only)	Concomitant Anti-rheumatic Theraphy
06	40/M	3	+ve	+ve/−ve	*Sal,Chl, Aur.	**Nap 1 g
07	54/F	7	−ve	+ve/−ve	DP,Myo, Sal,Aza, MTX.	Para 1–2 g Cod 16–32 mg
08	23/F	11	+ve	+ve/−ve	Chl,Myo, Sal,MTX, Aza.	Pred 7.5 mg Dicl 100 mg Para 1–2 g Dext 100–200 mg
09	51/F	15	−ve	+ve/+ve	Myo,Chl, DP,MTX.	Pred 7.5 mg Dicl 125 mg, Para 1–3 g

*Sal = Sulphasalazine; Chl = chloroquine or hydroxychloroquine; Aur = auranofin; DP = D-penicillamine; Myo = Myocrisin; Aza = azathioprine; MTX = methotrexate.

**Nap = naprosyn (dosage/day); Para = paracetamol; Cod = codeine phosphate; Pred = prednisolone; Dicl = diclofenac; Dext = dextropropoxyphene.

TABLE 7

Disease activity at entry for patients 1–5

Patient IDA Number	Morning Stiffness (mins)	Pain (0–10 cm on VAS)	Number Swollen Joints (0–28)	Ritchie Articular Index (0–69)	Grip Strength L/R (mm/Hg; max 300)	ESR (mm/hr normal ranges: F < 15; M < 10)	CRP (mg/l; normal range: < 10)	(range: 1–4)
01	60	3.9	19	30	108/107	35	5	2.67
02	20	2.7	25	31	67/66	18	14	2.0
03	90	4.9	14	16	230/238	48	44	2.5
04	30	6.9	17	12	204/223	24	35	2.33
05	90	5.7	28	41	52/89	87	107	3.0

TABLE 8

Disease activity at entry tor patients 6–9

Patient IDA Number	Morning Stiffness (mins)	Pain (0–10 cm on VAS)	Number Swollen Joints (0–28)	Ritchie Articular Index (0–69)	Grip Strength L/R (mm/Hg; max 300)	ESR (mm/hr normal ranges: F < 15; M < 10)	CRP (mg/l; normal range: < 10)	(range: 1–4)
06	120	5.0	3	4	260/280	23	33	2.33
07	105	7.4	27	31	59/80	25	10	2.83
08	270	9.3	17	37	73/125	35	31	3.17
09	180	4.5	20	26	53/75	15	33	2.5

All patients have tolerated the infusions of chimeric anti-CD4 and no serious adverse reactions have been observed. Specifically, no episodes of hemodynamic instability, fevers, or allergic reactions were observed in association with the infusions. Patients have not experienced any infections.

Although this is a non-blinded study, all patients experienced improvement in their clinical assessments of disease status, as well in biochemical parameters of inflammation measured in their serum.

Clinical assessments, including the duration of early morning stiffness; the assessment of pain on a visual analogue scale; total count of swollen joints; Ritchie articular index (a scaled score which assesses the total number of tender joints and the degree of joint tenderness); and Index of Disease Activity (a scaled score which incorporates several clinical and laboratory parameters), showed impressive improvements compared to controls. These improvements were typically in the range of an 80% drop from the baseline score; a degree of improvement which is well beyond the amount of improvement that can be attributed to placebo response. In addition, the duration of these improvements was for six to eight weeks in most cases, a duration of response far longer than would be anticipated from a placebo.

The improvements in clinical assessments were corroborated by improvements in biochemical inflammatory parameters measured in serum. The patients showed rapid drops of serum C-reactive protein, usually in the range of 80% from the baseline. Reductions in the erythrocyte sedimentation rate, usually in the range of 40%, were also observed. Circulating soluble TNF receptors were also decreased following therapy. The durations of the biochemical responses were similar to the duration of the clinical responses.

Preliminary assessment of immune responses to the chimeric anti-TNF antibody has shown no antibody response in the first four patients.

In summary, the preliminary evaluation of the results of this Phase I trial indicate that treatment of patients with advanced rheumatoid arthritis with anti-TNF MAb of the present invention is well tolerated and anti-TNF treatment is associated with rapid and marked improvement in clinical parameters of disease activity, including early morning stiffness, pain, and a number of tender and swollen joints; and is accompanied by improvement of biochemical parameters of inflammation.

Although this was an open label study, the magnitude of the clinical improvements is well beyond the degree of improvement that would be anticipated from a placebo response, such that the present invention is shown to have significant clinical efficacy for treating rheumatoid arthritis.

TABLE 9A

Flowchart for CHIMERIC ANTI-TNF STUDY C0168TRA
Group I (10 mg/kg at day 1 and day 14)

	Pre Scr	Screening	Wk d1	0 d2	Wk 1	Wk 2 d14	Wk 3	Wk 4	Wk 6	Wk 8
Consent	x									
Demography		x								
Physical Examination		x								x
Pregnancy Test		x								
Weight		x	x			x				x
Vital Signs		x	x*	x	x	x*	x	x	x	x
Anti-TNF Infusion			x			x				
Labs, see Chart		x	x'	x	x	x'	x	x	x	x
Clinical (Safety)				x	x	x'	x	x	x	x
Clinical (Response)		x	x'		x	x'	x	x	x	x
Synovial biopsy		x			x7					
Response evaluation										x

	Screening	Wk d1	0 d2	Wk 1	Wk 2 d14	Wk 3	Wk 4	Wk 6	Wk 8	
Hematology + ESR	x	x'		x	x'	x	x	x	x	
Biochemistry	x	x'		x	x'	x	x	x	x	
Urinalysis		x'		x	x'	x	x	x	x	
CRP + RF		x'		x	x'	x	x	x	x	
Serum Cytokines		x'		x	x'	x	x	x	x	
PBL		x	x	x						
Pharmacokinetics		x#	x#		x$					
HACA response		x'		x	x'	x	x	x	x	x

x* = vital signs will be obtained prior to infusion, every 30 minutes during the infusion and every 30 minutes for 2 hours after the infusion.
x' = Needs to be done prior to the infusion.
x# = Serum samples will be obtained prior to the infusion and at 1, 2, 4, 8, 12, and 24 hours after the end of the infusion.
x$ = Serum samples will be obtained to the infusion and at 2 hours

TABLE 9B

Flowchart for CHIMERIC ANTI-TNF STUDY C0168TRA
Group II2 (mg/kg every 4 days, 4 times total)

	Pre Scr	Screening	d1	wk d2	0 d5	+ d9	1 d13	wk 2	wk 3	wk 4	wk 6	wk 8	
Consent	x												
Demography		x											
Physical exam		x										x	
Pregnancy test		x											
Weight		x	x		x	x	x					x	
Vital signs		x	x*	x	x*	x*	x*	x	x	x	x	x	
Anti-TNF Infusion			x		x	x	x						
Labs, see chart		x	x'	x	x'	x'	x'	x	x	x	x	x	x
Clinical Safety				x	x'	x'	x'	x	x	x	x	x	
Clinical Response		x	x'			x'		x	x	x	x	x	
Synovial Biopsy		x					x7						
Response Evaluation												x	
Hematology + ESR		x	x'			x'		x	x	x	x	x	
Biochemistry		x	x'			x'		x	x	x	x	x	
Urinalysis			x'			x'		x	x	x	x	x	
CRP + RF			x'			x'		x	x	x	x	x	
Cytokines			x'			x'		x	x	x	x	x	
PBL			x	x		x				x			

TABLE 9B-continued

Flowchart for CHIMERIC ANTI-TNF STUDY C0168TRA
Group II2 (mg/kg every 4 days, 4 times total)

	Pre Scr	Screening	d1	wk d2	0 d5	+ d9	1 d13	wk 2	wk 3	wk 4	wk 6	wk 8
Pharmaco-kinetics			x#	x#	x$	x$	x$					
HACA Response			x'			x'		x	x	x	x	x

x* = Vital signs will be obtained prior to infusion, every 30 minutes during the infusion and every 30 minutes for 2 hours after infusion.
x' = Needs to be done prior to the infusion.
x# = Serum samples will be obtained to the infusion and at 1, 2, 4, 8, 12, and 24 hours after the end of the infusion.
x$ = Serum samples will be obtained prior to the infusion and at 2 hours after the end of the infusion.

TABLE 10

Measurement of the index of disease activity (DA)
Variables of Disease Activity

IDA score	Morning Stiffness (min)	Pain (VAS, cm)*	Grip Strength (mm-Hg)	Ritchie Articular Index	Hemoglobin (g/dl) Male	Hemoglobin (g/dl) Female	ESR
1	<10	0–2.4	>200	0	>14.1	>11.7	0–20
2	10–30	2.5–4.4	50–200	1–7	13–14	10.8–11.6	21–45
3	31–120	4.5–6.4	30–49	8–17	10–12.9	8.4–10.7	46–80
4	>120	6.5–10	<30	>18	<9.9	<8.3	>81

*Pain was measured on a visual analog scale (VAS) 0–10 cm.

Conclusions (1)

Safety of anti-TNF in RA

Anti-TNF was safe and very well tolerated:

no hemodynamic, febrile or allergic episodes;

no infections;

no clinical adverse events;

a single laboratory adverse event only, probably unrelated to anti-TNF.

Conclusions (2)

Efficacy of anti-TNF in RA

Anti-TNF therapy resulted in:

rapid and marked improvements in EMS, pain and articular index in most patients;

slower but marked improvement in swollen joint score, maximal by 3–4 weeks;

rapid and impressive falls in serum CRP, and a slower fall in ESR;

normalization of CRP and ESR in some patients;

rapid falls in serum C4d (a complement breakdown product) and IL-6 in patients where these indices were elevated at entry.

Duration of clinical improvements variable, with rebound in some patients at 6–8 weeks.

Accordingly, the present invention has been shown to have clinical efficacy in human patients for treating TNF involved pathologies using TNF MAbs of the present invention, such as for treating rheumatoid arthritis. Additionally, the human clinical use of TNF antibodies of the present invention in humans is also shown to correlate with in vitro data and in vivo animal data for the use of anti-TNF antibodies of the present invention for treating TNF-related pathologies.

EXAMPLE XXI

TREATMENT OF CROENIS DISEASE IN HUMANS USING ANTI-TNFα ANTIBODIES

Case History SB.

This 16 year old patient has a history of Crohn's disease since age 12. She was suffering from diarrhoea, rectal blood loss, abdominal pain, fever and weight loss. She showed perianal lesions, severe colitis and irregularity of the terminal ileum. She was treated with prednisolone (systemic and local) and pentasa. This resulted in remission of the disease, but she experienced extensive side effects of the treatment. She experienced severe exacerbations at age 12 and 12 yrs, 5 months, (Immuran™ added), 12 yrs, 9 months, 13 yrs, 5 months, and 14 yrs, 10 months. She experienced severe side effects (growth retardation, morbus Cushing, anemia, muscle weakness, delayed puberty, not able to visit school).

At 15 yrs, 11 months, she was diagnosed with a mass in the right lower quadrant. She had a stool frequency of 28 time per week (with as much as 10 times per day unproductive attempts). The Crohn's index wad 311, the pediatric score 77.5. The sedimentation rate was elevated. Albumen and hemoglobin reduced. Before the first treatment the score was 291 and pediatric score was 60, and she would possibly have to loose her colon. She was infused on compassionate grounds with 10 mg/kg cA2, without any side effects noticed. One week after treatment her sedimentation rate was reduced from 66 to 32 mm. The Crohn's index was 163 and pediatric score 30. She was reported to feel much better and the frequency of the stools was reduced greatly. Thee was apparently no more diarrhoea, but normal faeces. On October 15th, before the second infusion she had gained weight, had a sedimentation rate of 20 mm, an albumen of 46 h/l, Crohn's index 105, pediatric score 15. There seemed to be improvement on video endoscopy. A second infusion was performed at 16 yrs.

The patient was greatly improved after the second infusion. A endoscopy showed only 3 active ulcers and scar tissue.

This is in contrast with her colon on admission when the thought was that her colon should be removed. This case history shows a dramatic improvement of severe Crohn's disease upon treatment with cA2 anti-TNF antibody.

TABLE 11

CASE HISTORY SB

11 y, 8 m:	physical examination:	Diarrhoea, rectal blood loss, abdominal pain, fever (40%) weight loss perianal lesions
	sigmoidoscopy:	severe colitis, probably M. Crohn
	enterolysis:	irregularity terminal ileum
	Therapy:	prednisolone 10 mg 3 dd. Pentasa 250 mg 3 dd. enema (40 mg prednisone, 2 g 5 ASA) ml 1 dd.
	Result:	remission, however: extensive side effects of prednisone and stunting growth
	Action:	prednisone
11 y, 11 m		exacerbation same clinical picture as 11 y, 8 m
	sigmoidoscopy:	recurrence of colitis (grade IV) in last 60 cm and anus.
	Therapy:	prednisolone 40 mg 1 dd Pentasa 500 mg 3 dd enema 1 dd
	Result:	better
12 y, 5 m:		severe exacerbation; despite intensive treatment
	sigmoidoscopy:	extensive perianal and sigmoidal lesions; active disease
	Therapy:	continued + Immuran ™ 25 mg 1 dd
	Result:	slight improvement however still growth retardation, cushing, anaemia, muscle weakness.
	Action:	prednisone
12 y, 9 m:		exacerbation
	sigmoidoscopy:	extensive (active) colitis, polyps
	Action:	prednisone: 30 mg 1 dd, Immuran ™ 50 mg 1 dd, Pentasa 500 mg 3 dd, enema 2 dd
	Result:	still needs enema's with prednisone and oral prednisone. delayed puberty, stunting growth
14 y, 10 m:		severe exacerbation, weight loss, abdominal pain, fever.
	ileoscopy:	active colitis (grade IV), perianal lesions. Terminal ileum normal.
	Result:	No remission still fever, poor appetite, weight loss, diarrhea, not able to visit school
Important Findings:		
14 y, 11 m:		151.9 cm; 34 kg T = 38° C., Abdominal mass in right lower quadrant stool frequency 28 per week (however goes 10–15 times a day but most often without success) ESR 55 mm; Hb 6.2 mmol/1 Ht 0, 29 1/1; alb. 38.4 g/l Crohn's Dis. Act. Index: 311 Pediatric score: 77.5
14 y, 11.2 m: (before 1st infusion)		151, 8 cm: 34.6 kg
	Crohn's Dis Act Index:	291
	Pediatric score:	60
14 y, 11.4 m:		151, 8 cm: 34.6.kg ESR 32 mm; Hb 5.7 mmol/l
	Crohn's Dis Act Index:	163
	pediatric score:	30
15 y, 0 m (before 2nd infusion		152,1 cm: 34.8 kg Feels like she has never felt before. Parents also very enthusiastic ESR 30 mm: Hb 6, 3 mol/l Ht 0, 32 11; Alb 46 g/l
	Crohn Dis Act Index:	105
	Pediatric Score:	15
	Video-endoscopy:	Improvement

No problems or side effects observed during and following infusion.

Accordingly, anti-TNF antibodies according to the present invention, as exemplified by cA2, are shown to provide successful treatment of TNF related pathologies, as exemplified by Crohn's disease, in human patients with no or little side effects.

EXAMPLE XXII

TREATMENT OF ARTHRITIS IN HUMANS USING CHIMERIC IMMUNOGLOBULIN CHAIN OF THE PRESENT INVENTION

Patient Selection

Twenty patients were recruited, each of whom fulfilled the revised American Rheumatism Association criteria for the diagnosis of RA (Arnett et al., *Arthritis Rheum*. 31:315–324 (1988). The clinical characteristics of the patients are shown in Table 12. The study group comprised 15 females and 5 males, with a median age of 51 years (range 23–72), a median disease duration of 10.5 years (range 3–20) and a history of failed therapy with standard disease-modifying anti-rheumatic drugs (DMARDs; median number of failed DMARDs: 4, range 2–7). Seventeen were seropositive at entry or had been seropositive at some stage of their disease, all had erosions on X-Rays of hands or feet, and 3 had rheumatoid nodules. All patients had active disease at trial entry, as defined by an Index of Disease Activity (IDA; Mallya et al., *Rheumatol. Rehab*. 20:14–17 (1981) of at least 1.75, together with at least 3 swollen joints, and were classed as anatomical and functional activity stage 2 or 3 (Steinbrocker et al., *JAMA* 140:659–662 (1949). The pooled data for each of the clinical and laboratory indices of disease activity at the time of screening for the trial (up to 4 weeks prior to trial entry), and on the day of trial entry itself (week 0), are shown in Tables 13 and 14.

TABLE 12

Demographic features of 20 patients with refractory rheumatoid arthritis.

Patient	Age/ Sex	Disease Duration (years)	Previous DMARDs	Concomitant Therapy
1	48/F	7	SSZ, DP, GST, AU RMTX, AZA, HCQ	Pred 5 mg
2	63/F	7	SSZ, GST, DP	Para 1–2 g
3	59/M	3	AUR, HCQ, GST, MTX, SSZ	Pred 10 mg, Indo 225 mg
4	56/M	10	GST, DP, AZA, SS Z	Pred 12.5 mg, Ibu 2 g, Para 1–2 g
5	28/F	3	GST, SSZ, DP, AZ A	Pred 8 mg, Para 1–2 g, Cod 16 mg

TABLE 12-continued

Demographic features of 20 patients with refractory rheumatoid arthritis.

Patient	Age/ Sex	Disease Duration (years)	Previous DMARDs	Concomitant Therapy
6	40/M	3	SSZ, HCQ, AUR	Nap 1 g
7	54/F	7	DP, GST, SSZ, AZA MTX	Para 1–2 g, Cod 16–32 mg
8	23/F	11	HCQ, GST, SSZ, MTX AZA	Pred 7.5 mg, Dicl 100 mg, Para 1–2 g, Dex 100–200 mg
9	51/F	15	GST, HCQ, DP, MTX	Pred 7.5 mg, Dicl 125 mg, Para 1–3 g
10	47/F	12	SSZ, CYC, MTX	Ben 4 g
11	34/F	10	DP, SSZ, MTX	Pred 10 mg, Para 1.5 g, Cod 30–90 mg
12	57/F	12	GST, MTX, DP, AUR	Asp 1.2 g
13	51/F	7	SSZ, AZA	Para 1–4 g
14	72/M	11	GST, DP, AZA, MTX	Pred 5 mg, Para 1–4 g, Cod 16–64 mg
15	51/F	17	HCQ, DP, SSZ, MTX	Asp 0.3 g
16	62/F	16	GST, DP, SSZ, MTX	Para 1–4 g,
17	56/F	11	SSZ, DP, GST, MTX HCQ, AZA	Cod 16–64 mg Pred 7.5 mg, Eto 600 mg, para 1–2 g, Dext 100–200 mg
18	48/F	14	GST, MTX, DP, SSZAUR, AZA	Pred 7.5 mg, Indo 100 mg, Para 1–3 g
19	42/F	3	SSZ, MTX	Fen 450 mg, Ben 6 g, Cod 30 mg
20	47/M	20	GST, DP, SSZ, AZA	Pred 10 mg, Para 1–3 g

*DMARDs = disease - modifying anti-rheumatic drugs SSZ = sulphasalazine; DP = D-penicillamine; GST = gold sodium thiomalate; AUR = auranofin; MTX = methotrexate; AZA = azathioprine; HCQ = (hydroxy) chloroquine; CYC = cyclophosphamide. Pred = prednisolone (dose/day); Para = paracetamol; Indo = Indomethacin; Ibu = ibuprofen; Cod = codeine phosphate; Nap = naprosyn; Dicl = diclofenac; Dext = dextropropoxyphene; Ben = benorylate; Asp = asprin; eto = etodolac; Fen = fenbufen.

TABLE 13

Changes in clinical assessments following treatment of rheumatoid arthritis patients with cA2.

Week of Trial	Morning Stiffness min	Pain Score (0–10) cm	Ritchie Index (0–69)	Swollen Joints (0–28) number	Grip Strength (L) (0–300) mm Hg	Grip Strength (R) (0–300) mm Hg	IDA (1–4)	Patient Assessment (grades improved 0–3)
Screen	135(0–600)	7.4(4–9.7)	23(4–51)	16(4–28)	84(45–300)	96(57–300)	3(2.3–3.3)	NA
p value 0	180(20–600)	7.1(2.7–9.7)	28(4–52)	18(3–27)	77(52–295)	92(50–293)	3(2–3.5)	NA
p value 1	20(0–180)	2.6(0.6–7.8)	13(2–28)	13.5(1–25)	122(66–300)	133(57–300)	2(1.5–3.3)	1(1–3)
	<0.001	<0.001	<0.001; <0.002	>0.05	>0.05	>0.05	<0.001	NA
p value 2	15(0–150)	3.0(0.3–6.4)	13(1–28)	11.5(1–22)	139(75–300)	143(59–300)	2(1.5–3.2)	1.5(1–3)
	<0.001	<0.001	<0.001	<0.003; <0.02	<0.03; >0.05	>0.05	<0.001	NA
p value 3	5(0–150)	2.2(0.2–7.4)	8(0–22)	6(1–19)	113(51–300)	142(65–300)	2(1.2–3.2)	2(1–2)
	<0.001	<0.001	<0.001	<0.001; <0.002	>0.05	>0.05	<0.001	NA
p value 4	15(0–90)	1.9(0.1–5.6)	10(0–17)	6(0–21)	124(79–300)	148(64–300)	1.8(1.3–2.7)	2(1–2)
	<0.001	<0.001	<0.001	<0.001; <0.002	<0.02; >0.05	<0.03; >0.05	<0.001	NA
p value 6	5(0–90)	1.9(0.1–6.2)	6(0–18)	5(1–14)	119(68–300)	153(62–300)	1.7(1.3–2.8)	2(1–2)
	<0.001	<0.001	<0.001	<0.001	<0.04; >0.05	<0.05; >0.05	<0.001	NA
p value 8	15(0–60)	2.1(0.2–7.7)	8(1–28)	7(1–18)	117(69–300)	167(53–300)	1.8(1.5–2.8)	2(1–3)
	<0.001	<0.001	<0.001	<0.001	<0.03; >0.05	<0.03; >0.05	<0.001	NA

Datas are expressed as the median (range) of values from 20 patients; data from patient 15 were not included after week 2 (dropout); P values show significance by Mann-Whitney test ccmpared with week 0 values; adjusted for multiple statistical comparisons. IDA = Index of disease activity; NA = not applicable.

TABLE 14

Changes in laboratory measures following treatment of rheumatoid arthritis patients with cA2.

Week of Trial	Hgb g/liter	WBC × 10/liter	Platelet Count × 10/liter	ESR mm/hour	CRP mg/liter	SAA mg/ml	RF Inverse titer
Screen	117(98–146)	7.9(3.9–15.2)	352(274–631)	59(18–87)	42(9–107)	ND	ND
p value 0	113(97–144)	9.0(4.9–15.7)	341(228–710)	55(15–94)	39.5(5–107)	245(18–1900)	2,560 (160–10,240)
p value 1	114(96–145) >0.05	8.5(3.6–13.6) >0.05	351(223–589) >0.05	26(13–100) >0.05	5(0–50) <0.001	58(0–330) <0.001; <0.003	ND
p value 2	112(95–144) >0.05	8.2(4.3–12.7) >0.05	296(158–535) <0.04; >0.05	27(10–90) <0.02; >0.05	5.5(0–80) <0.001; <0.003	80(11–900 <0.02; <0.04	ND
p value 3	110(89–151) >0.05	9.0(3.7–14.4) >0;05	289(190–546) <0.03; >0.05	27(12–86) <0.04; >0.05	7(0–78) <0.001; <0.002	ND	ND
p value 4	112(91–148) >0.05	8.2(4.7–13.9) >0.05	314(186–565) >0.05	23(10–87) <0.04; >0.05	10(0–91) <0.004; <0.02	ND	ND
p value 6	116(91–159 >0.05	9.1(2.9–13.9) >0.05	339(207–589) >0.05	23(12–78) <0.03; >0.05	8(0–59) <0.001	ND	ND
p value 8	114(94–153) >0.05	7.6(4.2–13.5) >0.05	339(210–591) >0.05	30(7–73) >0.05	6(0–65) <0.001	ND	480(40–05,120) >0.05

Data are expressed as the median (range) of values from 20 patients; data from patient 15 were not included after week 2 (dropout). For rheumatoid factor (RF), only those patients with week 0 titers > 1/160 in the particle agglutination assay were included (No. = 14). P values show significance by Mann-Whitney test compared with week 0 values; adjusted for multiple statistical comparisons; ND = not done. Normal ranges: hemoglobin (Hgb) 120–160 g/liter (F), 135–175 g/liter (M); white blood cell count (WBC) 4–11 × 10^9/liter; platelet count 150–400 × 10^9/liter; erythrocyte sedimentation rate (ESR) < 15 mm/hour (F), < 10 mm/hour (M); C-reactive protein (CRP) < 10 mg/liter; serum amyloid A (SAA) < 10 mg/ml.

TABLE 16

260793	270793	280793	290793	020893	200893	270893
ESR-77	ESR-47	BSR-58	ESR-77	ESR-77	ESR-46	ESR-38

All DMARDs were discontinued at least 1 month prior to trial entry. Patients were allowed to continue on a non-steroidal anti-inflammatory drug and/or prednisolone (<12.5 mg/day) during the trial. The dosage of these agents was kept stable for 1 month prior to trial entry and during the course of the trial, and no parenteral corticosteroids were allowed during these periods. Simple analgesics were allowed ad libitum. Patients with other serious medical conditions were excluded. Specific exclusions included serum creatinine>150 umol/liter (normal range 60–120 umol/liter), hemoglobin (Hgb)<90 gm/liter (normal range 120–160 gm/liter [females]; 135–175 gm/liter [males]), white blood cell count (WBC)<4×10^9/liter (normal range 4–11×10^9/liter), platelet count<100×10^9/liter (normal range 150–400×10^9/liter), and abnormal liver function tests or active pathology on chest X-Ray.

All patients gave their informed consent for the trial, and approval was granted by the local ethics committee.

Treatment

The cA2 antibody was stored at 4° C. in 20 ml vials containing 5 mg of cA2 per milliliter of 0.01 M phosphate buffered saline in 0.15M sodium chloride at a pH of 7.2 and was filtered through a 0.2 um sterile filter before use. The appropriate amount of cA2 was then diluted to a total volume of 300 ml in sterile saline and administered intravenously via a 0.2 um in-line filter over a 2 hour period.

Patients were admitted to hospital for 9–24 hours for each treatment, and were mobile except during infusions. The trial was of an open, uncontrolled design, with a comparison of two treatment schedules. Patients 1 to 5 and 11 to 20 received a total of 2 infusions, each of 10 mg/kg cA2, at entry to the study (week 0) and 14 days later (week 2). Patients 6 to 10 received 4 infusions of 5 mg/kg activity included complete blood counts, C-reactive protein (CRP; by rate nephelometry) and the erythrocyte sedimentation rate (ESR; Westergren). Follow-up assessments were made at monthly intervals after the conclusion of the formal trial period, in order to assess the duration of response.

Analysis of improvement in individual patients was made using two separate indices. Firstly, an index of disease activity (IDA) was calculated for each time point according to the method of Mallya and Mace (Mallya et al., *Rheumatol. Rehab.* 20:14–17 (1981), with input variable of morning stiffness, pain score, Ritchie Index, grip strength, ESR and Hgb. The second index calculated was that of Paulus (Paulus et al., *Arthritis Rheum.* 33:477–484 (1990) which uses input variables of morning stiffness, ESR, joint pain/tenderness, joint swelling, patient's and physician's global assessment of disease severity. In order to calculate the presence or otherwise of a response according to this index, two approximations were made to accommodate our data. The 28 swollen joint count used by us (nongraded; validated in Fuchs et al., *Arthritis Rheum.* 32:531–537 (1989)) was used in place of the more extensive graded count used by Paulus, and the patient's and physician's global assessments of response recorded by us were approximated to the global assessments of disease activity used by Paulus infra. In addition to determining response according to these published indices, we selected 6 disease activity assessments of interest (morning stiffness, pain score, Ritchie index, swollen joint count, ESR and CRP) and calculated their mean percentage improvement. We have used FIGS. **24** and **25** to give an indication of the degree of improvement seen in responding patients.

Immunological Investigations—Rheumatoid factors were measured using the rheumatoid arthritis particle agglutination assay (RAPA, FujiBerio Inc., Tokyo, Japan), in which titers of 1/160 or greater were considered significant. Rheumatoid factor isotypes were measured by ELISA (Cambridge Life Sciences, Ely, UK). The addition of cA2 at concentrations of up to 200 ug/ml to these assay cA2, at entry, and days 4, 8 and 12. The total dose received by the 2 patient groups was therefore the same at 20 mg/kg.

Assessment

Safety Monitoring—Vital signs were recorded every 15 to 30 minutes during infusions, and at intervals for up to 24 hours post infusion. Patients were questioned concerning possible adverse events before each infusion and at weeks 1, 2, 3, 4, 6, and 8 of the trial. A complete physical examination was performed at screening and week 8. In addition, patients were monitored by standard laboratory tests including complete blood count, C3 and C4 components of complement, IgG, IgM and IgA, serum electrolytes, creatinine, urea, alkaline phosphatase, aspartate transaminase and total bilirubin. Sample times for these tests were between 0800 and 0900 hours (pre-infusion) and 1200–1400 hours (24 hours post completion of the infusion). Blood tests subsequent to 15 day 1 were performed in the morning, usually between 0700 and 1200 hours. Urine analysis and culture were also performed at each assessment point.

Response Assessment—The patients were assessed for response to cA2 at weeks 1, 2, 3, 4, 6 and 8 of the trial. the assessments were all made between 0700 and 1300 hours by the same observer. The following clinical assessments were made: duration of morning stiffness (minutes), paid score (0 to 10 cm on a visual analog scale), Ritchie Articular Index (maximum 69; Ritchie et al., *Quart. J. Med.* 147:393–406 (1968)), number of swollen joints (28 joint count; validated in Fuchs et al., *Arthritis Rheum.* 32:531–537 (1989), grip strength (0 to 300 mm Hg, mean of 3 measurements per hand by sphygmomanometer cuff) and an assessment of function (the Stanford Health Assessment Questionnaire (HAG) modified for British patients; 34). In addition, the patients' global assessments of response were recorded on a 5-point scale (worse, no response, fair response, good response, excellent response). Routine laboratory indicators of disease systems did not alter assay results (data not shown). Antinuclear antibodies were detected by immunofluorescence on HEpo 2 cells (Biodiagnostics, Upton, Worcs. UK) and antibodies to extractable nuclear antigens were measured by counter immunoelectrophoresis with poly-antigen extract (Biodiagnostics). Sera positive by immunofluorescence were also screened for antibodies to DNA by the Farr assay (Kodak Diagnostics, Amersham, UK). Anti-cardiolipin antibodies were measured by ELISA (Shield Diagnostics, Dundee, Scotland). Serum amyloid A (SAA) was measured by sandwich ELISA (Biosource International, Camarillo, Calif., USA). Antiglobulin responses to the infused chimeric antibody were measured by an in-house ELISA, using cA2 as a capture reagent.

Cytokine Assays—Bioactive TNF was measured in sera using the WEHI 164 clone 13 cytotoxicity assay (Espevik et al., *J. Imm. Methods* 95:99–105 (1986). Total IL-6 was measured in sera using a commercial immunoassay (Medgenix Diagnostics, SA, Belgium) and by a sandwich ELISA developed 'in house' using monoclonal antibodies provided by Dr. F. di Padova (Basel, Switzerland). Microtiter plates were coated with monoclonal antibody LNI 314-14 at a concentration of 3 ug/ml for 18 hours at 4° C. and blocked with 3% bovine serum albumin in 0.1M phosphate buffered saline, pH 7.2. Undiluted sera or standards (recombinant hIL-6, 0–8.1 ug/ml) were added to the wells in duplicate and incubated for 18 hours at 4° C. Bound IL-6 was detected by incubation with monoclonal antibody LNI 110-14 for 90 minutes at 37° C., followed by biotin—labeled goat anti-murine IgG2b for 90 minutes at 37° C. (Southern Biotechnology, Birmingham, Ala.). The assay was developed using streptavidin—alkaline phosphatase (Southern Biotechnology) and p-nitrophenylphosphate as a substrate and the optical density read at 405 nm.

Statistics—Comparisons between week 0 and subsequent time points were made for each assessment using the Mann-Whitney test. For comparison of rheumatoid factor (RAPA) titers, the data were expressed as dilutions before applying the test.

This was an exploratory study, in which pre-judgements about the optimal times for assessment were not possible. Although it has not been common practice to adjust for multiple statistical comparisons in such studies, a conservative statistical approach would require adjustment of p values to take into account analysis at several time points. The p values have therefore been presented in two forms: unadjusted, and after making allowance for analysis at multiple time points by use of the Bonferroni adjustment. Where p values remained <0.001 after adjustment, a single value only is given. A p value of <0.05 is considered significant.

Results

Safety of cA2—the administration of cA2 was exceptionally well tolerated, with no headache, fever, hemodynamic disturbance, allergy or other acute manifestation. No serious adverse events were recorded during the 8-week trial. Two minor infective episodes were recorded, patient 15 presented at week 2 with clinical features of bronchitis and growth of normal commensals only on sputum culture. She had a history of smoking and of a similar illness 3 years previously. The illness responded promptly to treatment with amoxicillin, but her second cA2 infusion was withheld and the data for this patient are therefore not analyzed beyond week 2. Patient 18 showed significant bacteriuria on routine culture at week 6($>10^5$/ml; lactose fermenting coliform), but was asymptomatic. This condition also responded promptly to amoxicillin.

Routine analysis of blood samples showed no consistent adverse changes in hematological parameters, renal function, liver function, levels of C3 or C4 or immunoglobulins during the 8 weeks of the trial. Four minor, isolated and potentially adverse laboratory disturbances were recorded. Patient 2 experienced a transient rise in blood urea, from 5.7 mmol/liter to 9.2 mmol/liter (normal range 2.5 to 7 mmol/liter), with no change in serum creatinine. This change was associated with the temporary use of a diuretic, prescribed for a non-rheumatological disorder. The abnormality normalized within 1 week and was classified as 'probably not' related to cA2. Patient 6 experienced a transient fall in the peripheral blood lymphocyte count, from 1.6 to 0.8×10^9/liter (normal range 1.0–4.8×10^9/liter). This abnormality normalized by the next sample point (2 weeks later), was not associated with any clinical manifestations and was classified as 'possible related' to cA2. Patients 10 and 18 developed elevated titers of anti-DNA antibodies at weeks 6 and 8 of the trial, with elevated anti-cardiolipin antibodies being detected in patient 10 only. Both patients had a pre-existing positive antinuclear antibody and patient 10 had a history of borderline lymphocytopenia and high serum IgM. There were no clinical features of systemic lupus erythematosus and the laboratory changes were judged 'possibly related' to cA2.

Efficacy of cA2

Disease Activity—The pattern of response for each of the clinical assessments of disease activity and the derived IDA are shown in Table 13. All clinical assessments showed improvement following treatment with cA2, with maximal responses from week 3. Morning stiffness fell from a median of 180 minutes at entry to 5 minutes at week 6 ($p<0.001$, adjusted), representing an improvement of 73%. Similarly, the Ritchie Index improved from 28 to 6 at week 6, ($p<0.001$, adjusted, 79% improvement) and the swollen joint count fell from 18 to 5, ($p<0.001$, adjusted, 72% improvement). The individual swollen joint counts for all time points are shown in FIG. **24**. Grip strength also improved; the median grip strength rose from 77 (left) and 92 (right) mm Hg at entry to 119 (left) and 153 (right) mmHg at week 6 ($p<0.04$, $p<0.05$, left and right respectively; $p>0.05$ after adjustment for multiple comparisons). The IDA showed a fall from a median of 3 at entry to 1.7 at week 6 ($p<0.001$, adjusted). Patients were asked to grade their responses to cA2 on a 5 point scale. No patient recorded a response of 'worse' or 'no change' at any point in the trial. 'fair', 'good' and 'excellent' responses were classed as improvements of 1, 2 and 3 grades respectively. At week 6, the study group showed a median of 2 grades of improvement (Table 13).

We also measured changes in the patients' functional capacity, using the HAQ modified for British patients (range 0–3). The median (range) HAQ score improved from 2(0.9–3) at entry to 1.1 (0–2.6) by week 6, ($p<0.001$; $p<0.002$ adjusted).

The changes in the laboratory tests which reflect disease activity are shown in Table 14. the most rapid and impressive changes were seen in serum CRP, which fell from a median of 39.5 mg/liter at entry to 8 mg/liter by week 6 of the trial ($p<0.001$, adjusted; normal range<10 mg/liter), representing an improvement of 80%. Of the 19 patients with elevated CRP at entry, 17 showed falls to the normal range at some point during the trial. The improvement in CRP was maintained in most patients for the assessment period (Table 14 and FIG. **25**); the exceptions with high values at 4 and 6 weeks tended to be those with the highest starting values (data not shown). The ESR also showed improvement, with a fall from 55 mm/hour at entry to 23 mm/hour at week 6 ($p<0.03$; $p>0.05$ adjusted; 58% improvement; normal range<10 mm/hour, <15 mm/hour, males and females respectively). SAA levels were elevated in all patients at trial entry, and fell from a median of 245 mg/ml to 58 mg/ml at week 1 ($p<0.003$, adjusted; 76% improvement; normal range<10/mg/ml) and to 80 mg/ml at week 2 ($p<0.04$, adjusted). No significant changes were seen in Hgb, WBC or platelet count at week 6, although the latter did improve at weeks 2 and 3 compared with trial entry (Table 14).

The response data have also been analyzed for each individual patient. The majority of patients had their best overall responses at week 6, at which time 13 assessed their responses as 'good' while 6 assessed their responses as 'fair'. Eighteen of the 19 patients who completed the treatment schedule achieved an improvement in the index of Disease Activity (Mallya et al., *Rheumatol. Rehab.* 20:14–17 (1981) of 0.5 or greater at week 6, and 10 achieved an improvement of 1.0 or greater. All patients achieved a response at week 6 according to the index of Paulus (Paulus et al., *Arthritis Rheum.* 33:477–484 (1990). Finally, all patients showed a mean improvement at week 6 in the 6 selected measures of disease activity (as presented above) of 30% or greater, with 18 of the 19 patients showing a mean improvement of 50% or greater.

Although the study was primarily designed to assess the short-term effects of cA2 treatment, follow-up clinical and laboratory data are available for those patients followed for sufficient time (number=12). The duration of response in these patients, defined as the duration of a 30% (or greater) mean improvement in the 6 selected disease activity measures, was variable, ranging from 8 to 25 (median 14) weeks.

Comparison of the clinical and laboratory data for patients treated with 2 infusions of cA2 (each at 10 m/kg) compared with those treated with 4 infusions (each at 5 mg/kg) showed no significant differences in the rapidity or extent of response (data not shown).

Inununological Investigations and cytokines—Measurement of rheumatoid factor by RAPA showed 14 patients with significant tiers (>1/160) at trial entry. Of these, 6 patients showed a fall of at least 2 titers on treatment with cA2, while the remaining patients showed a change of 1 titer or less. No patient showed a significant increase in RF titer during the trial. The median RF titer in the 11 patients fell from ½, 560 at entry to 1/480 by week 8 ($p>0.05$; Table 14). Specific RF isotypes were measured by ELISA, and showed falls in the 6 patients whose RAPA had declined significantly, as well as in some other patients. Median values for the three RF isotypes in the 14 patients seropositive at trial entry were 119, 102 and 62 IU/ml (IgM, IgG and IgA isotypes respectively) and at week 8 were 81, 64 and 46 IU/ml ($p>0.05$).

We tested sera from the first 9 patients for the presence of bioactive TNF, using the WEHI 164 clone 13 cytotoxicity assay (Espevik et al., *J. Imm. Methods* 95:99–105 (1986). In 8 patients, serum sets spanning the entire trial period were tested, while for patient 9, one pre-trial, one intermediate and the last available sample only were tested. The levels of bioactive TNF were below the limit of sensitivity of the assay in the presence of human serum (1 pg/ml). Since production of CRP and SAA are thought to be regulated in large part by IL-6, we also measured serum levels of this cytokine, using 2 different assays which measure total IL-6. In the Medgenix assay, IL-6 was significantly elevated in 17 of the 20 patients at entry. In this group, levels fell from 60 (18–500) pg/ml to 40 (0–230) pg/ml at week 1 ($p>0.05$; normal range<10 pg/ml) and to 32 (0–210) pg/ml at week 2 ($p<0.005$, $p<0.01$, adjusted). These results were supported by measurement of serum IL-6 in the first 16 patients in a separate ELISA developed in-house. IL-6 was detectable in 11 of the 16, with median (range) levels falling from 210 (25–900) pg/ml at entry to 32 (0–1,700) pg/ml at week 1 ($p<0.02$, $p<0.04$, adjusted; normal range<10 pg/ml) and to 44 (0–240) pg/ml at week 2 ($p<0.02$, $p<0.03$, adjusted).

We tested sera from the first 10 patients for the presence of anti-globulin responses to the infused chimeric antibody, but none were detected. In many patients however, cA2 was still detectable in serum samples taken at week 8 and this can have interfered with the ELISA.

Discussion

This is the first report describing the use of anti-TNFα antibodies in human autoimmune disease. Many cytokines are produced in rheumatoid synovium, but we chose to target specifically TNFα because of mounting evidence that it was a major molecular regulator in RA. The study results presented here support that view and allow three important conclusions to be drawn.

First, treatment with cA2 was safe and the infusion procedure was well tolerated. Although fever, headache, chills and hemodynamic disturbance have all been reported following treatment with anti CD4 or anti CDw52 in RA,

such features were absent in our patients. Also notable was the absence of any allergic event despite repeated treatment with the chimeric antibody, although the interval between initial and repeat infusions can have been too short to allow maximal expression of any anti-globulin response. The continuing presence of circulating cA2 at the conclusion of the trial amy have precluded detection of antiglobulin responses, but also implied that any such responses were likely to be of low titre and/or affinity. Although we recorded 2 infective episodes amongst the study group, these were minor and the clinical courses were unremarkable. TNFα has been implicated in the control of listeria and other infections in mice (Havell et al., *J. Immunol.* 143:2894–2899 (1989), but our limited experience does not suggest an increased risk of infections after TNFα blockade in man.

The second conclusion concerns the clinical efficacy of cA2. The patients we treated had long-standing, erosive, and for the most part seropositive disease, and had each failed therapy with several standard DMARDs. Despite this, the major clinical assessments of disease activity and outcome (morning stiffness, paid score, Ritchie index, swollen joint count and HAQ score) showed statistically significant improvement, even after adjustment for multiple comparisons. All patients graded their response as at least 'fair', with the majority grading it as 'good'. In addition, all achieved a response according to the criteria of Paulus and showed a mean improvement of at least 30% in 6 selected disease activity measures.

The improvements in clinical assessments following treatment with cA2 appear to be at least as good as those reported following treatment of similar patients with anti-leukocyte antibodies. The two therapeutic approaches can already be distinguished, however, by their effects on the acute phase response, since in several studies of anti-leukocyte antibodies, no consistent improvements in CRP or ESR were seen. In contrast, treatment with cA2 resulted in significant falls in serum CRP and SAA, with normalization of values in many patients. The changes were rapid and marked, and in the case of CRP, sustained for the duration of the study (Table 14). The falls in ESR were less marked, achieving statistical significance only when unadjusted (Table 14).

These results are consistent with current concepts that implicate TNFα in the regulation of hepatic acute phase protein synthesis, either directly, or by control of other, secondary, cytokines such as IL-6 (Fong et al., *J. Exp. Med.* 170:1627–1633 (1989), Guerne et al., *J. Clin. Invest.* 83:585–592 (1989)). In order to investigate the mechanism of control of the acute phase response in our patients, we measured serum TNFα and IL-6 before and after cA2 treatment. Bioactive TNFα was not detectable in baseline or subsequent sera. We used 2 different assays for IL-6, in view of previous reports of variability between different immunoassays in the measurement of cytokines in biological fluids (Roux-Lombard et al., *Clin. Exp. Rheum.* 10:515–520 (1992), and both demonstrated significant falls in serum IL-6 by week 2. These findings support the other objective laboratory changes induced by cA2, and provide in vivo evidence that TNFα is a regulatory cytokine for IL-6 in this disease. Amongst the other laboratory tests performed, rheumatoid factors fell significantly in 6 patients.

Neutralization of TNFα can have a number of beneficial consequences, including a reduction in the local release of cytokines such as IL-6 and other inflammatory mediators and modulation of synovial endothelial/leukocyte interactions. cA2 can also bind directly to synovial inflammatory cells expressing membrane TNFα, with subsequent in situ cell lysis.

The results obtained in this small series have important implications, both scientifically and clinically. At the scientific level, the ability of the neutralizing antibody, cA2, to reduce acute phase protein synthesis, reduce the production of other cytokines such as IL-6, and significantly improve the clinical state demonstrates that it is possible to interfere with the cytokine network in a useful manner without untoward effects. Due to the many functions and overlapping effects of cytokines such as IL-1 and TNFα, and the fact that cytokines induce the production of other cytokines and of themselves, there had been some pessimism as to whether targeting a single cytokine in vivo would have any beneficial effect (Kingsley et al., *Immunol. Today* 12:177–179 (1991), Trenthan, *Curr. Opin. Rheumatol.* 3:369–372 (1991)). This view is clearly refuted. On the clinical side, the results of short-term treatment with cA2 are significant and confirm that TNFα is useful as a new therapeutic target in RA.

EXAMPLE XXIII

TREATMENT WITH CHIMERIC ANTI-TNF IN A PATIENT WITH SEVER ULCERATIVE COLITIS

The patient is a 41 year old woman with long term ulcerative colitis, which was diagnosed by endoscopy and histology. She has a pancolitis, but the main disease activity was left-sided. There were no extra-intestinal complications in the past. Maintenance therapy consisted of Asacol(TM). Only one sever flair-up occurred 4 years previously and was successfully treated with steroids.

At beginning month one, she was admitted elsewhere because of a very sever flair-up of the ulcerative colitis. Treatment consisted of high doses of steroids intravenously, antibiotics, asacol and Total Parental Nutrition. Her clinical condition worsened and a colectomy was considered.

At end of month one, she was admitted at the internal ward of the AMC. Her main complaints consisted of abdominal pains, frequent water stools with blood and mucopus and malaise.

Medication: ASACOL 2 dd 500 mg, orally
Di-Adresone-T 1 dd 100 mg, intravenously
Flagyl 3 dd 500 mg, intravenously
Fortum 3 dd 1 gram, intravenously
Total parental nutrition via central venous catheter

On physical examination the patient was moderately ill with a weight of 55 kg and a temperature of 36° C. Jugular venous pressure was not elevated. Blood pressure was 110/70 mm Hg with a pulse rate of 80 per minute. No lymphadenopathy was found. Oropharynx was normal. Central venous catheter was inserted in situ with no signs of inflammation at the place of insertion. Normal auscultation of the lungs and heart. The abdomen was slightly distended and tender. Bowel sounds where reduced. Liver and spleen where not enlarged. No signs of peritonitis. Rectal examination was normal.

All cultures of the stools where negative.

Plain x-ray of the abdomen; slightly dilated colon. No thumb-printing, no free air, no toxic megacolon.

Sigmoidoscopy; (video-taped) Very severe inflammation with deep ulcers. Dilated rectum and sigmoid. Because of danger of perforation the color, the endoscopy was limited to the racto-sigmoid. No biopsies where taken.

Conclusion at time of admission: Severe steroid resistant flair-up of ulcerative colitis.

Antibiotics where stopped, because no improvement was noticed and there was no temperature.

After informed consent of the patient, treatment was started with 10 mg/kg bodyweight (a 550 mg) of cA2 chimeric monoclonal anti-TNF (Centocor) given intravenously over 2 hours (according the protocol of cA2 used in severe Crohn's disease).

During the infusion there were no complaints. Vital signs were monitored and were all normal. Before and after infusion blood samples where drawn. Two days after infusion she had less abdominal pain, the stool frequency decreased and no blood was seen in the stools any more. However she developed high temperature (40° C.). Blood-cultures where positive for *Staphylococcus epidermidis*. Infection of the central venous catheter was suspected. The catheter was removed and the same Staphylococcus was cultured from the tip of the central venous catheter. During this period she was treated with antibiotics for three days. After this her temperature dropped and she recovered substantially. Steroids were tapered off to 40 mg of prednisone daily.

After 14 days sigmoidoscopy was repeated and showed a remarkable improvement of the mucosa with signs of re-epithelization. There were no signs of bleeding, less mucous and even some normal vascular structures were seen.

At four months she was discharged.

At the outpatient clinic further monitoring was done weekly. Patient is still improving. Stool frequency is two times per day without blood or mucopus. Her laboratory improved, but there is still anaemia, probably due to iron deficiency. A colonoscopy is planned in the nearby future.

Our conclusion is that this patient had a very severe flair-up of her ulcerative colitis. She was refractory to treatment and a total colectomy was seriously considered. After infusion of cA2 the clinical course improved dramatically in spite of the fact that there was a complication of a sepsis which was caused by the central venous catheter.

EXAMPLE XXIV

p55 Fusion Protein Structure

The extracellular domains of the p55 and p75 receptors were expressed as Ig fusion proteins from DNA constructs designed to closely mimic the structure of naturally occurring, rearranged Ig genes. Thus, the fused genes included the promoter and leader peptide coding sequence of a highly expressed chimeric mouse-human antibody (cM-T412, Looney et all, *Hum. Antibody Hybridomas* 1992, 3, 191–200) on the 5' side of the TNF receptor insert, and codons for eight amino acids of human J sequence and a genomic fragment encoding all three constant domains of human IgG1 on the 3' side of the receptor insert position (FIGS. **27** and **28**).

Minor changes were introduced at the N-terminal ends of the heavy chain fusion proteins so that the first two amino acids would be identical or similar to the first two amino acids (Gln-Ile) encoded by the cM-T412 antibody gene (from which the leader peptide originated). This was done to increase the likelihood that any interactions between the N-terminal end of the mature protein and the leader peptide would still result in efficient transport into the lumen of the endoplasmic reticulum. Boyd et al., *Cell* 1990, 62, 1031–1033. Therefore, the Asp^1 and Ser^2 residues of naturally-occurring p55 were replaced with a Gln residue, and the Leu^1 residue of p75 was preceded by a Gln residue in all p75 constructs. No amino acid changes were introduced at the N-terminal end of the p55 light chain fusion.

Expression Vectors

PCR methodology was used to engineer cloned genes. Oligonucleotides were purchased from National Biosciences (Plymouth, Minn.). PCR amplification kits were from Perkin-Elmer (CA) and DNA sequencing kits from U.S. Biochemical Corporation (Cleveland, Ohio). Alkaline phosphatase-conjugated goat anti-human IgG was purchased from Jackson ImmunoResearch (West Grove, Pa.). ^{125}I-labeled human TNF was obtained from Du Pont Company, NEN (Boston, Mass.) and unlabeled recombinant human TNF from R&D Systems (Minneapolis, Minn.). Protein A-Sepharose beads was purchased from PHARMACIA (Piscataway, N.J.).

PCR methodology was used to engineer two cloned genes encoding the heavy chain or light chain of an efficiently expressed murine antibody, cM-T412 (see Looney et al.), for the purpose of directing the expression of foreign genes in a mammalian cell system. The approaches were to effectively delete the coding region of the antibody variable region and to place a unique restriction site in its place (StuI for the heavy chain vector and EcoRV for the light chain vector).

The resulting vector contained 2.5 kb of 5' flanking genomic DNA, the promoter, the leader peptide coding sequence (including the leader intron), a StuI cloning site to introduce inserts, coding sequence for eight amino acids of human J sequence Gly Thr Leu Val Thr Val Ser Ser (SEQ ID NO:6) followed by genomic sequences for the human IgG1 constant region. An analogous vector was made from the cM-T412 light chain gene except that an EcoRV cloning site was introduced at the carboxyl terminal end of the light chain leader peptide and a different human J sequence was encoded by the vector Gly Thr Lys Leu Glu Ile Lys (SEQ ID NO:7). Both vectors are based on plasmid pSV2-gpt and subsequent vector derivatives that contain genomic sequences for either the heavy chain or light chain constant regions. See Mulligan et al., *Science* 209:1422–1427 (1980). The *E. coli* gpt gene allows selection of transfected cells with mycophenolic acid.

Heavy Chain Vector A previously cloned EcoRI fragment containing the cM-T412 heavy chain gene (Goeddel et al., *Cold Spring Harbor Symp. Quant. Biol.* 1986, 51, 597–609) was subcloned into pUC19. This recombinant plasmid was used as a template for two PCR reactions. In one reaction, an oligo corresponding to the "reverse" primer of the pUC plasmids and the 3' oligo 5'-CCTGGATACCTGTGAAAAGA-3' (SEQ ID NO:8) (bold marks half of a StuI site; oligo was phosphorylated prior to the PCR reaction) were used to amplify a fragment containing 3 kb of 5' flanking DNA, the promoter, transcription start site and leader peptide coding sequence (including the leader intron). In the second reaction, the 5' oligo 5'-CCTGGTACCTTAGTCACCGTCTCCTCA-3' (SEQ ID NO:9) (bold marks half of a StuI site; oligo phosphorylated prior to the PCR reaction) and an oligo corresponding to the "forward" primer of pUC plasmids amplified a fragment encoding eight amino acids of human J sequence Gly Thr Leu Val Thr Val Ser Ser (SEQ ID NO:6) and a splice donor to allow splicing to the human constant region coding sequence provided in another vector. The two PCR fragments were digested with EcoRI and then simultaneously ligated into EcoRI-digested pUC19 to make pHC684 (FIG. **27**).

Because the StuI site formed at the junction of the two PCR fragments was followed by a 'GG' dinucleotide sequence, a dcm methylation site was formed preventing StuI from digesting that site when the DNA was grown in HB101 strain of *E coli*. Therefore, the plasmid DNA was

introduced into dcm-JM110 *E. coli* cells and reisolated. StuI was then able to cut at the junction but a second StuI site in the 5' flanking DNA was a apparent (DNA sequencing showed that StuI site to also he followed by a GG dinucleotide and therefore also methylated). To make the StuI cloning site at the junction be unique, a 790 bp XbaI fragment that included only one of the two StuI sites was subcloned into pUC19 to make the vector pHC707 (FIG. **27**A) which was then grown in JM110 cells. The StuI cloning site formed at the junction of the two PCR fragments second and third nucleotides (i.e., 'CA') of the last codon (Ala) of the signal sequence in order to maintain the appropriate translation reading frame (FIG. **27**).

A PCR fragment encoding a protein of interest can then be ligated into the unique StuI site of pHC707. The insert can include a translation stop codon that would result in expression of a "non-fusion" protein. Alternatively, a fusion protein could be expressed by the absence of a translation stop codon, thus allowing translation to proceed through additional coding sequences positioned downstream of the StuI cloning site. Coding sequences in the StuI site of pHC707 would not be fused directly to the IgG1 coding sequences in pHC730 but would be separated by an intron sequence that partially originates from pHC707 and partially from pHC730. These intron sequences would be deleted in the cell following transcription resulting in an RNA molecule that is translated into a chimeric protein with the protein of interest fused directly to the IgG1 constant domains.

The plasmid pHC730 was a modified form of an IgG1 expression, pSV2 gpt-hCyl vector described previously (Goeddel et al., *Cold Spring Harbor Symp. Quant. Biol.* 1986, 51, 597–609) (FIG. **28**). The modifications were (1) removal of the unique Sal1 and XbaI sites upstream of the constant region coding sequence, (2) insertion of a Sal1 linker into the unique BamHI site to allow use of Sal1 to linearize the plasmid prior to transfections, and (3) ligation into the unique EcoRI site the cloned cM-T412 EcoRI fragment but with the XbaI fragment flanking the V gene deleted (FIG. **29**). The resulting expression vector had a unique XbaI site for inserting the XbaI fragments from pHC707.

Light Chain Vector

A previously cloned HindIII fragment containing the cM-T412 light chain gene (Goeddel et al., *Cold Spring Harbor Symp. Quant. Biol.* 1986, 51, 597–609) was subcloned into pUC19 and the resulting plasmid used as template for PCR reactions. In one PCR reaction the "reverse" pUC primer and the 3' oligo 5'-AATAGATATCTCCTTCAACACCTGCAA-3' (SEQ ID NO:10) (EcoRV site is in bold) were used to amplify a 2.8 kb fragment containing 5' flanking DNA, the promoter, transcription start site and leader peptide coding sequence (including the leader intron) of the cloned light chain gene. This fragment was then digested with HindIII and EcoRV. In a second PCR reaction, the 5' oligo 5'-ATCGGGACAAAGTTGGAAATA-3' (SEQ ID NO:11) (bold marks half of an EcoRV site) and the "forward" pUC primer were used to amplify a fragment encoding seven amino acids of human J sequence (Gly Thr Lys Leu Glu Ile Lys) and an intron splice donor sequence. This fragment was digested with HindIII and ligated along with the other PCR fragment into pUC cut with HindIII. The resulting plasmid, pLC671 (FIG. **29**), has a unique EcoRV cloning site at the junction of the two PCR fragments with the EcoRV site positioned such that the first three nucleotides of the EcoRV site encoded the first amino acid of the mature protein (Asp).

The pLC671 HindIII insert was designed to be positioned upstream of coding sequences for the human kappa light chain constant region present in a previously described expression vector, pSV2gpt-hCk (FIG. **30**). However, pSV2gpt-hCk contained an EcoRV site it its gpt gene. Because it was desired that the EcoRV site in the pLC671 HindIII fragment be a unique cloning site after transferring the fragment into pSV2gpt-hCk, the EcoRV site in pSV2gpt-hCk was first destroyed by PCR mutagenesis. Advantage was taken of the uniqueness of this EcoRV site in pSV2gpt-hCk and a Kpn1 site 260 bp upstream of the EcoRV site. Therefore, the 260 bp Kpn1-EcoRV fragment was removed from pSV2gpt-hCk and replaced with a PCR fragment that has identical DNA sequence to the 260 bp fragment except for a single nucleotide change that destroys the EcoRV site. The nucleotide change that was chosen was a T to a C in the third position of the EcoRV recognition sequence (i.e., GATATC changed to GACATC). Because the translation reading frame is such that GAT is a codon and because both GAT and GAG codons encode an Asp residue, the nucleotide change does not change the amino acid ended at that position. Specifically, pSV2gpt-hCk was used as template in a PCR reaction using the 5' oligo 5'-GGCGGTCTGGTACCGG-3' (SEQ ID NO:12) (Kpn1 site is in bold) and the 3' oligo 5'-GTCAACAACATAGTCATCA-3' (SEQ ID NO:13) (bold marks the complement of the ASP codon). The 260 bp PCR fragment was treated with the Klenow fragment of DNA polymerase to fill-in the DNA ends completely and then digested with Kpn1. The fragment was ligated into pSV2gpt-hCk that had its Kpn1-EcoRV fragment removed to make pLC327 (FIG. **30**).

The HindIII fragment of pLC671 was cloned into the unique HindIII site of pLC327 to make the light chain expression vector, pLC690 (FIG. **30**). This plasmid can be introduced into cells without further modifications to encode a truncated human kappa light chain, JCk, that contains the first two amino acids of the cM-T412 light chain gene, seven amino acids of human J sequences, and the light chain constant region. Alternatively, coding sequence of interest can be introduced into the unique EcoRV site of pLC690 to make a light chain fusion protein.

TNF Receptor DNA Constructs

For the p55 heavy chain fusion, amino acids 3–159 of the p55 extracellular domain were encoded in a PCR fragment generated using the 5' oligo 5'-CACAGGTGTGTCCCCAAGGAAAA-3' (SEQ ID NO:14) (bold marks the Val^3 codon) and the 3' oligo 5'-AATCTGGGGTAGGCACAA-3' (SEQ ID NO:15) (bold marks the complement of the Ile^{159} codon). For the p55 light chain fusion, amino acids 2–159 were encoded in a PCR fragment made Using the 5' oligo 5'-AGTGTGTGTCCCCAAGG-3' (SEQ ID NO:16) (bold marks the Ser^2 codon) and the same 3' oligo shown above. The light chain vector contained the codon for Asp^1 of p55. The DNA template for these PCR reactions was a previously reported human p55 cDNA clone. Gray et al., *Proc. Natl. Acad. Sci. USA* 1990, 87, 7380–7384.

A truncated light chain that lacked a variable region was expressed by transfecting cells with the light chain vector with no insert in the EcoRV cloning site. The resulting protein, termed JC_K, consisted of the first two amino acids of the cM-T412 light chain gene, seven amino acids of human J sequence (Gly Thr Lys Leu Glu Ile Lys) (SEQ ID NO:7), and the human light chain constant region.

A non-fusion form of p55 (p55-nf) was expressed in CHO-K1 cells using the CMV-major immediate early promoter after introducing a translation stop codon immediately after Ile^{159}. Secreted p55 was purified by affinity chromatography on a TNFα column.

Transfections and ELISA Assays

All plasmids were linearized with a restriction enzyme prior to introducing them into cells. Cells of the myeloma cell line X63-Ag8.653 were transfected with 12 μg of DNA by electroporation. Cell supernatants were assayed for IgG domains. Briefly, supernatants were incubated in plates coated with anti-human IgG Fc and then bound protein detected using alkaline phosphatase-conjugated anti-human and light chains.

Purification of Fusion Proteins

Cell supernatants were clarified by centrifugation followed by passage through a 0.45 micron filter. Supernatants were adjusted to 20 mM Tris-HCl, pH 8.3, 150 mM NaCl, and 1 mM EDTA (1×protein A buffer) and passed over a column of protein A-Sepharose beads. The column was washed in 1×protein A buffer followed by 100 mM Na Citrate, pH 5.0 to elute bound bovine IgG originating from the cell media. Bound fusion protein was then eluted in 100 mM Na Citrate, pH 3.5, neutralized with 0.2 volumes 1 M Tris, and dialyzed against PBS.

TNF Cytotoxicity Assays

TNF-sensitive WEHI-164 cells (Espevik et al., *J. Immunol. Methods* 1986, 95, 99–105) were plated in 1 μg/ml actinomycin D at 50,000 cells per well in 96-well microtiter plates for 3–4 hours. Cells were exposed to 40 pM TNFα or TNFβ and varying concentrations of fusion protein. The mixture was incubated overnight at 37° C. Cell viability was determined by adding 3-[4,5-dimethyl-thiazol-2-yl]-2, 5-diphenyltetrazolium bromide dye (MTT) to a final concentration of 0.5 mg/ml, incubating for 4 hours at 37° C., lysing the cells in 0.1 N HCl, 0.1% SDS and measuring the optical density at 550 nm wavelength.

Saturation Binding Analyses

Fusion proteins were captured while at a concentration of 10 ng/ml in 96-well microtiter plates coated with goat anti-human Fc antibodies. Varying concentrations of ^{125}I-TNF (34.8 μCi/μg) were added in PBS/1% BSA and allowed to bind for two hours at room temperature. Plates were washed and bound cpm determined. Non-specific binding was determined using an irrelevant antibody.

Several different versions of the p55 fusion proteins were expressed. Unlike what was reported for CD4 (Capon et al., *Nature* 1989, 337, 525–531) and IL-2 (Landolfi, *J. Biol. Chem.* 1991, 146, 915–919) fusion proteins that also included the C_H1 domain of the heavy chain, inclusion of a light chain proved to be necessary to get secretion of the Ig heavy chain fusion proteins from the murine myeloma cells. The light chain variable region was deleted to enable the TNF R domain on the heavy chain to bind TNF without stearic hindrance from the light chain.

The "double fusion" (df) protein, p55-df2, has p55 fused to both the heavy chain and light chain and is therefore tetravalent with regard to p55. p55-sf3 has the p55 receptor (and the same eight amino acids of human J sequence present in p55-sf2 and p55-df2) linked to the hinge region, i.e., the C_H1 domain of the constant region is deleted.

After one or two rounds of subcloning, spent cell supernatant from the various cell lines were yielding 20 μg/ml (for p55-sf2) of fusion protein. The proteins were purified from the spent supernatant by protein A column chromatography and analyzed by SDS-PAGE with or without a reducing agent. Each fusion protein was clearly dimeric in that their M_r estimates from their migration through a non-reducing gel was approximately double the estimated M_r from a reducing gel. However, two bands were seen for p55-sf2 and p55-df2. Two lines of evidence indicated that, in each case, the lower bands did not include a light chain while the upper bands did include a light chain. First, when p55-sf2 containing both bands were passed over an anti-kappa column, the upper band bound to the column while the lower band passed through the column. Second, Western blots have shown that only the upper bands were reactive with anti-kappa antibodies.

It is believed that the versions of these fusion proteins that do not have a light chain (k) were not secreted to a significant degree but rather were primarily released from dead cells because 1) supernatants from cells transfected with the p55 heavy chain fusion gene and no light chain gene did not have detectable fusion protein until after there was significant cell death, and 2) the ratio of the k– to k+ versions of p55-sf2 increased as cell cultures went from 95% viability to 10% viability.

WEHI Cytotoxicity Assays

The ability of the various fusion proteins to bind and neutralize human TNFα or TNFβ was tested in a TNF-mediated cell killing assay. Overnight incubation of the murine fibrosarcoma cell line, WEHI 164 (Espevik et al., *J. Immunol. Methods* 1986, 95, 99–105), with 20 pM (1 ng/ml) TNFα results in essentially complete death of the culture. When the fusion proteins were pre-incubated with TNFα (FIG. **31**A) or TNFβ (FIG. **31**B and Table 1 above) and the mixture added to cells, each fusion protein demonstrated dose-dependent protection of the cells from TNF cytotoxicity. Comparison of the viability of control cells not exposed to TNF to cells incubated in both TNF and fusion protein showed that the protection was essentially complete at higher concentrations of fusion protein.

Tetravalent p55-df2 showed the greatest affinity for TNFα requiring a concentration of only 55 pM to confer 50% inhibition of 39 pM (2 ng/ml) TNFα (FIG. **31**A and Table 1). Bivalent p55-sf2 and p75P-sf2 were nearly as efficient, requiring concentrations of 70 pM to half-inhibit TNFα. Approximately two times as much p75-sf2 was required to confer 50% inhibition compared to p55-sf2 at the TNF concentration that was used. The monomeric, non-fusion form of p55 was much less efficient at inhibiting TNFα requiring a 900-fold molar excess over TNFα to inhibit cytotoxicity by 50%. This much-reduced inhibition was also observed with a monomeric, Fab-like p55 fusion protein that was required at a 2000-fold molar excess over TNFα to get 50% inhibition. The order of decreasing inhibitory activity was therefore p55-df2>p55-sf2=p75P-sf2>p75-sf2>>>monomeric p55.

EXAMPLE XXV p75

To make a p75 heavy chain fusion (p75-sf2), amino acids 1–235 (Smith et al., *Science* 1990, 248, 1019–1023 and Kohno et al., *Proc. Natl. Acad. Sci.* 1990, 87, 8331–8335) were encoded in a fragment prepared using the 5' oligo 5'-CACAGCTGCCCGCCCAGGTGGCAT-3' (SEQ ID NO:17) (bold marks the Leu^1 codon) and the 3' oligo 5'-GTCGCCAGTGCTCCCTT-3' (SEQ ID NO:18) (bold marks the complement of the Asp^{235} codon). Two other p75 heavy chain fusions (p75P-sf2 and p75P-sf3) were made using the same 5' oligo with the 3' oligo 5'-ATCGGACGTGGACGTGCAGA-3' (SEQ ID NO:19). The resulting PCR fragment encoded amino acids 1–182. The PCR fragments were blunt-end ligated into the StuI or EcoRV site of the appropriate vector and checked for the absence of errors by sequencing the inserts completely.

Several different versions of the p75 fusion proteins were also expressed. p75-sf2 has the complete extracellular

domain of p75 fused to the heavy chain while p75P-sf2 lacks the C-terminal 53 amino acids of the p75 extracellular domain. p75P-sf3 is the same as p75P-sf2 except that it lacks the C_H1 domain. The region deleted in p75P-sf2 and -sf3 contains sites of O-linked glycosylation and a proline-rich region, neither of which is present in the extracellular domain of p55. Seckinger et al., *Proc. Nat. Acad. Sci. USA* 1990, 87, 5188–5192.

Similar to p55-sf2, two bands were seen for p75-sf2 and p75P-sf2 (FIG. **32**B, lane **8**).

Surprisingly, the order of decreasing inhibitory activity was different for TNFβ, as presented in FIG. **32**. p75P-sf2 was most efficient at inhibition requiring a concentration of 31 pM to half-inhibit human TNFβ at 2 pM. Compared to p75P-sf2, three times as much p75-sf2 and three times as much p55-sf2 were necessary to obtain the same degree of inhibition. The order of decreasing inhibitory activity was therefore p75P-sf2>p75-sf2=p55-sf2.

Affinity Measurements

A comparison was made of the binding affinity of various fusion proteins and TNFα by saturation binding (FIGS. **33**A and **33**B) and Scatchard analysis (FIGS. **33**C–H). A microtiter plate was coated with excess goat anti-Fc polyclonal antibody and incubated with 10 ng/ml of fusion protein in TBST buffer (10 mM Tris-HCl, pH 7.8, 150 NaCl, 0.05% Tween-20) for 1 hour. Varying amounts of ^{125}I labeled TNFα (specific activity—34.8 μCi/μg) was then incubated with the captured fusion protein in PBS (10 mM Na Phosphate, pH 7.0, 150 mM NaCl) with 1% bovine serum albumin for 2 hours. Unbound TNFα was washed away with four washes in PBS and the cpm bound was quantitated using a y-counter. All samples were analyzed in triplicate. The slope of the lines in (FIGS. **33**C–H) represent the affinity constant, K_a. The dissociation constant (K_d) values (see Table 1) were derived using the equation K_d=1/K.

EXAMPLE XXVI

In vivo Results

C3H mice were challenged with 5 μg of human TNFα after treatment with an immunoreceptor molecule of the invention. The effect of the treatment was compared with two control treatments. The first control, cA2, is a chimeric mouse/human IgG_1 monoclonal antibody that binds human TNF, and thus is a positive control. The second control, c17-1A, is a chimeric mouse/human IgG_1 irrelevant monoclonal antibody and is thus a negative control. The results of the treatments were as presented in the following Table 17.

TABLE 17

Treatment	Dead Fraction	% Dead
1 μg cA2	5/14	36%
10 μg CA2	1/15	7%
50 μg c17-1A	13/15	87%
1 μg p55-sf2	8/15	53%
10 μg p55-sf2	0/15	0%
50 μg p55-sf2	0/15	0%

Mice were injected with 25 μg of p55 fusion protein or a control antibody and 1 hour later were challenged with 1 μg lipopolysaccharide (type J5). Mice were checked 24 hours later. The results are presented in the following Table 18.

TABLE 18

Treatment	Dead Fraction	% Dead
Control Antibody	11/11	100%
p55-sf2	0/13	0%

All references cited herein, including journal articles or abstracts, published or corresponding U.S. or foreign patent applications, issued U.S. or foreign patents, or any other references, are entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited references. Additionally, the entire contents of the references cited within the references cited herein are also entirely incorporated by reference.

Reference to known method steps, conventional methods steps, known methods or conventional methods is not in any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the relevant art.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance presented herein, in combination with the knowledge of one of ordinary skill in the art.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 19

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 157 amino acids

-continued

(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val
 1               5                  10                  15

Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg
            20                  25                  30

Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu
        35                  40                  45

Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe
    50                  55                  60

Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile
65                  70                  75                  80

Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala
                85                  90                  95

Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys
            100                 105                 110

Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu Lys
        115                 120                 125

Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp Phe
    130                 135                 140

Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu
145                 150                 155
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 321 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..321

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
GAC ATC TTG CTG ACT CAG TCT CCA GCC ATC CTG TCT GTG AGT CCA GGA       48
Asp Ile Leu Leu Thr Gln Ser Pro Ala Ile Leu Ser Val Ser Pro Gly
  1               5                  10                  15

GAA AGA GTC AGT TTC TCC TGC AGG GCC AGT CAG TTC GTT GGC TCA AGC       96
Glu Arg Val Ser Phe Ser Cys Arg Ala Ser Gln Phe Val Gly Ser Ser
            20                  25                  30

ATC CAC TGG TAT CAG CAA AGA ACA AAT GGT TCT CCA AGG CTT CTC ATA      144
Ile His Trp Tyr Gln Gln Arg Thr Asn Gly Ser Pro Arg Leu Leu Ile
        35                  40                  45

AAG TAT GCT TCT GAG TCT ATG TCT GGG ATC CCT TCC AGG TTT AGT GGC      192
Lys Tyr Ala Ser Glu Ser Met Ser Gly Ile Pro Ser Arg Phe Ser Gly
    50                  55                  60

AGT GGA TCA GGG ACA GAT TTT ACT CTT AGC ATC AAC ACT GTG GAG TCT      240
Ser Gly Ser Gly Thr Asp Phe Thr Leu Ser Ile Asn Thr Val Glu Ser
65                  70                  75                  80

GAA GAT ATT GCA GAT TAT TAC TGT CAA CAA AGT CAT AGC TGG CCA TTC      288
Glu Asp Ile Ala Asp Tyr Tyr Cys Gln Gln Ser His Ser Trp Pro Phe
                85                  90                  95

ACG TTC GGC TCG GGG ACA AAT TTG GAA GTA AAA                          321
```

-continued

```
Thr Phe Gly Ser Gly Thr Asn Leu Glu Val Lys
            100                 105


(2) INFORMATION FOR SEQ ID NO:3:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 107 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Asp Ile Leu Leu Thr Gln Ser Pro Ala Ile Leu Ser Val Ser Pro Gly
  1               5                  10                  15

Glu Arg Val Ser Phe Ser Cys Arg Ala Ser Gln Phe Val Gly Ser Ser
             20                  25                  30

Ile His Trp Tyr Gln Gln Arg Thr Asn Gly Ser Pro Arg Leu Leu Ile
         35                  40                  45

Lys Tyr Ala Ser Glu Ser Met Ser Gly Ile Pro Ser Arg Phe Ser Gly
     50                  55                  60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Ser Ile Asn Thr Val Glu Ser
 65                  70                  75                  80

Glu Asp Ile Ala Asp Tyr Tyr Cys Gln Gln Ser His Ser Trp Pro Phe
                 85                  90                  95

Thr Phe Gly Ser Gly Thr Asn Leu Glu Val Lys
            100                 105


(2) INFORMATION FOR SEQ ID NO:4:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 357 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (ix) FEATURE:
          (A) NAME/KEY: CDS
          (B) LOCATION: 1..357

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GAA GTG AAG CTT GAG GAG TCT GGA GGA GGC TTG GTG CAA CCT GGA GGA       48
Glu Val Lys Leu Glu Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
  1               5                  10                  15

TCC ATG AAA CTC TCC TGT GTT GCC TCT GGA TTC ATT TTC AGT AAC CAC       96
Ser Met Lys Leu Ser Cys Val Ala Ser Gly Phe Ile Phe Ser Asn His
             20                  25                  30

TGG ATG AAC TGG GTC CGC CAG TCT CCA GAG AAG GGG CTT GAG TGG GTT      144
Trp Met Asn Trp Val Arg Gln Ser Pro Glu Lys Gly Leu Glu Trp Val
         35                  40                  45

GCT GAA ATT AGA TCA AAA TCT ATT AAT TCT GCA ACA CAT TAT GCG GAG      192
Ala Glu Ile Arg Ser Lys Ser Ile Asn Ser Ala Thr His Tyr Ala Glu
     50                  55                  60

TCT GTG AAA GGG AGG TTC ACC ATC TCA AGA GAT GAT TCC AAA AGT GCT      240
Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asp Ser Lys Ser Ala
 65                  70                  75                  80

GTC TAC CTG CAA ATG ACC GAC TTA AGA ACT GAA GAC ACT GGC GTT TAT      288
Val Tyr Leu Gln Met Thr Asp Leu Arg Thr Glu Asp Thr Gly Val Tyr
                 85                  90                  95

TAC TGT TCC AGG AAT TAC TAC GGT AGT ACC TAC GAC TAC TGG GGC CAA      336
Tyr Cys Ser Arg Asn Tyr Tyr Gly Ser Thr Tyr Asp Tyr Trp Gly Gln
```

-continued

```
            100                 105                 110

GGC ACC ACT CTC ACA GTC TCC                                         357
Gly Thr Thr Leu Thr Val Ser
        115
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 119 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Glu Val Lys Leu Glu Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
  1               5                  10                  15

Ser Met Lys Leu Ser Cys Val Ala Ser Gly Phe Ile Phe Ser Asn His
             20                  25                  30

Trp Met Asn Trp Val Arg Gln Ser Pro Glu Lys Gly Leu Glu Trp Val
         35                  40                  45

Ala Glu Ile Arg Ser Lys Ser Ile Asn Ser Ala Thr His Tyr Ala Glu
     50                  55                  60

Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asp Ser Lys Ser Ala
 65                  70                  75                  80

Val Tyr Leu Gln Met Thr Asp Leu Arg Thr Glu Asp Thr Gly Val Tyr
                 85                  90                  95

Tyr Cys Ser Arg Asn Tyr Tyr Gly Ser Thr Tyr Asp Tyr Trp Gly Gln
            100                 105                 110

Gly Thr Thr Leu Thr Val Ser
        115
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Gly Thr Leu Val Thr Val Ser Ser
  1               5
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Gly Thr Lys Leu Glu Ile Lys
  1               5
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

-continued

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CCTGGATACCTGTGAAAAGA 20

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCTGGTACCTTAGTCACCGTCTCCTCA 27

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AATAGATATCTCCTTCAACACCTGCAA 27

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ATCGGGACAAAGTTGGAAATA 21

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GGCGGTCTGGTACCGG 16

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

-continued

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTCAACAACATAGTCATCA 19

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CACAGGTGTGTCCCCAAGGAAAA 23

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AATCTGGGGTAGGCACAA 18

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

AGTGTGTGTCCCCAAGG 17

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CACAGCTGCCCGCCCAGGTGGCAT 24

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GTCGCCAGTGCTCCCTT 17

-continued

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

ATCGGACGTGGACGTGCAGA 20

What is claimed is:

1. A chimeric antibody comprising at least part of a human immunoglobulin constant region and at least part of a non-human immunoglobulin variable region, said antibody capable of binding an epitope specific for human tumor necrosis factor TNFα, wherein the non-human immunoglobulin variable region comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 3 and SEQ ID NO: 5.

2. An immunoassay method for detecting human TNF in a sample, comprising:

(a) contacting said sample with an antibody according to claim **1**, or a TNF binding fragment thereof, in detectably labeled form; and

(b) detecting the binding of the antibody to said TNF.

3. A chimeric antibody comprising at least part of a human immunoglobulin constant region and at least part of a non-human immunoglobulin variable region, said antibody capable of binding an epitope specific for human tumor necrosis factor TNFα, wherein the non-human immunoglobulin variable region comprises a polypeptide encoded by a nucleic acid sequence selected from the group consisting of SEQ ID NO: 2 and SEQ ID NO: 4.

4. An immunoassay method for detecting human TNF in a sample, comprising:

(a) contacting said sample with an antibody according to claim **3**, or a TNF binding fragment thereof, in detectably labeled form; and

(b) detecting the binding of the antibody to said TNF.

5. A chimeric antibody, comprising two light chains and two heavy chains, each of said chains comprising at least part of a human immunoglobulin constant region and at least part of a non-human immunoglobulin variable region, said variable region capable of binding an epitope of human tumor necrosis factor hTNFα, wherein said light chains comprise variable regions comprising SEQ ID NO: 3 and said heavy chains comprise variable regions comprising SEQ ID NO: 5.

6. A chimeric antibody according to claim **5**, wherein the human immunoglobulin constant region is an IgG1.

7. A chimeric antibody comprising at least part of a human IgG1 constant region and at least part of a non-human immunoglobulin variable region, said antibody capable of binding an epitope specific for human TNFα, wherein the non-human immunoglobulin variable region comprises a polypeptide encoded by a nucleic acid sequence selected from the group consisting of SEQ ID NO: 2 and SEQ ID NO: 4.

8. A polypeptide comprising the amino acid sequence of SEQ ID NO: 3, wherein said polypeptide binds to hTNFα and competitively inhibits the binding of monoclonal antibody cA2 to hTNFα.

9. A polypeptide comprising the amino acid sequence of SEQ ID NO: 5, wherein said polypeptide binds to hTNFα and competitively inhibits the binding of monoclonal antibody cA2 to hTNFα.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

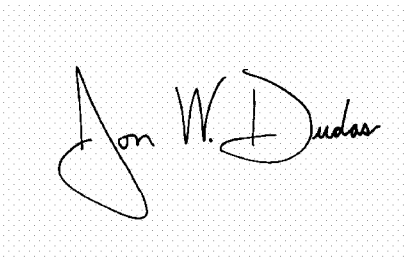
PATENT NO. : 6,284,471 B1
APPLICATION NO. : 08/192093
DATED : September 4, 2001
INVENTOR(S) : Junming Le et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title Page; Item (54);
On Page 1 in the title, please replace "ANTI-TNFA ANTIBODIES AND ASSAYS EMPLOYING ANTI-TNFA ANTIBODIES" with --ANTI-TNFα ANTIBODIES AND ASSAYS EMPLOYING ANTI-TNFα ANTIBODIES--.

Signed and Sealed this

Twenty-third Day of October, 2007



JON W. DUDAS
Director of the United States Patent and Trademark Office

EXHIBIT B

US007223396B2

(12) **United States Patent**
Le et al.

(10) **Patent No.: US 7,223,396 B2**
(45) **Date of Patent: May 29, 2007**

(54) **METHODS OF TREATMENT OF FISTULAS IN CROHN'S DISEASE WITH ANTI-TNF ANTIBODIES**

(75) Inventors: **Junming Le**, Jackson Heights, NY (US); **Jan Vilcek**, New York, NY (US); **Peter Daddona**, Menlo Park, CA (US); **John Ghrayeb**, Thorndale, PA (US); **David Knight**, Berwyn, PA (US); **Scott Siegel**, Westborough, MA (US)

(73) Assignees: **Centocor, Inc.**, Malvern, PA (US); **New York University**, New York, NY (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 620 days.

(21) Appl. No.: **10/319,011**

(22) Filed: **Dec. 12, 2002**

(65) **Prior Publication Data**

US 2003/0194402 A1 Oct. 16, 2003

Related U.S. Application Data

(60) Continuation of application No. 09/756,398, filed on Jan. 8, 2001, now Pat. No. 6,835,823, which is a division of application No. 09/133,119, filed on Aug. 12, 1998, now Pat. No. 6,277,969, which is a division of application No. 08/570,674, filed on Dec. 11, 1995, now abandoned, which is a continuation-in-part of application No. 08/324,799, filed on Oct. 18, 1994, now Pat. No. 5,698,195.

(51) **Int. Cl.**
A61K 39/395 (2006.01)
C07K 16/28 (2006.01)

(52) **U.S. Cl.** **424/145.1**; 424/130.1; 424/133.1; 424/141.1; 424/158.1; 530/387.1; 530/387.3; 530/388.1; 530/388.2; 530/388.23

(58) **Field of Classification Search** ..... None
See application file for complete search history.

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(57) **ABSTRACT**

Anti-TNF antibodies, fragments and regions thereof which are specific for human tumor necrosis factor-α (TNFα) and are useful in vivo diagnosis and therapy of a number of TNFα-mediated pathologies and conditions, as well as polynucleotides coding for murine and chimeric antibodies, methods of producing the antibody, methods of use of the anti-TNF antibody, or fragment, region or derivative thereof, in immunoassays and immunotherapeutic approaches are provided.

32 Claims, 37 Drawing Sheets

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U.S. Appl. No. 11/401,391, filed Apr. 10, 2006, Junming Le, Jan Vilcek, Peter Daddona, John Ghrayeb, David M. Knight and Scott Siegel.
U.S. Appl. No. 11/400,787, filed Apr. 7, 2006, Junming Le, Jan Vilcek, Peter Daddona, John Ghrayeb, David Knight and Scott Siegel.
U.S. Appl. No. 11/501,162, filed Aug. 8, 2006, Junming Le, Jan Vilcek, Peter Daddona, John Ghrayeb, David Knight and Scott Siegel.

* cited by examiner

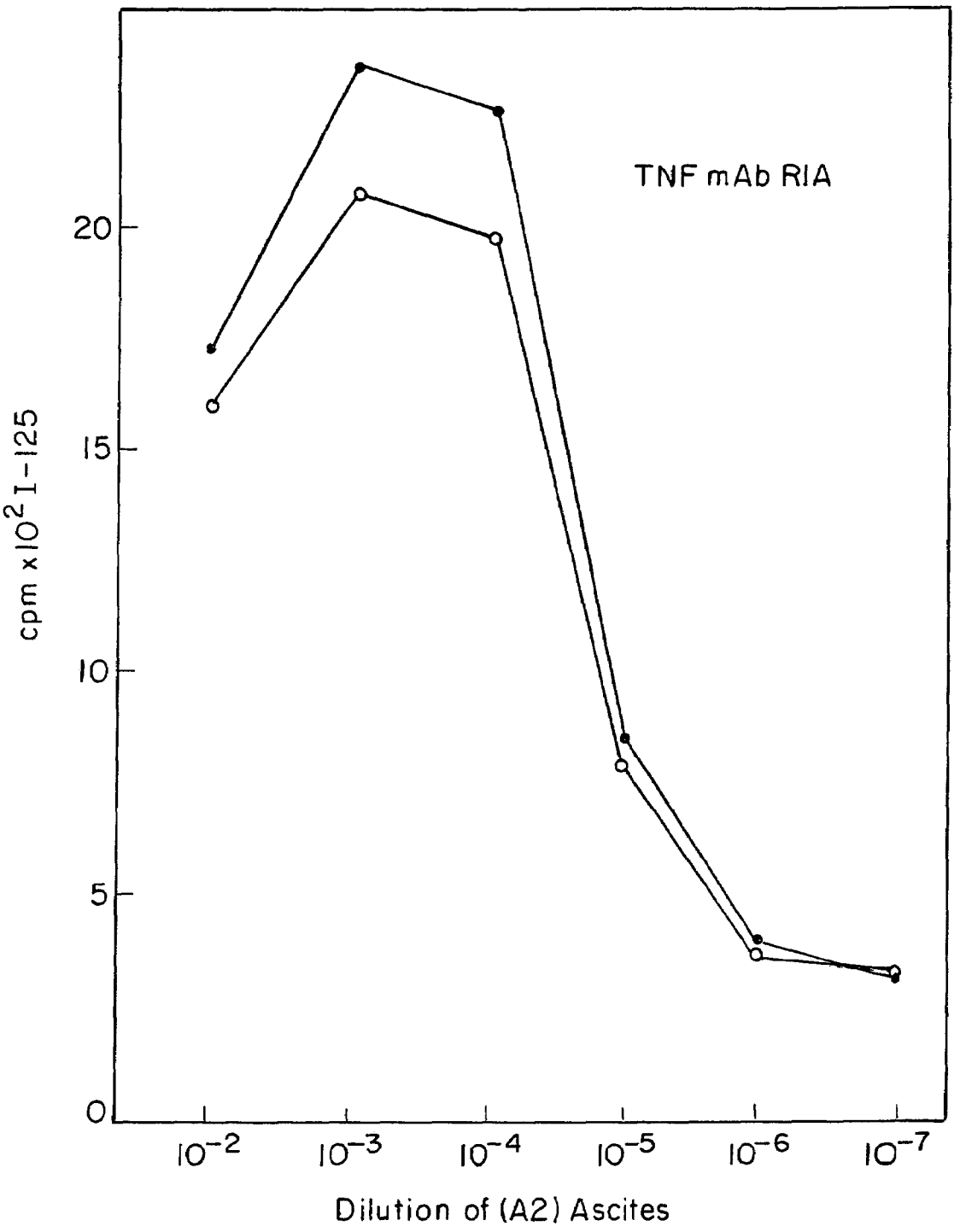

TNF mAb RIA
cpm x10² I-125
20
15
10
5
0
10⁻²
10⁻³
10⁻⁴
10⁻⁵
10⁻⁶
10⁻⁷
Dilution of (A2) Ascites

FIG. 1

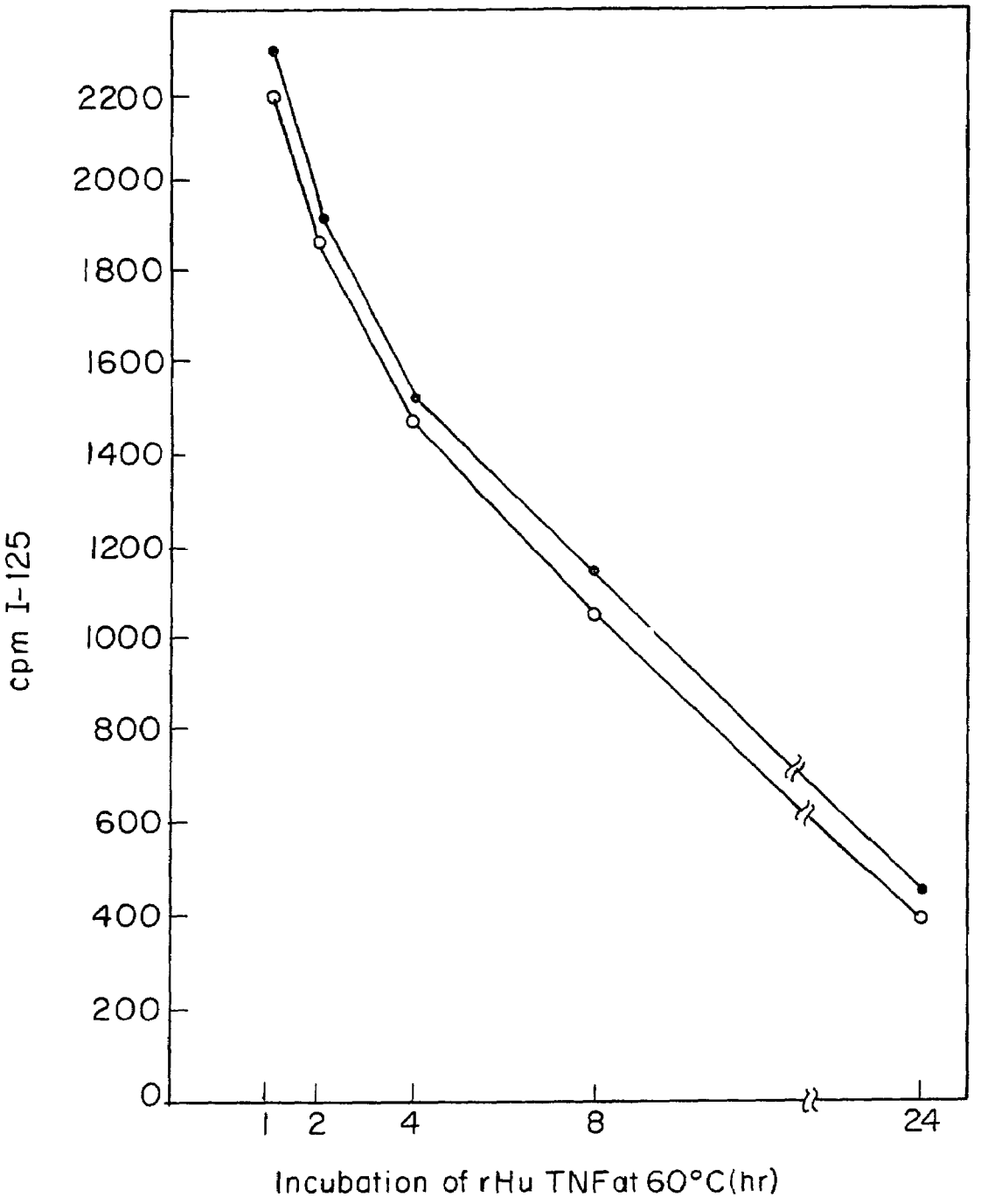

2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
cpm I-125
1
2
4
8
24
Incubation of rHu TNF at 60°C (hr)

FIG. 2

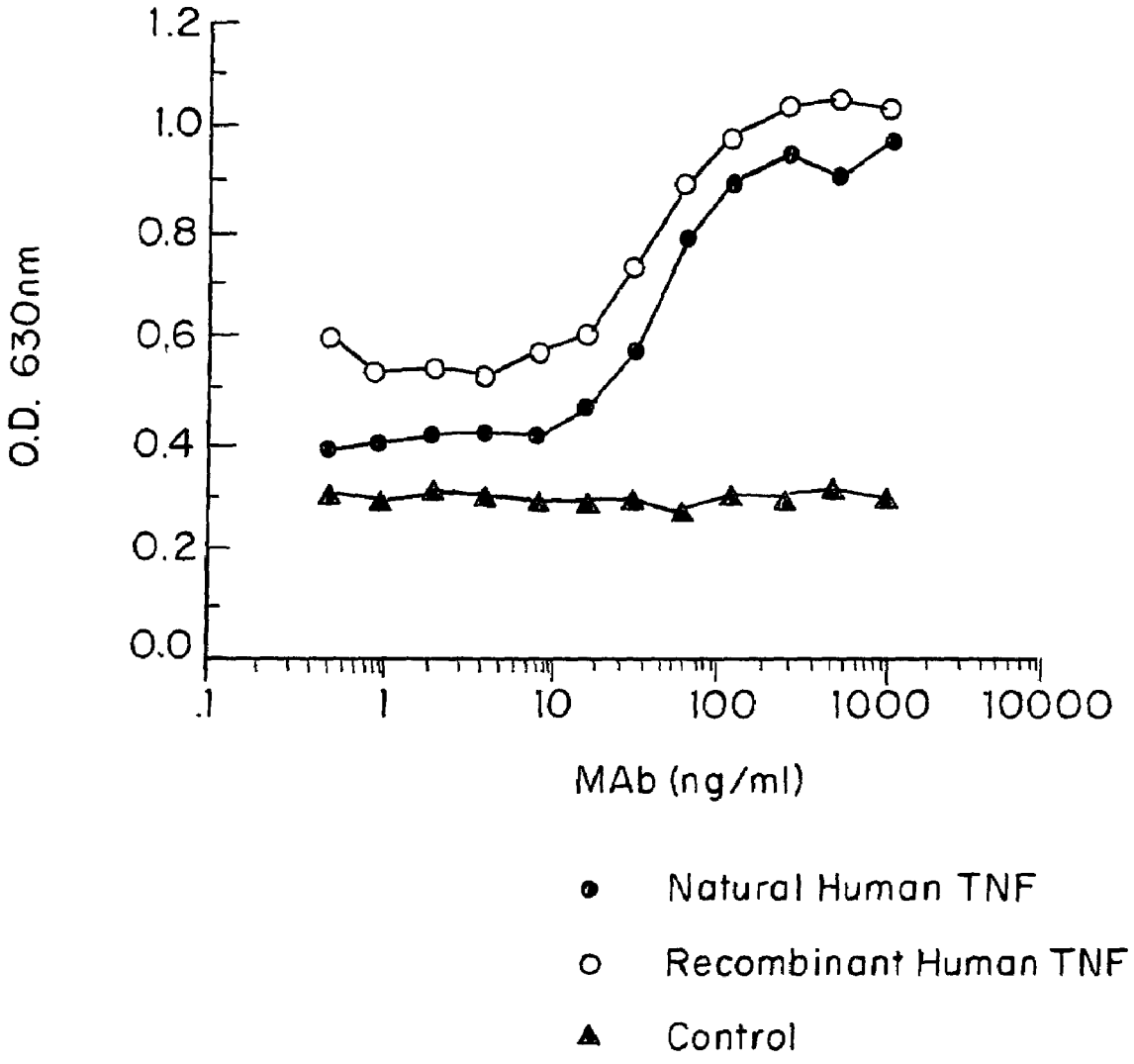

1.2
1.0
0.8
0.6
0.4
0.2
0.0
O.D. 630nm
.1
1
10
100
1000
10000
MAb (ng/ml)
Natural Human TNF
Recombinant Human TNF
Control

FIG. 3

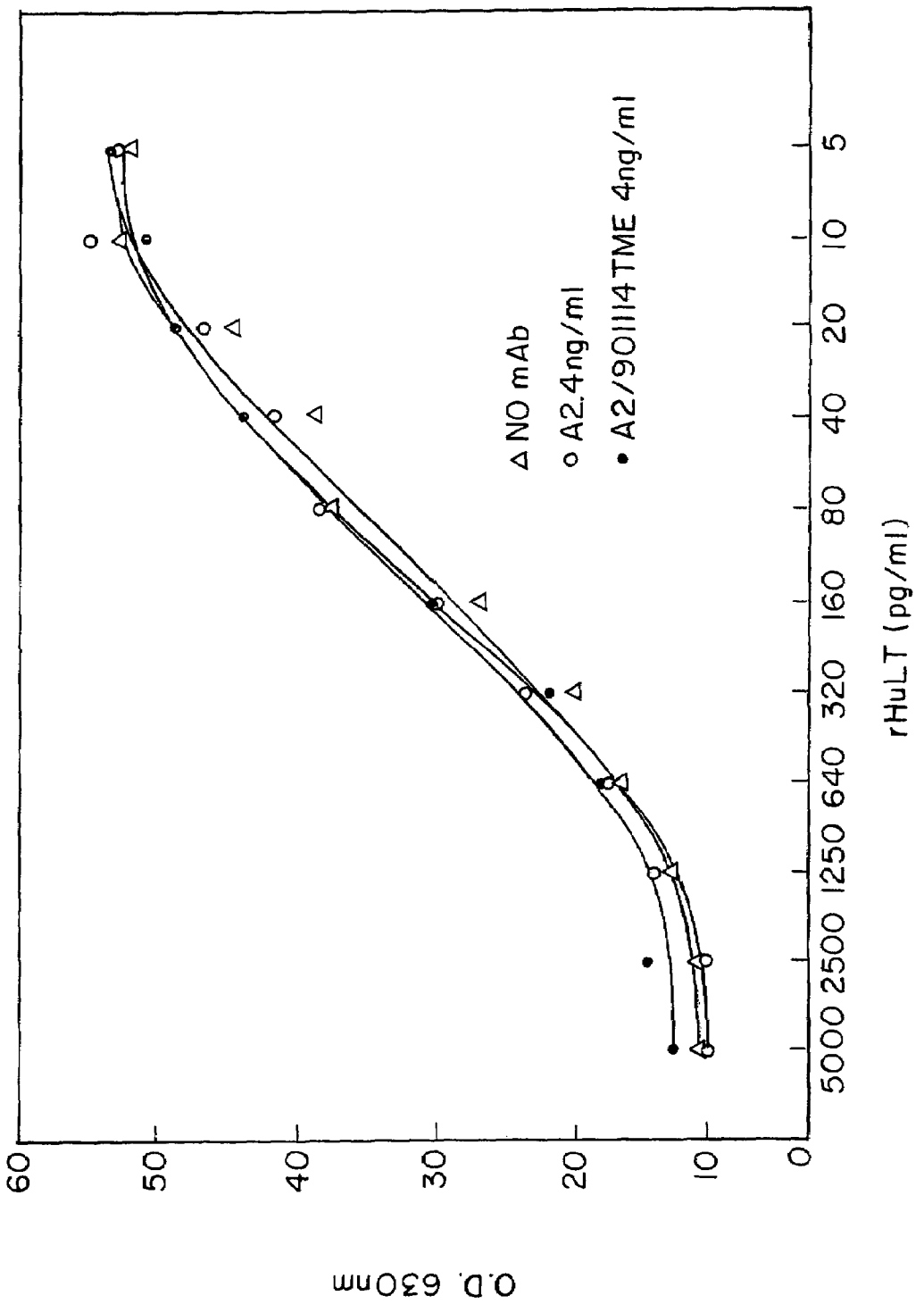

O.D. 630nm
0
10
20
30
40
50
60
5000 2500 1250 640 320 160 80 40 20 10 5
rHuLT (pg/ml)
Δ NO mAb
o A2.4ng/ml
• A2/9011l4 TME 4ng/ml

FIG. 4

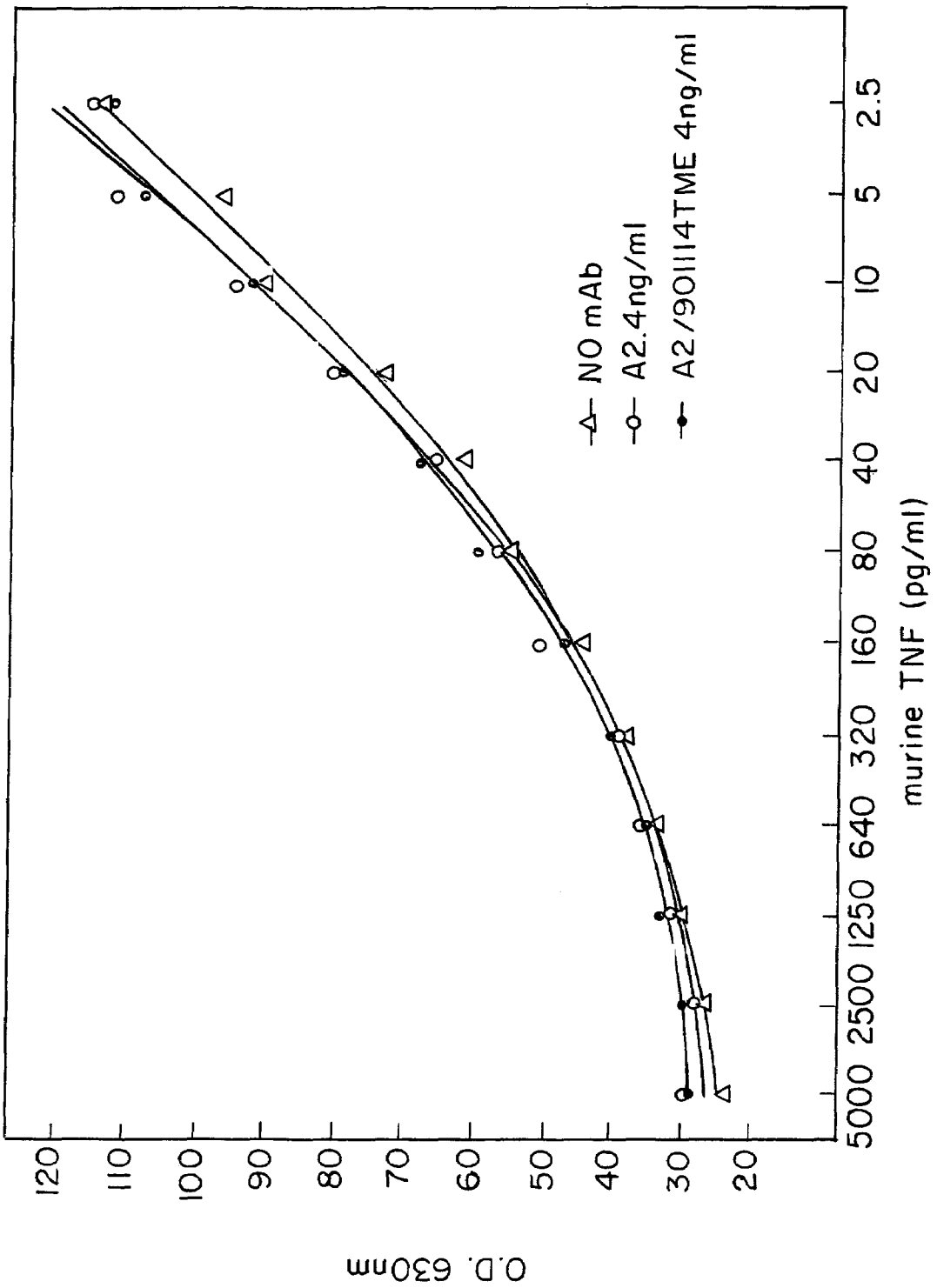

O.D. 630nm
120
110
100
90
80
70
60
50
40
30
20
5000 2500 1250 640 320 160 80 40 20 10 5 2.5
murine TNF (pg/ml)
NO mAb
A2.4ng/ml
A2/9011114TME 4ng/ml

FIG. 5

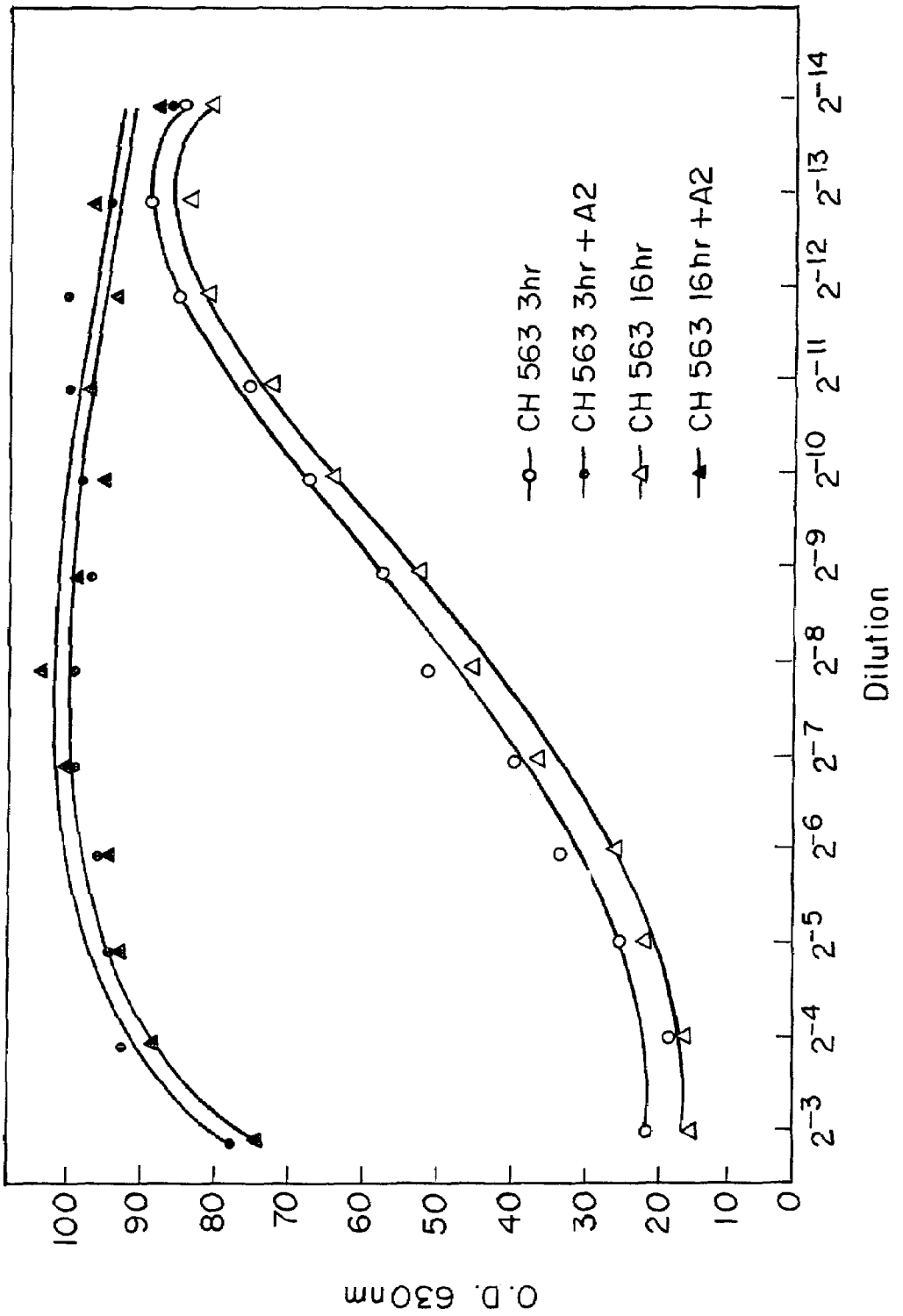

O.D. 630nm
100
90
80
70
60
50
40
30
20
10
0
2^{-3} 2^{-4} 2^{-5} 2^{-6} 2^{-7} 2^{-8} 2^{-9} 2^{-10} 2^{-11} 2^{-12} 2^{-13} 2^{-14}
Dilution
CH 563 3hr
CH 563 3hr + A2
CH 563 16hr
CH 563 16hr + A2

FIG. 6

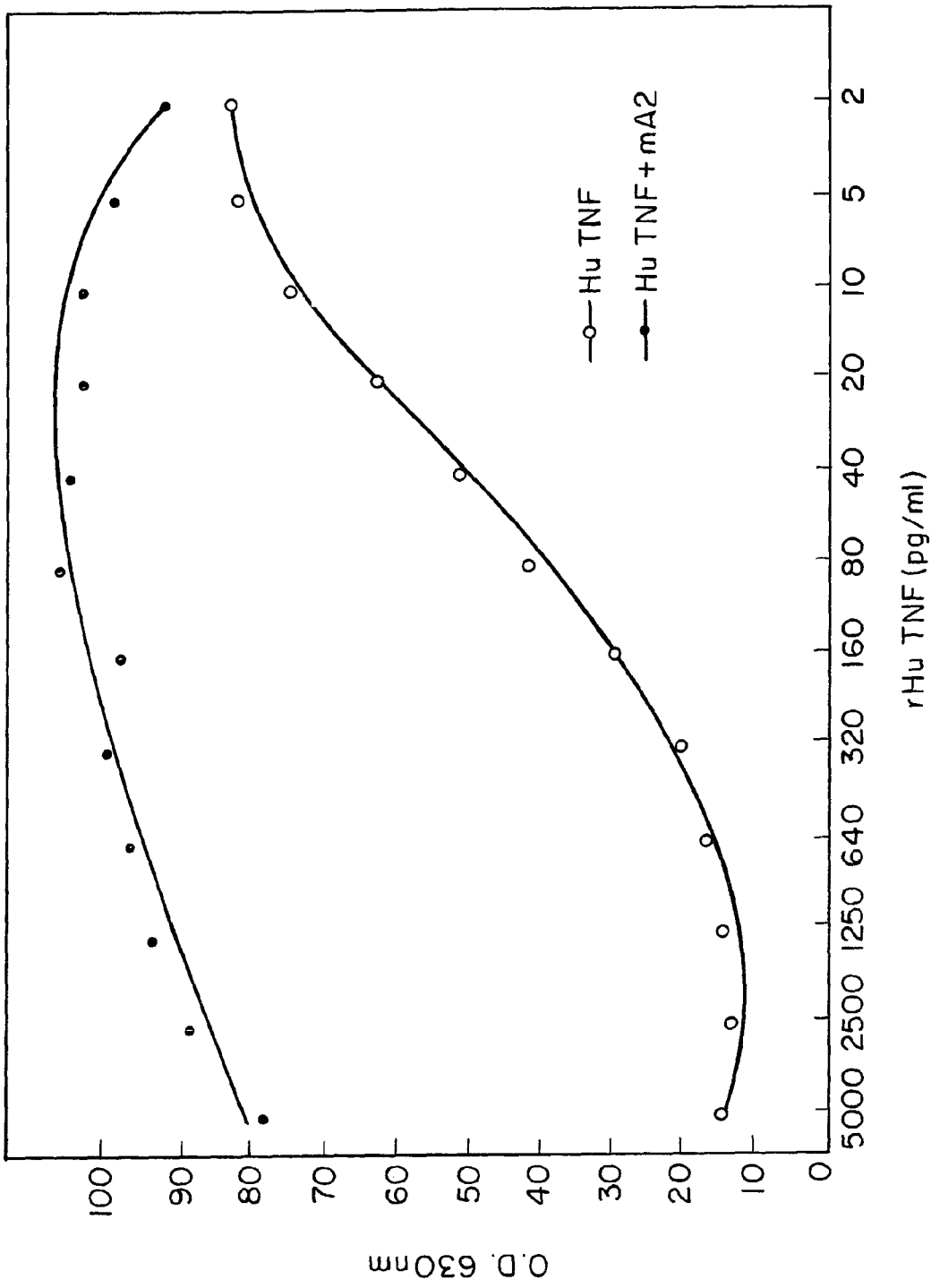
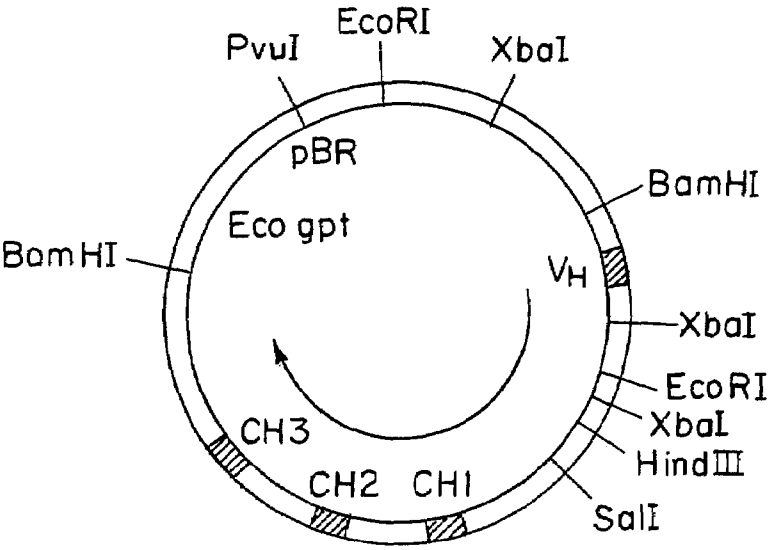
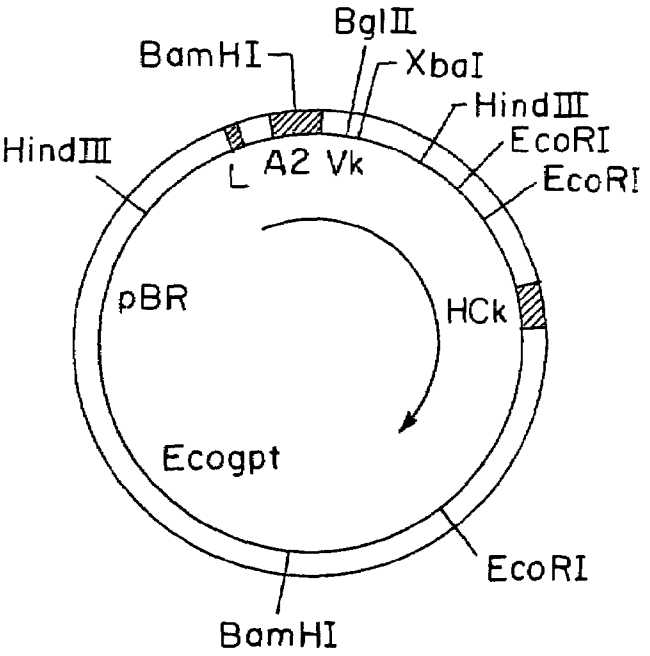

O.D. 630nm
100
90
80
70
60
50
40
30
20
10
0
5000 2500 1250 640 320 160 80 40 20 10 5 2
rHu TNF (pg/ml)
Hu TNF
Hu TNF + mA2

FIG. 7



pA2HG1apgpt

FIG. 8A



pA2HuKapgpt

FIG. 8B

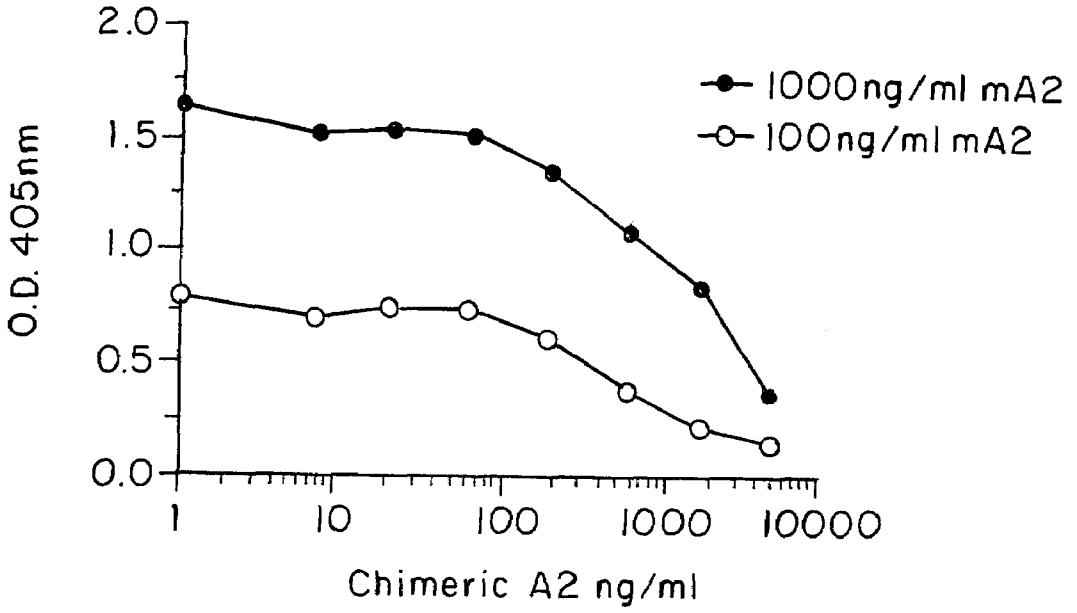

2.0
1.5
1.0
0.5
0.0
O.D. 405nm
1000ng/ml mA2
100ng/ml mA2
1
10
100
1000
10000
Chimeric A2 ng/ml

FIG. 9A

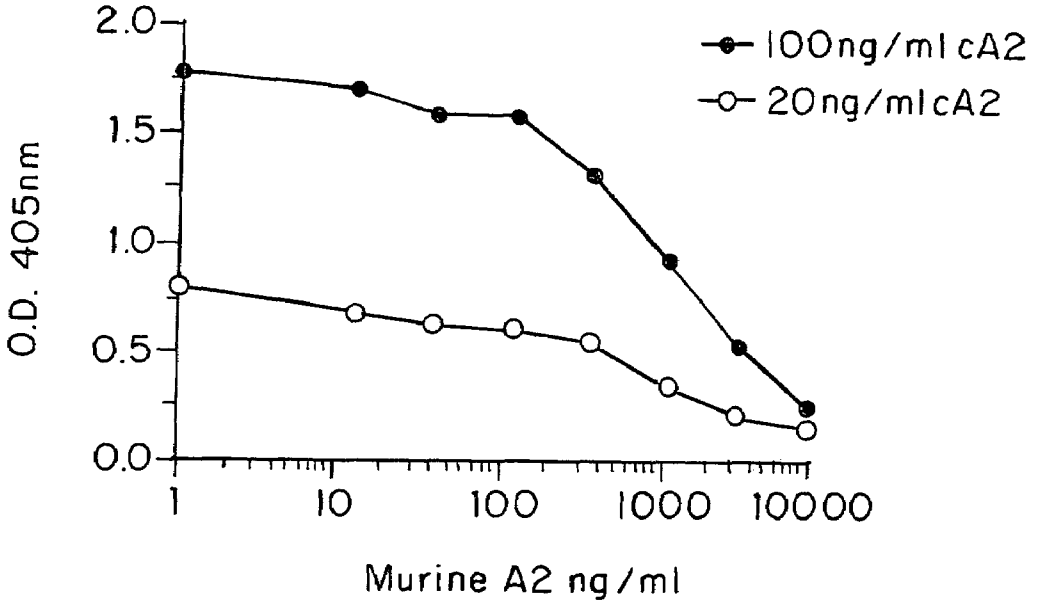

2.0
1.5
1.0
0.5
0.0
O.D. 405nm
100ng/ml cA2
20ng/ml cA2
1
10
100
1000
10000
Murine A2 ng/ml

FIG. 9B

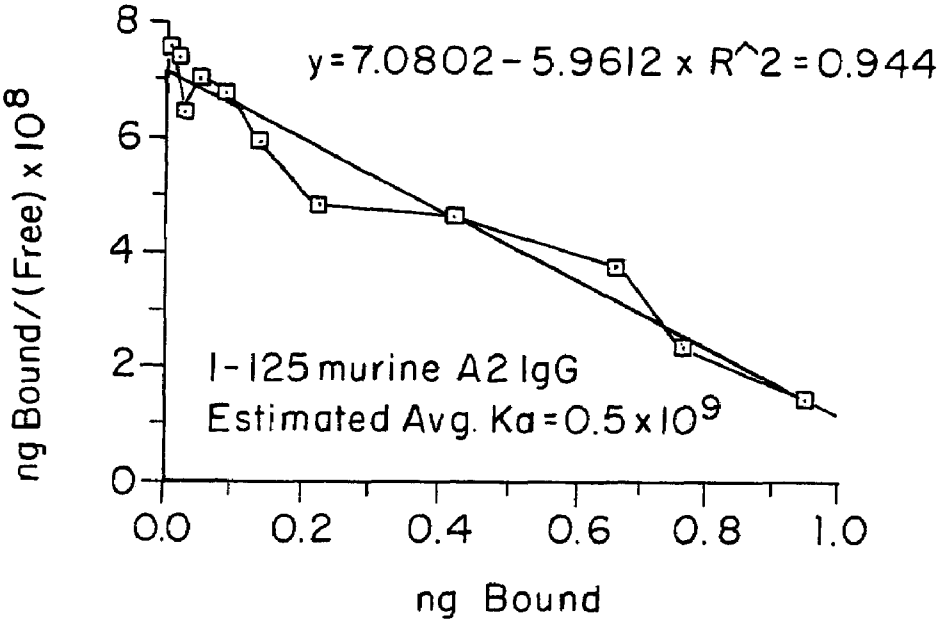


FIG. 10A

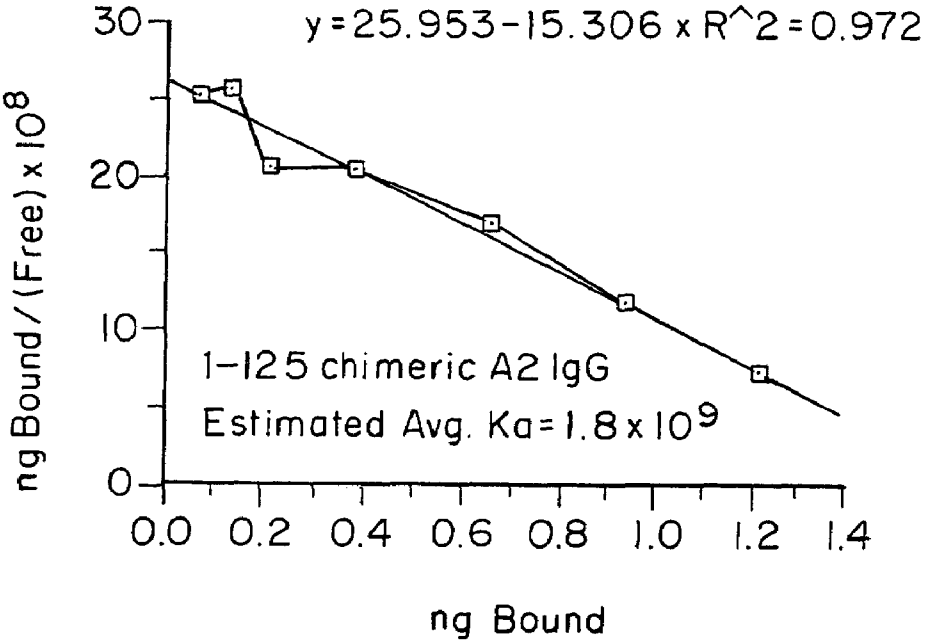


FIG. 10B

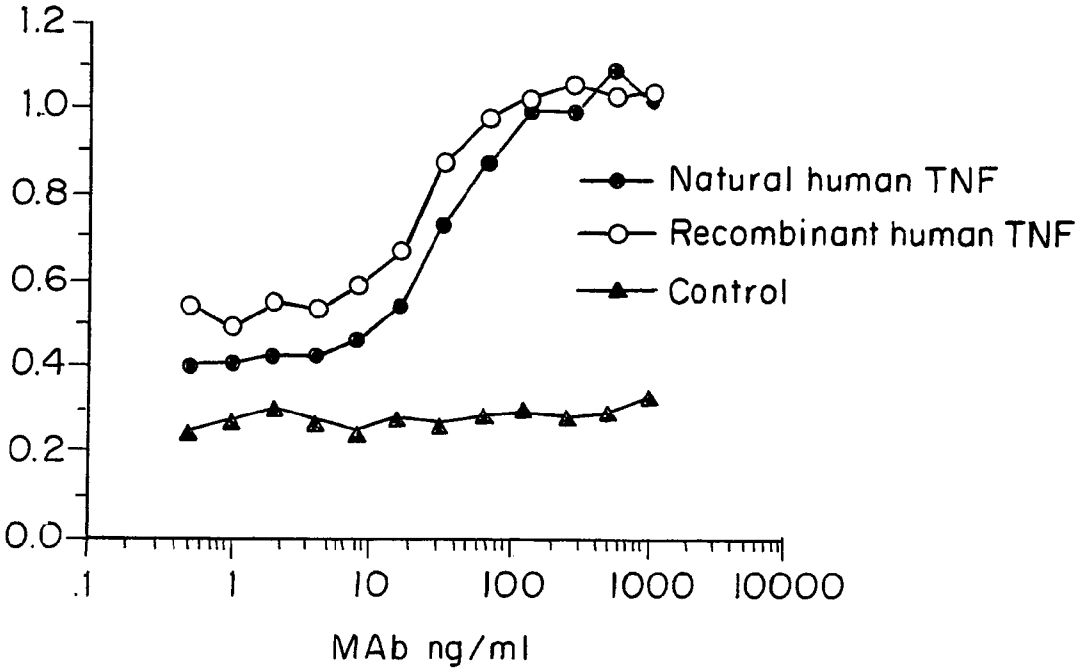

1.2
1.0
0.8
0.6
0.4
0.2
0.0
.1
1
10
100
1000
10000
MAb ng/ml
Natural human TNF
Recombinant human TNF
Control

FIG. 11

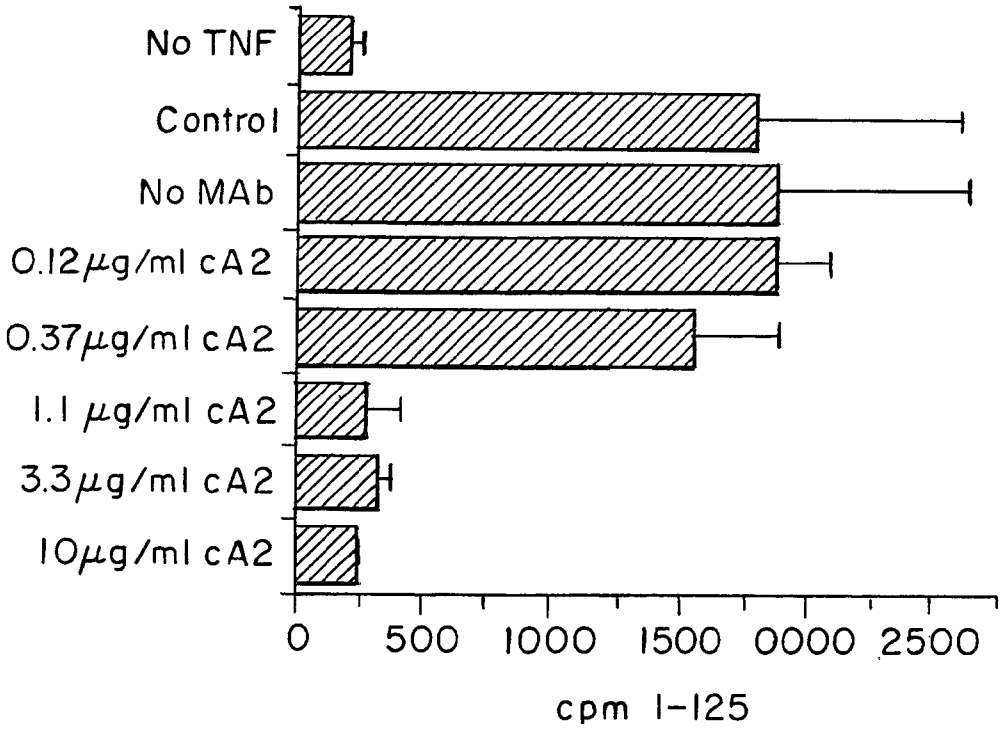

No TNF
Control
No MAb
0.12μg/ml cA2
0.37μg/ml cA2
1.1 μg/ml cA2
3.3μg/ml cA2
10μg/ml cA2
0
500
1000
1500
0000
2500
cpm I-125

FIG. 12

```
1                                                  10
Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val Val Ala Asn Pro

21                 30
Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg Ala Asn Ala Leu Leu Ala Asn Gly

41                 50
Val Glu Leu Arg Asp Asn Gln Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser

61                 70
Gln Val Leu Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile

81                 90
Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala Ile Lys Ser Pro

101                110
Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys Pro Trp Tyr Glu Pro Ile Tyr Leu

121                130
Gly Gly Val Phe Gln Leu Glu Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp

141                150
Tyr Leu Asp Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu
```

FIG. 13

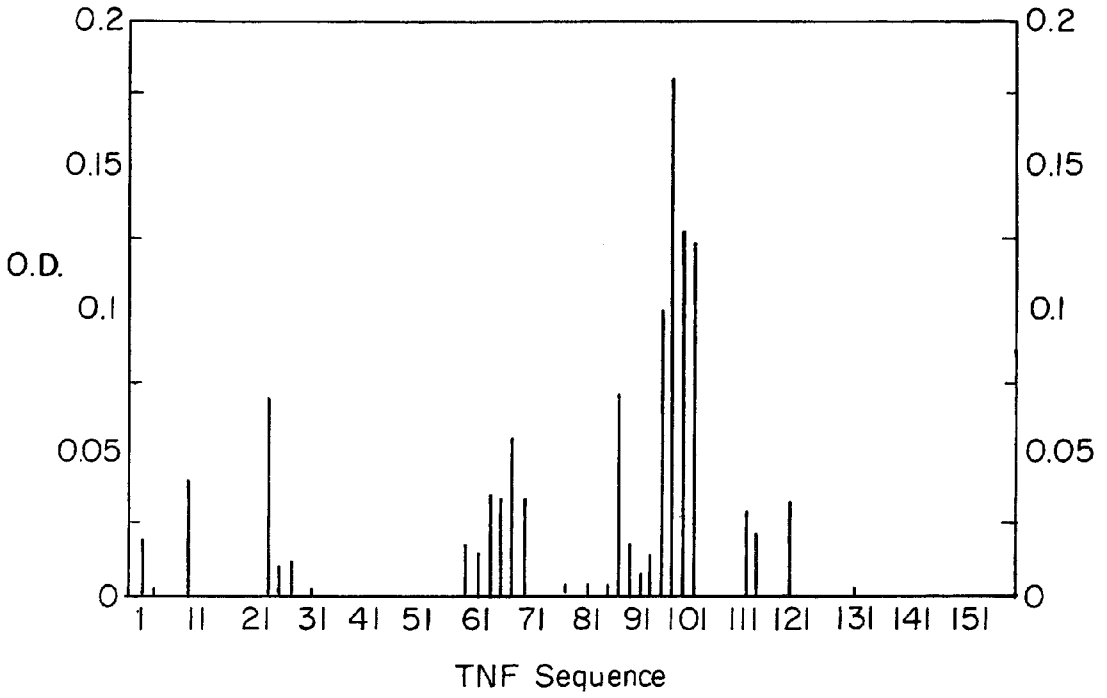


FIG. 14A

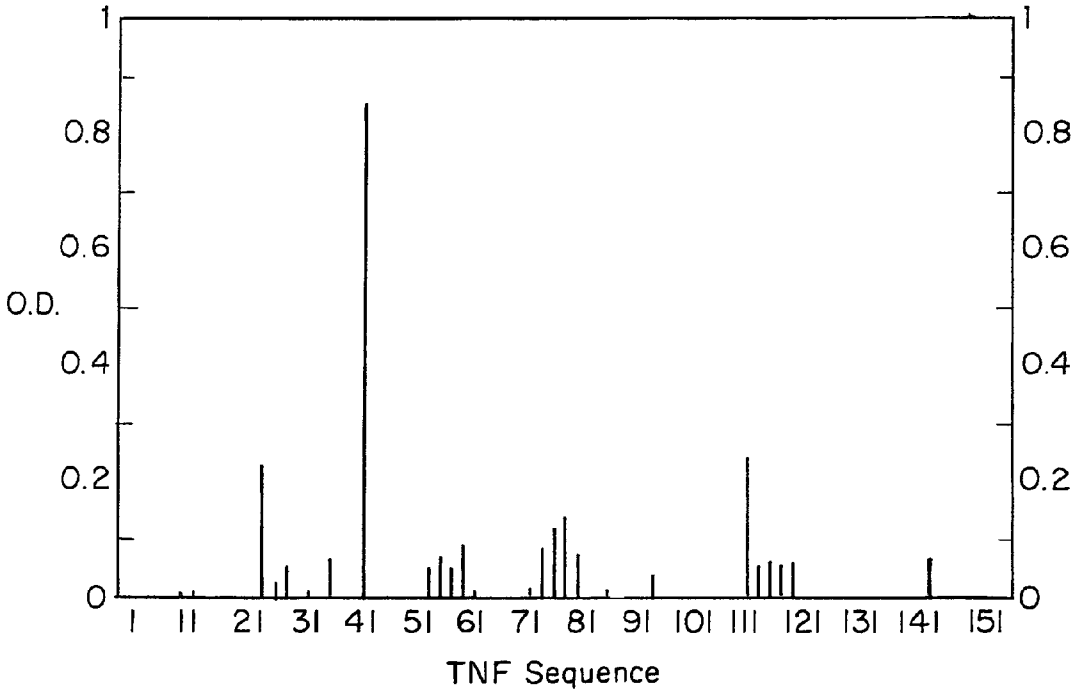


FIG. 14B

```
1
Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val Val Ala Asn Pro
                10
21
Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg Ala Asn Ala Leu Leu Ala Asn Gly
                30
41
Val Glu Leu Arg Asp Asn Gln Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser
                50
61
Gln Val Leu Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile
                70
81
Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala Ile Lys Ser Pro
                90
101
Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys Pro Trp Tyr Glu Pro Ile Tyr Leu
                110
121
Gly Gly Val Phe Gln Leu Glu Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp
                130
141
Tyr Leu Asp Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu
                150
```

FIG. 15

```
GACATCTTGCTGACTCAGTCTCCAGCCATCCTGTCTGTGAGTCCAGGAGAAAGAGTCAGT
AspIleLeuLeuThrGlnSerProAlaIleLeuSerValSerProGlyGluArgValSer

TTCTCCTGCAGGGCCAGTCAGTTCGTTGGCTCAAGCATCCACTGGTATCAGCAAAGAACA
PheSerCysArgAlaSerGlnPheValGlySerSerIleHisTrpTyrGlnGlnArgThr

AATGGTTCTCCAAGGCTTCTCATAAAGTATGCTTCTGAGTCTATGTCTGGGATCCCTTCC
AsnGlySerProArgLeuLeuIleLysTyrAlaSerGluSerMetSerGlyIleProSer

AGGTTTAGTGGCAGTGGATCAGGGACAGATTTTACTCTTAGCATCAACACTGTGGAGTCT
ArgPheSerGlySerGlyThrAspPheThrLeuSerIleAsnThrValGluSer

GAAGATATTGCAGATTATTACTGTCAAGAAAGTCATAGCTGGCCATTCACGTTCGGCTCG
GluAspIleAlaAspTyrTyrCysGlnGlnSerHisSerTrpProPheThrPheGlySer

GGGACAAATTTGGAAGTAAAA
GlyThrAsnLeuGluValLys
```

FIG. 16A

```
GAAGTGAAGCTTGAGGAGTCTGGAGGAGGCTTGGTGCAACCTGGAGGATCCATGAAACTC
GluValLysLeuGluGluSerGlyGlyGlyLeuValGlnProGlyGlySerMetLysLeu

TCCTGTGTTGCCTCTGGATTCATTTTCAGTAACCACTGGATGAACTGGGTCCGCCAGTCT
SerCysValAlaSerGlyPheIlePheSerAsnHisTrpMetAsnTrpValArgGlnSer

CCAGAGAAGGGGCTTGAGTGGGTTGCTGAAATTAGATCAAATCTATTAATTCTGCAACA
ProGluLysGlyLeuGluTrpValAlaGluIleArgSerLysSerIleAsnSerAlaThr

CATTATGCGGAGTCTGTGAAAGGGAGGTTCACCATCTCAAGAGATGATTCCAAAAGTGCT
HisTyrAlaGluSerValLysGlyArgPheThrIleSerArgAspAspSerLysSerAla

GTGTACCTGCAAATGACCGACTTAAGAACTGAAGACACTGGCGTTTATTACTGTTCCAGG
ValTyrLeuGlnMetThrAspLeuArgThrGluAspThrGlyValTyrTyrCysSerArg

AATTACTACGGTAGTACCTACGACTACTGGGGCCAAGGCACCACTCTCACAGTGTCC
AsnTyrTyrGlySerThrTyrAspTyrTrpGlyGlnGlyThrThrLeuThrValSer
```

FIG. 16B

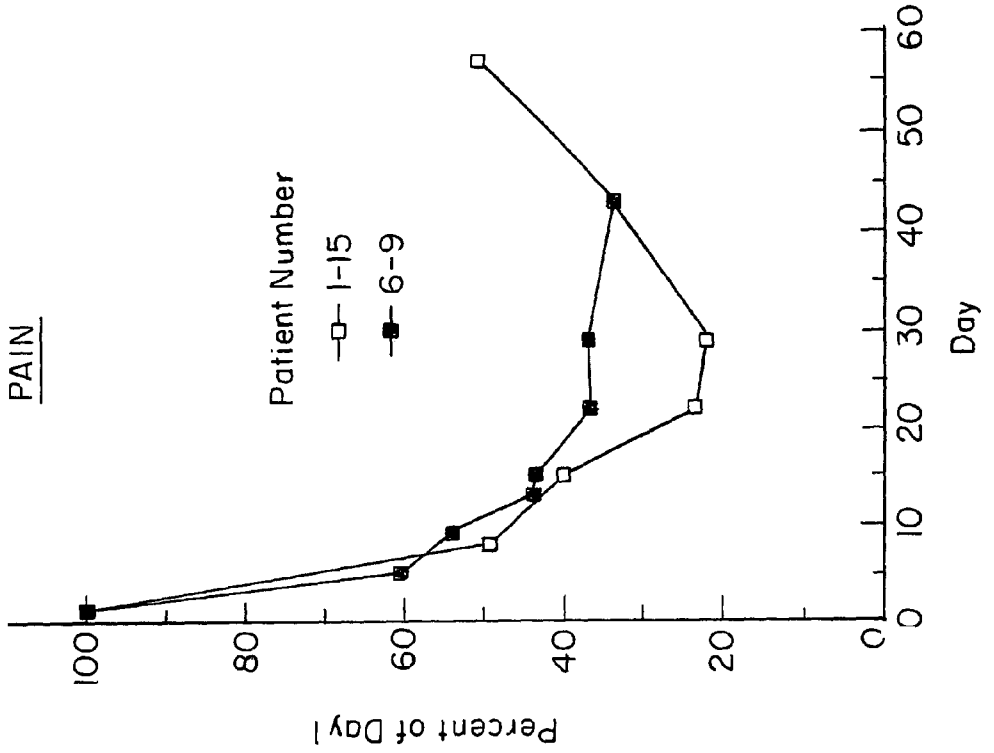

PAIN
Patient Number
1-15
6-9
Percent of Day 1
0
20
40
60
80
100
0
10
20
30
40
50
60
Day

FIG. 18

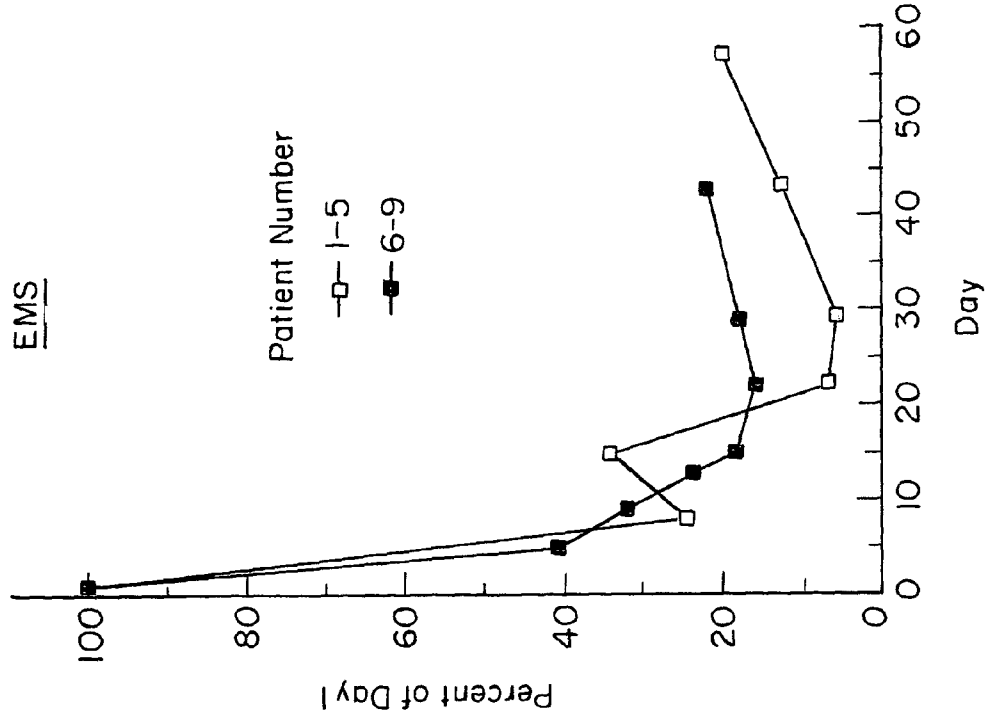

EMS
Patient Number
1-5
6-9
Percent of Day 1
0
20
40
60
80
100
0
10
20
30
40
50
60
Day

FIG. 17

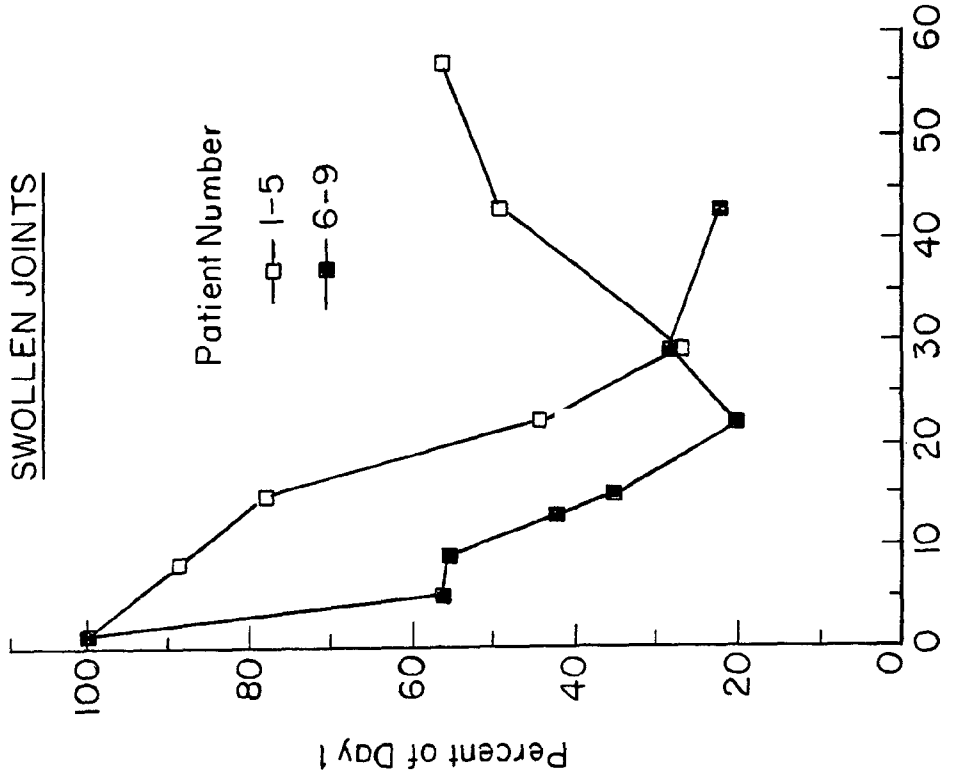

SWOLLEN JOINTS
Patient Number
1-5
6-9
Percent of Day 1
Day
0
20
40
60
80
100
0
10
20
30
40
50
60

FIG. 20

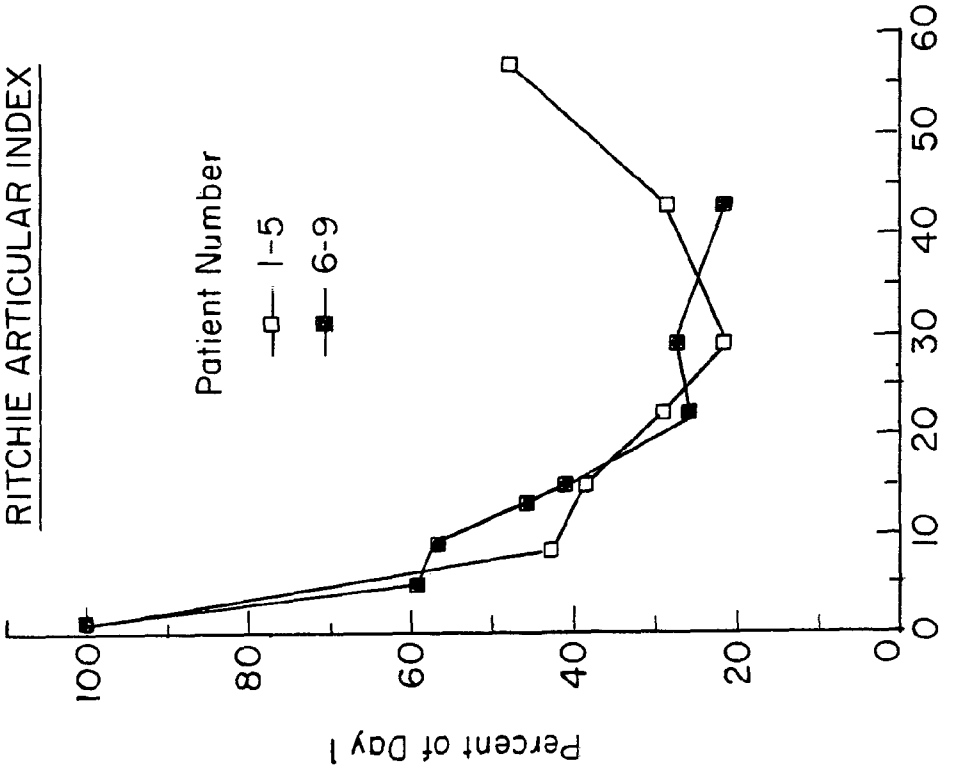

RITCHIE ARTICULAR INDEX
Patient Number
1-5
6-9
Percent of Day 1
Day
0
20
40
60
80
100
0
10
20
30
40
50
60

FIG. 19

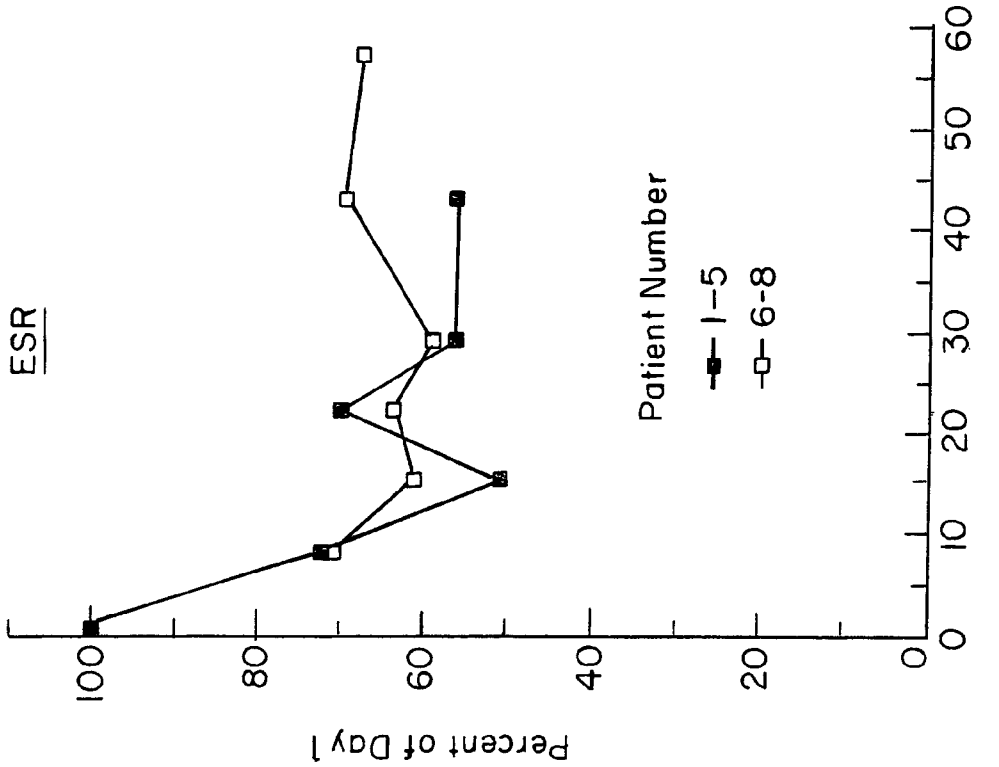

ESR
Percent of Day 1
100
80
60
40
20
0
0
10
20
30
40
50
60
Day
Patient Number
1-5
6-8

FIG. 22

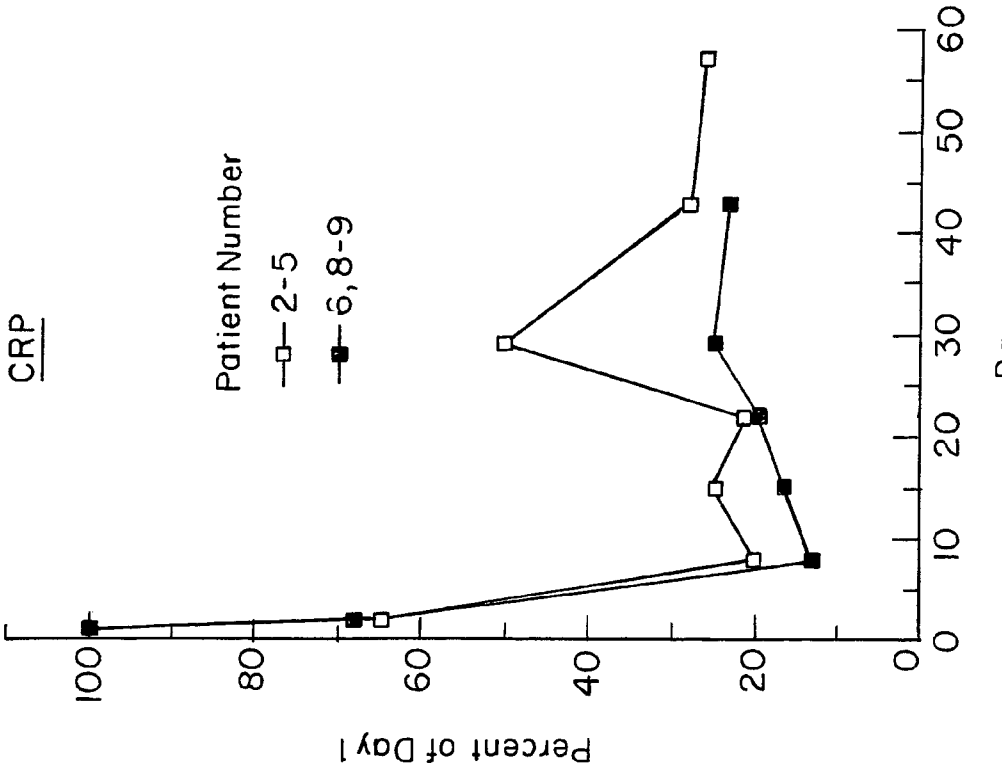

CRP
Percent of Day 1
100
80
60
40
20
0
0
10
20
30
40
50
60
Day
Patient Number
2-5
6, 8-9

FIG. 21

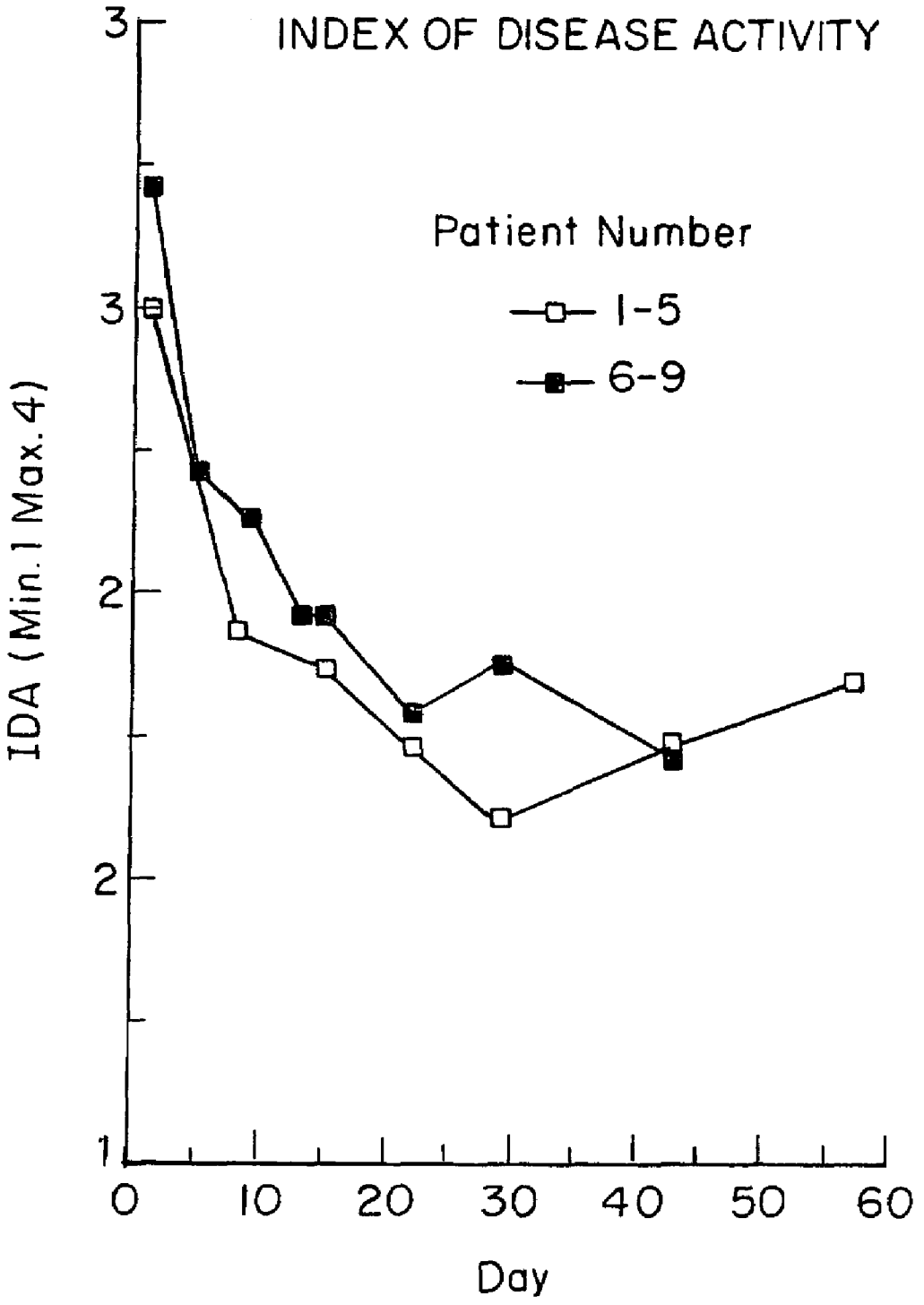

INDEX OF DISEASE ACTIVITY
Patient Number
1-5
6-9
IDA (Min. 1 Max. 4)
3
3
2
2
1
0
10
20
30
40
50
60
Day

FIG. 23

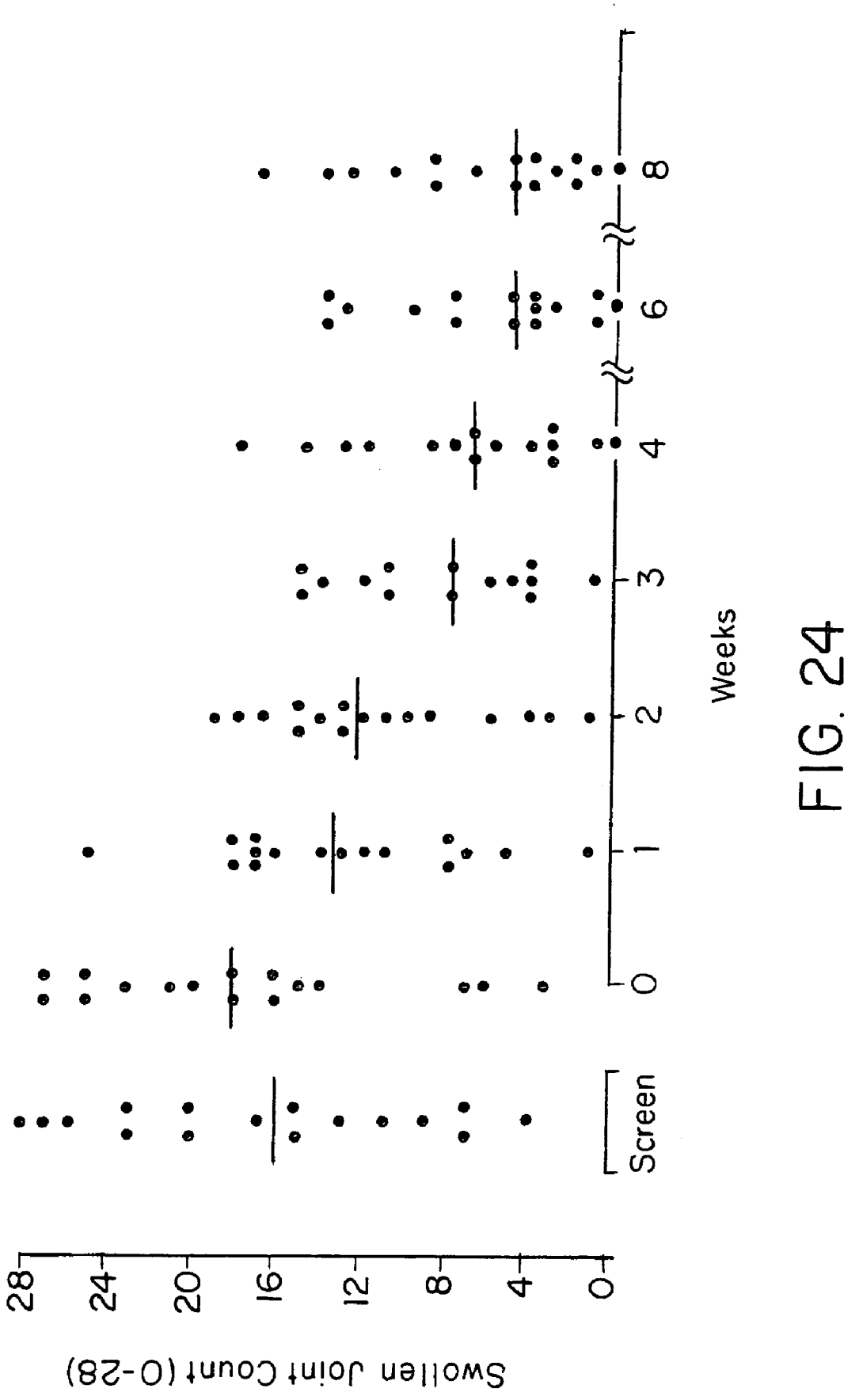

Swollen Joint Count (0-28)
28
24
20
16
12
8
4
0
Screen
0
1
2
3
4
6
8
Weeks

FIG. 24

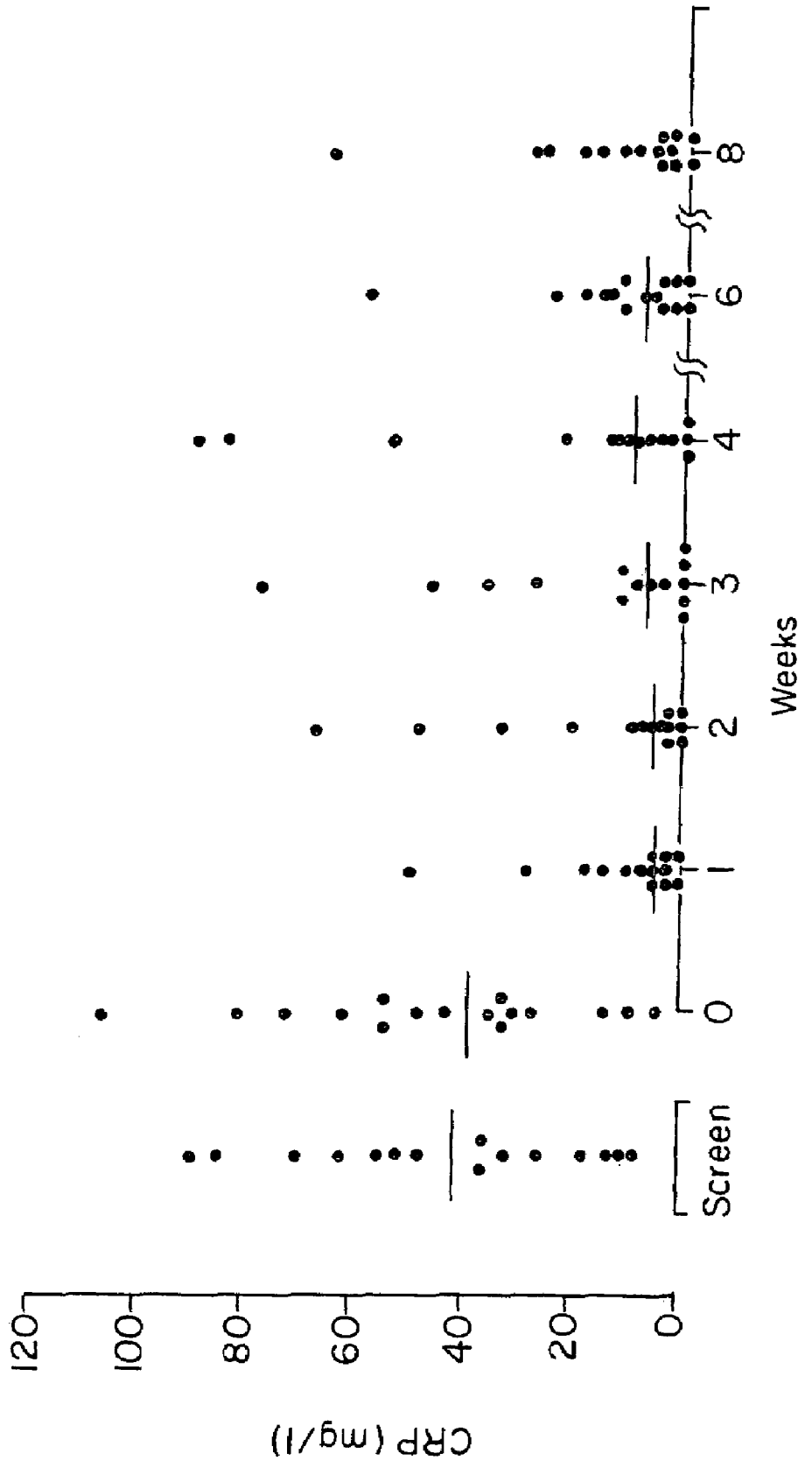
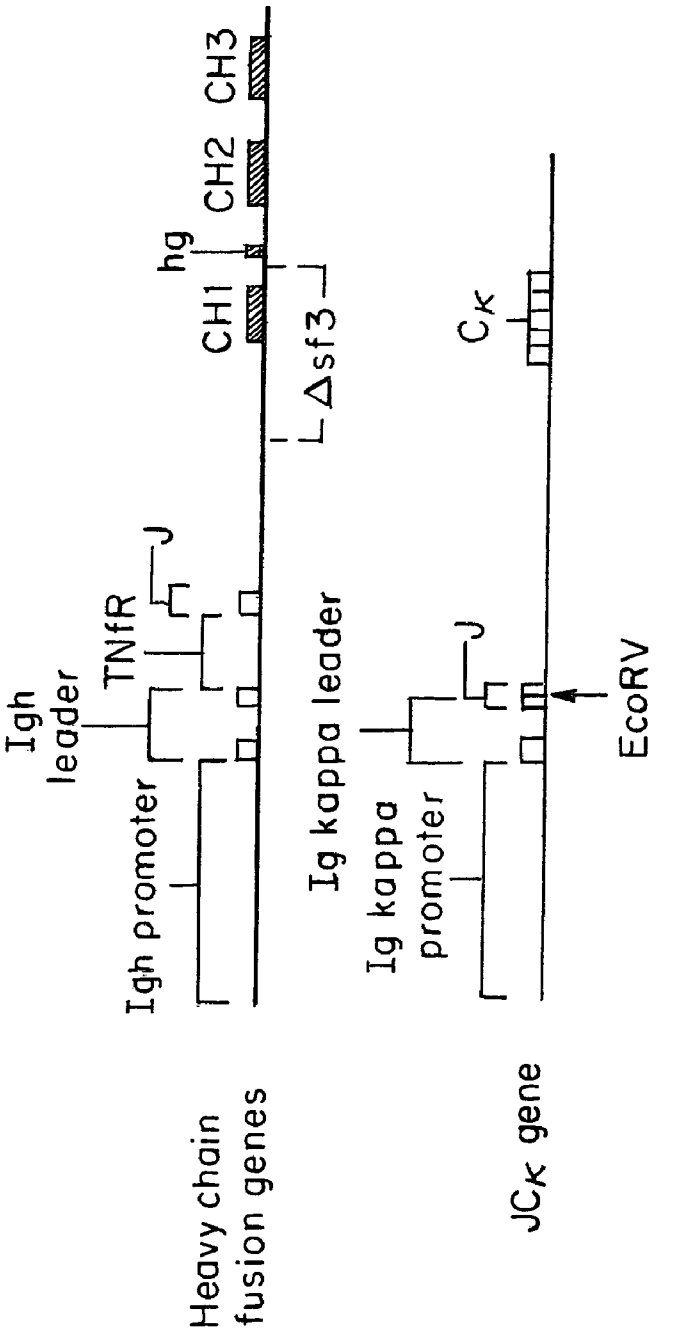


FIG. 25

FIG. 26A


Igh leader
TNfR
J
Igh promoter
hg
CH1
CH2
CH3
Δsf3
Heavy chain fusion genes
Ig kappa leader
J
Ig kappa promoter
Cκ
EcoRV
JCκ gene

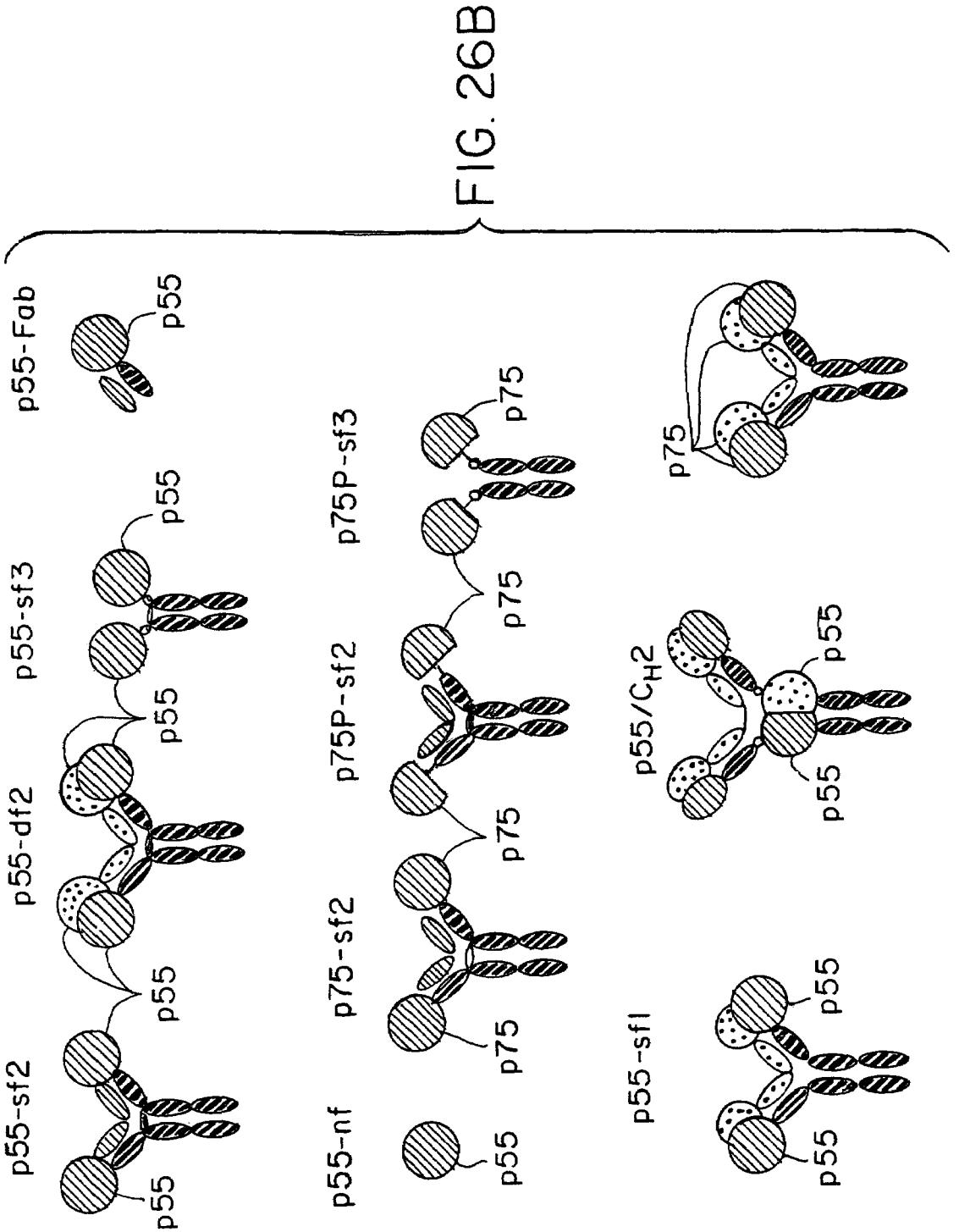


FIG. 26B
p55-Fab
p55
p55-sf3
p55-df2
p55-sf2
p75P-sf3
p75P-sf2
p75-sf2
p55-nf
p75
p55/CH2
p55-sf1

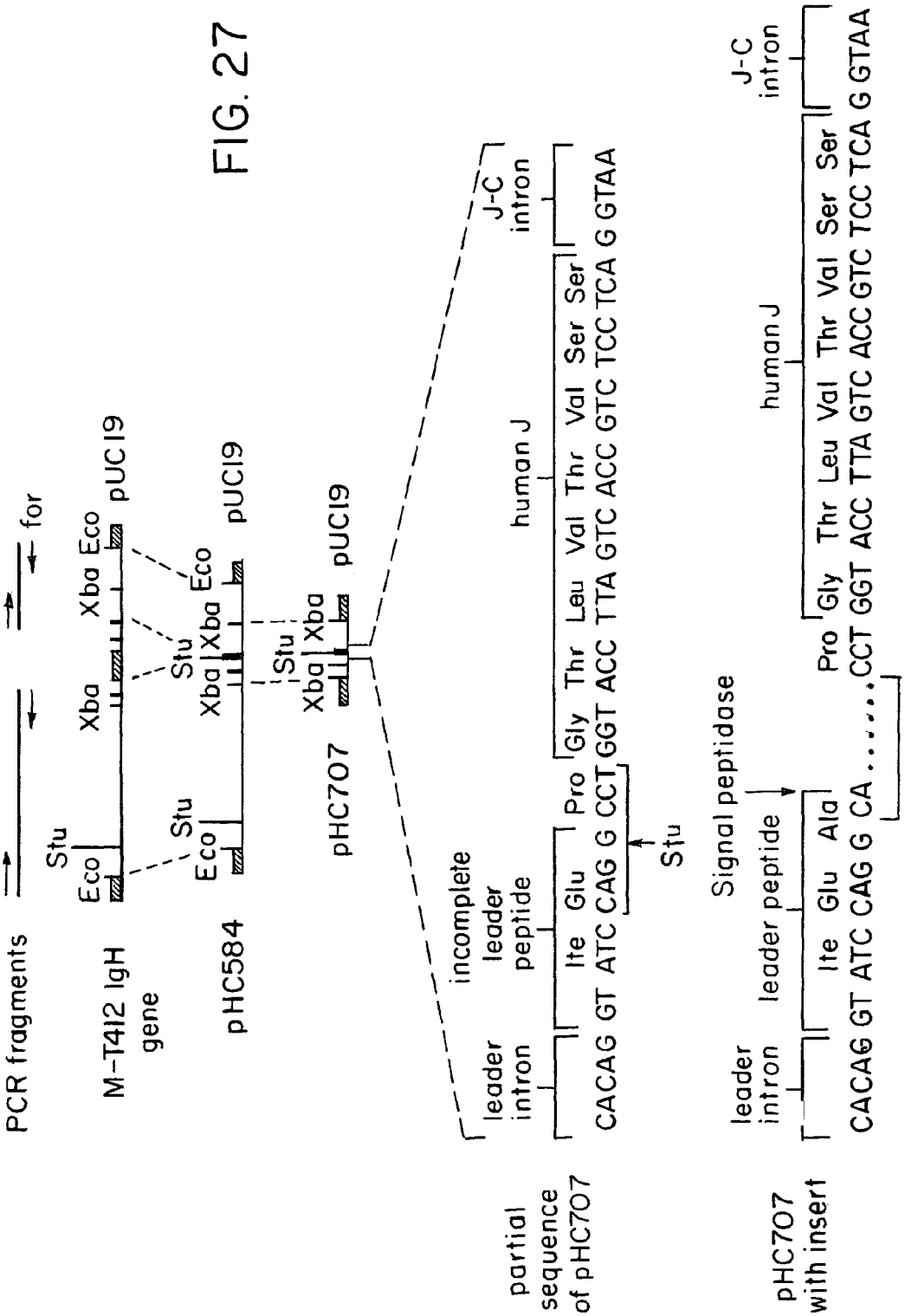

PCR fragments
rev
for
Stu
Eco
Xba
Xba Eco
pUC19
M-T412 IgH gene
Stu
Eco
Stu
Xba
Xba Eco
pUC19
pHC584
Stu
Xba
Xba
pUC19
pHC707
incomplete leader peptide
leader intron
human J
J-C intron
Ile Glu Pro Gly Thr Leu Val Thr Val Ser Ser
CACAG GT ATC CAG G CCT GGT ACC TTA GTC ACC GTC TCC TCA G GTAA
Stu
partial sequence of pHC707
Signal peptidase
leader peptide
leader intron
human J
J-C intron
Ile Glu Ala Pro Gly Thr Leu Val Thr Val Ser Ser
CACAG GT ATC CAG G CA ..... CCT GGT ACC TTA GTC ACC GTC TCC TCA G GTAA
insert
pHC707 with insert

FIG. 27

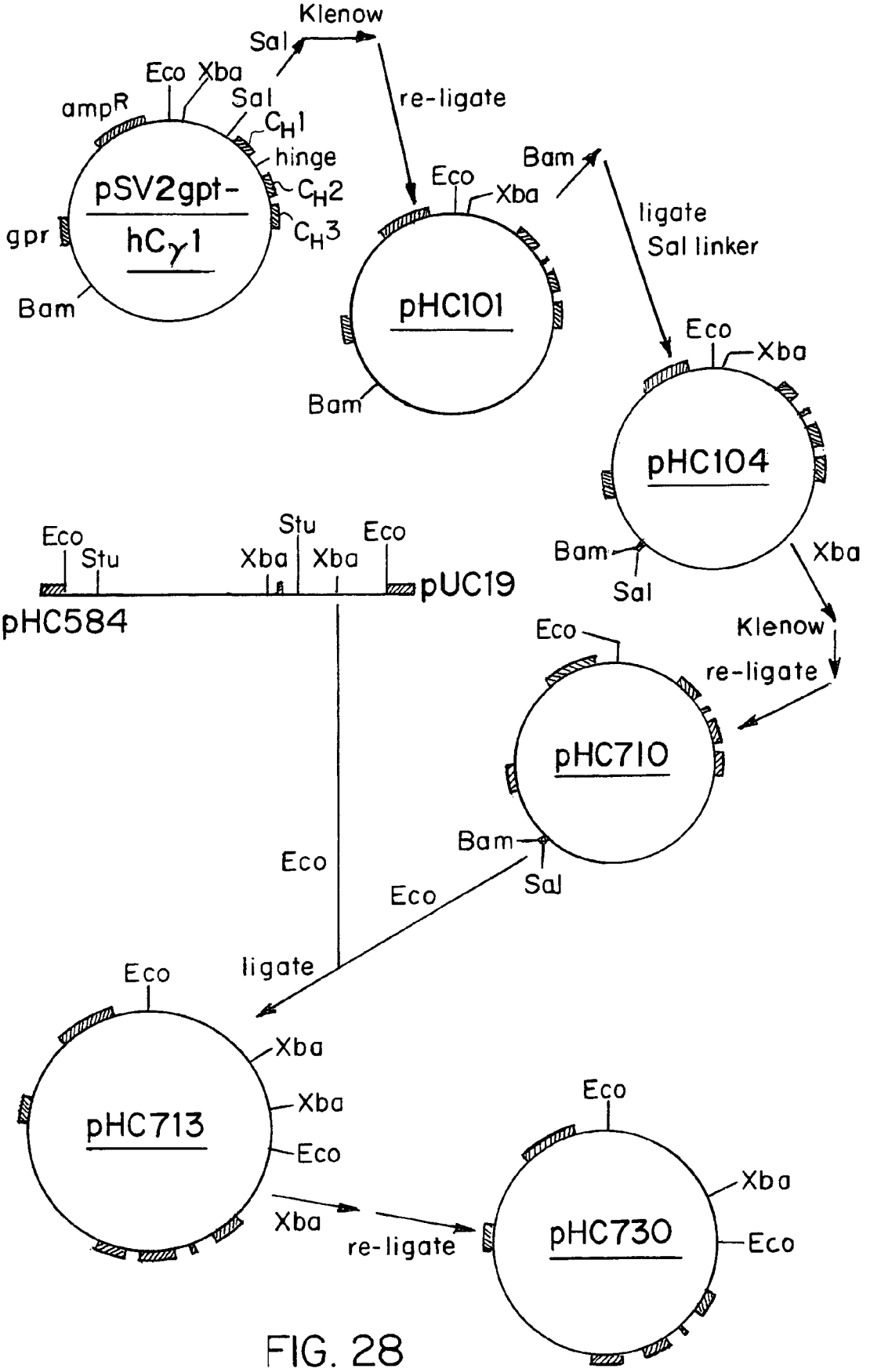

Klenow
Sal
Eco Xba
Sal
ampR
C_H1
hinge
C_H2
C_H3
pSV2gpt-
$hC_\gamma1$
gpr
Bam
re-ligate
Eco
Xba
pHC101
Bam
Bam
ligate
Sal linker
Eco
Xba
pHC104
Bam
Sal
Xba
Eco
Stu
Xba
Xba
Eco
pUC19
pHC584
Klenow
re-ligate
Eco
pHC710
Bam
Sal
Eco
Eco
ligate
Eco
Xba
Xba
pHC713
Eco
Xba
re-ligate
Eco
pHC730
Xba
Eco

FIG. 28

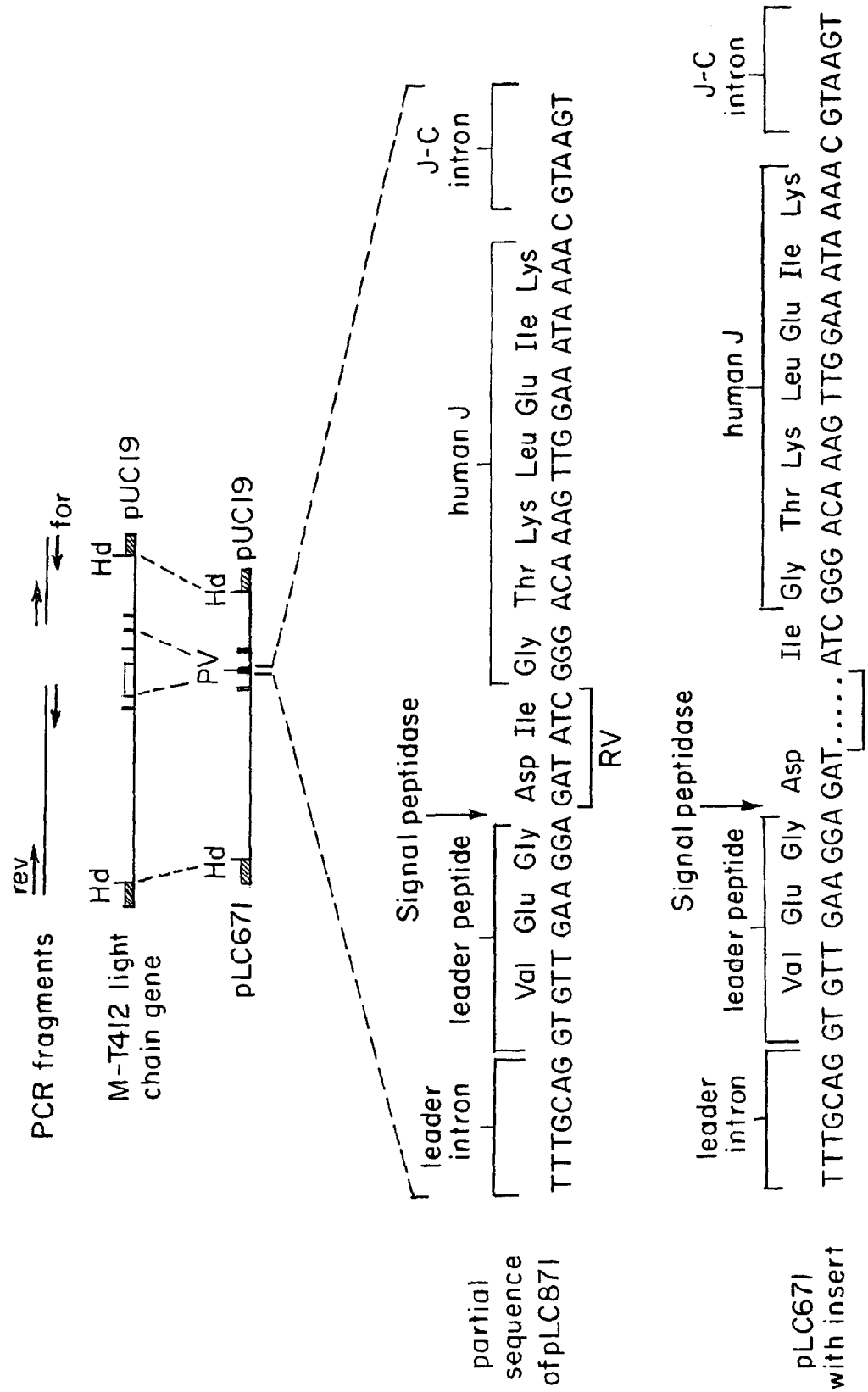
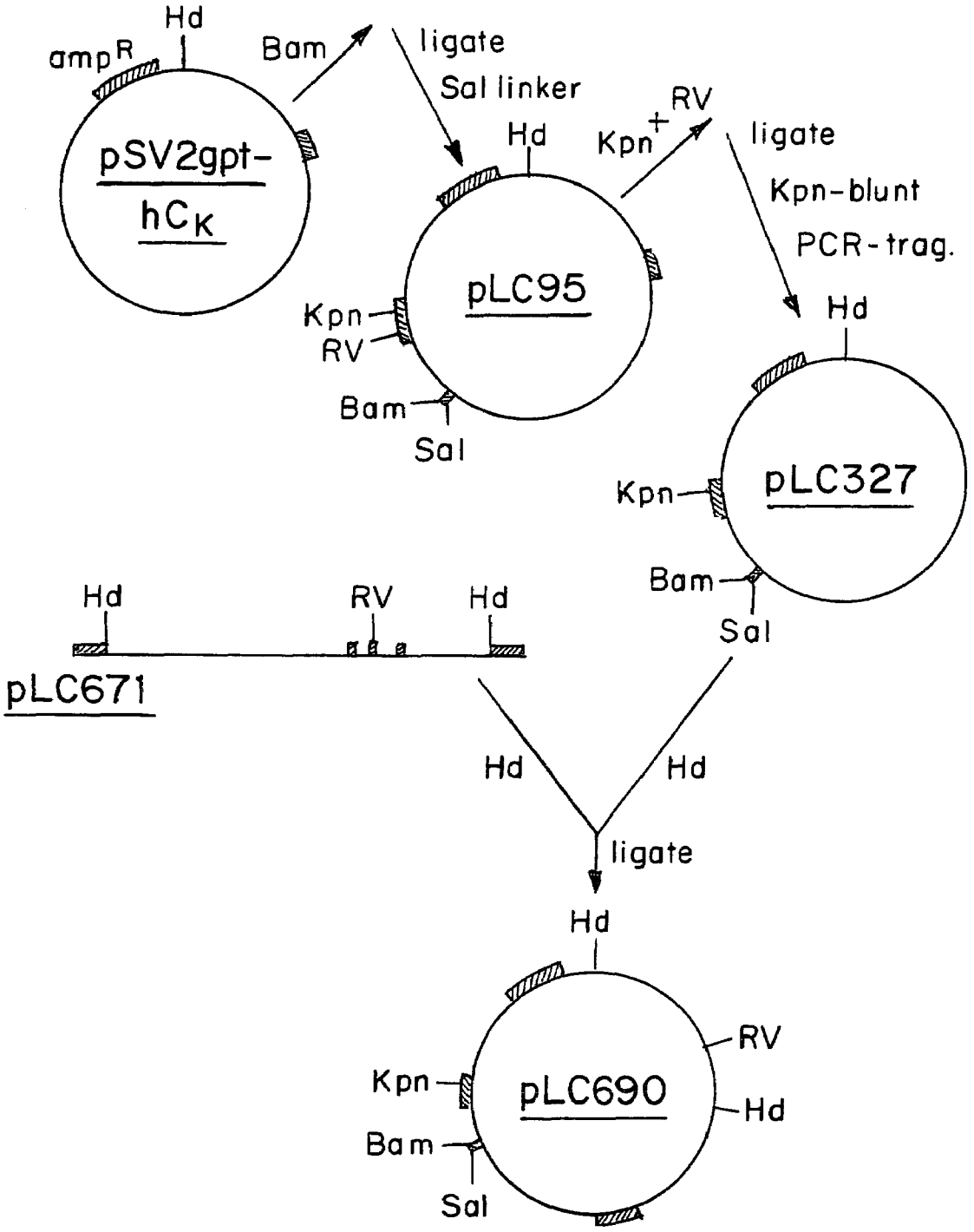

PCR fragments
rev
for
M-T412 light chain gene
Hd
Hd
pUC19
PV
Hd
Hd
pUC19
pLC671
partial sequence ofpLC871
leader intron
Signal peptidase
leader peptide
human J
J-C intron
Val Glu Gly Asp Ile Gly Thr Lys Leu Glu Ile Lys
TTTGCAG GT GTT GAA GGA GAT ATC GGG ACA AAG TTG GAA ATA AAA C GTAAGT
RV
pLC671 with insert
leader intron
Signal peptidase
leader peptide
human J
J-C intron
Val Glu Gly Asp Ile Gly Thr Lys Leu Glu Ile Lys
TTTGCAG GT GTT GAA GGA GAT.....ATC GGG ACA AAG TTG GAA ATA AAA C GTAAGT
insert

FIG. 29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ampR
Bam
ligate
Sal linker
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Hd
Kpn+
RV
ligate
Kpn-blunt
PCR-trag.
pLC95
Kpn
RV
Bam
Sal
Hd
pLC327
Kpn
Bam
Sal
Hd
RV
Hd
pLC671
Hd
Hd
ligate
Hd
RV
Kpn
pLC690
Hd
Bam
Sal

FIG. 30

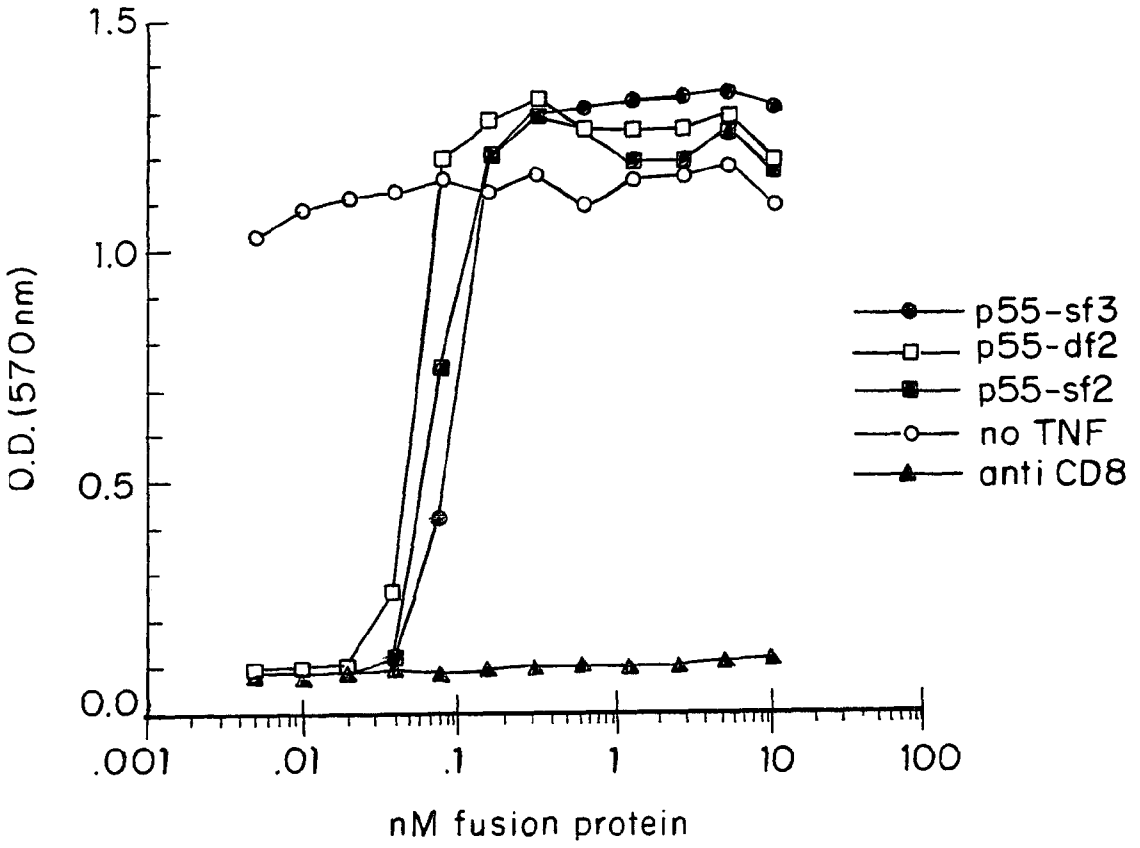
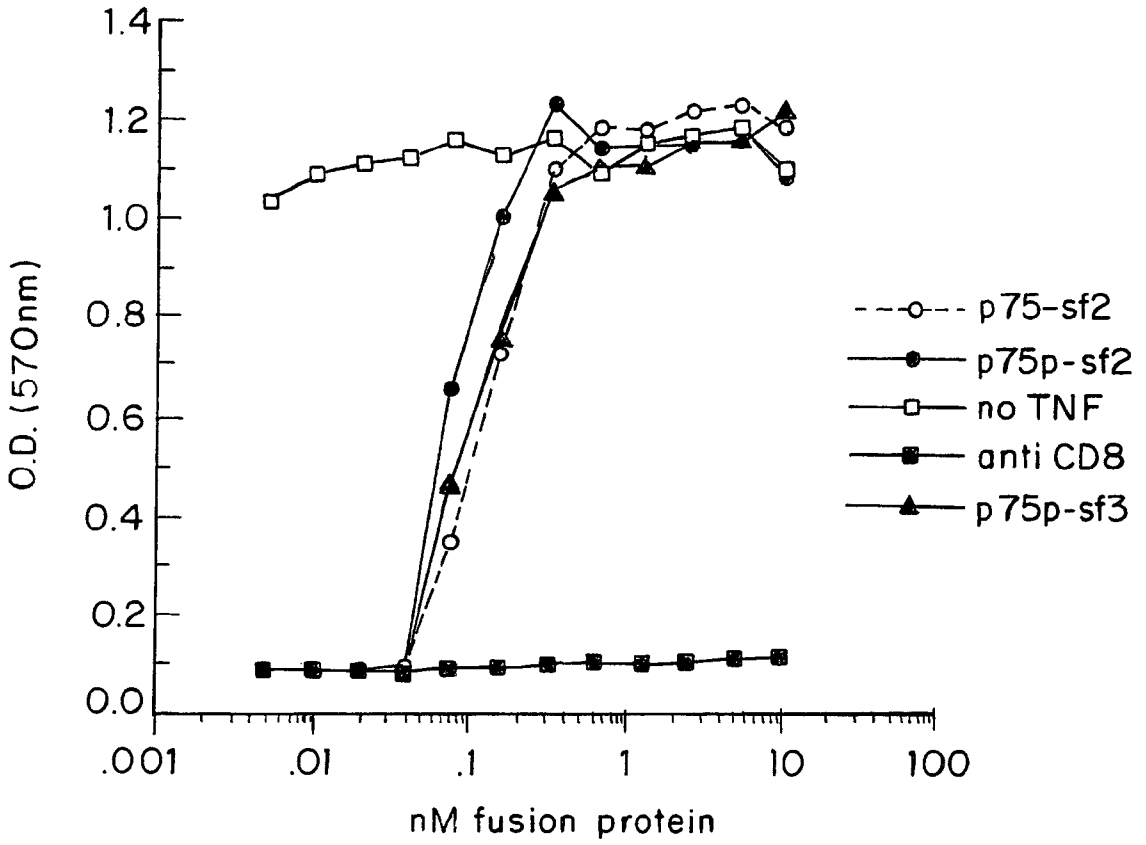

1.5
1.0
0.5
0.0
O.D.(570nm)
.001
.01
.1
1
10
100
nM fusion protein
p55-sf3
p55-df2
p55-sf2
no TNF
anti CD8

FIG. 31A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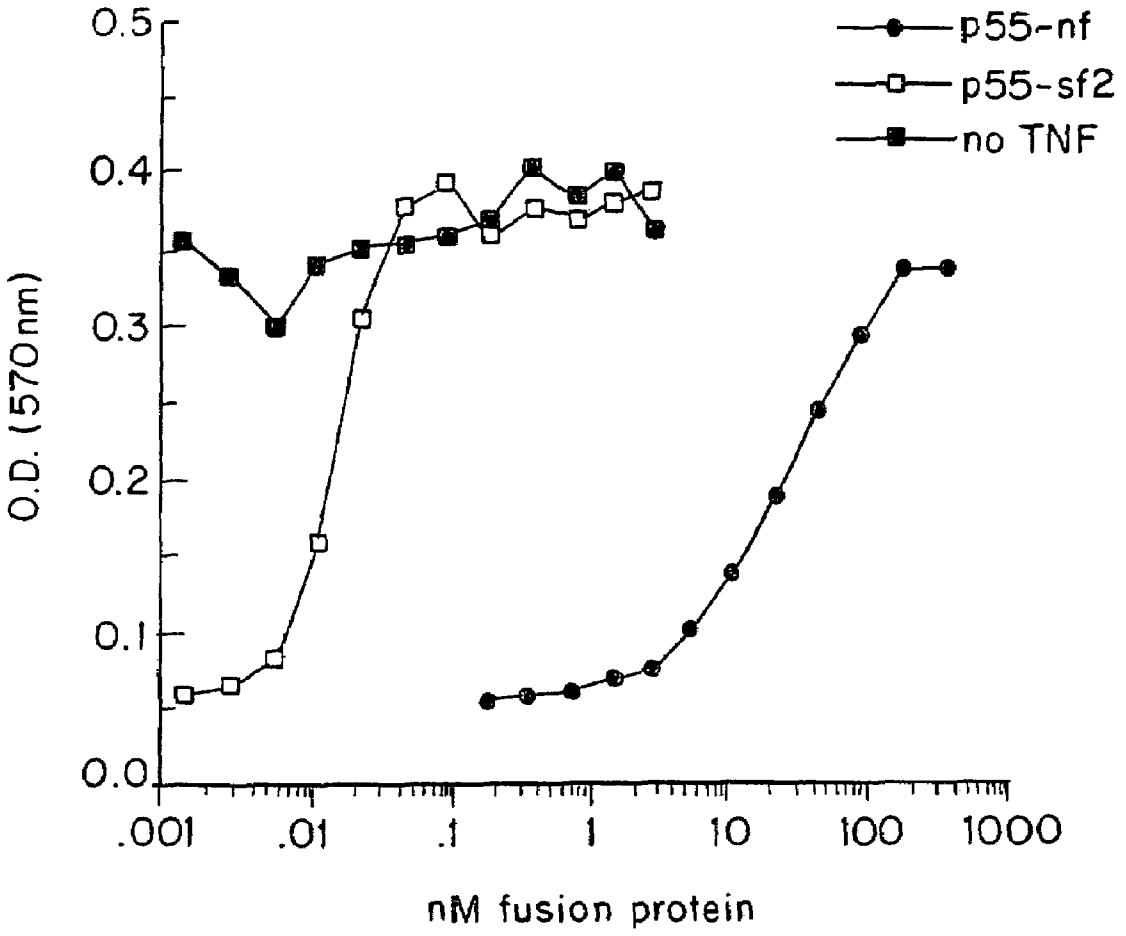
1.0
0.8
0.6
0.4
0.2
0.0
O.D. (570nm)
.001
.01
.1
1
10
100
nM fusion protein
p75-sf2
p75p-sf2
no TNF
anti CD8
p75p-sf3

FIG. 31B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p55-sf2
no TNF
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0.4
0.3
0.2
0.1
0.0
O.D. (570nm)
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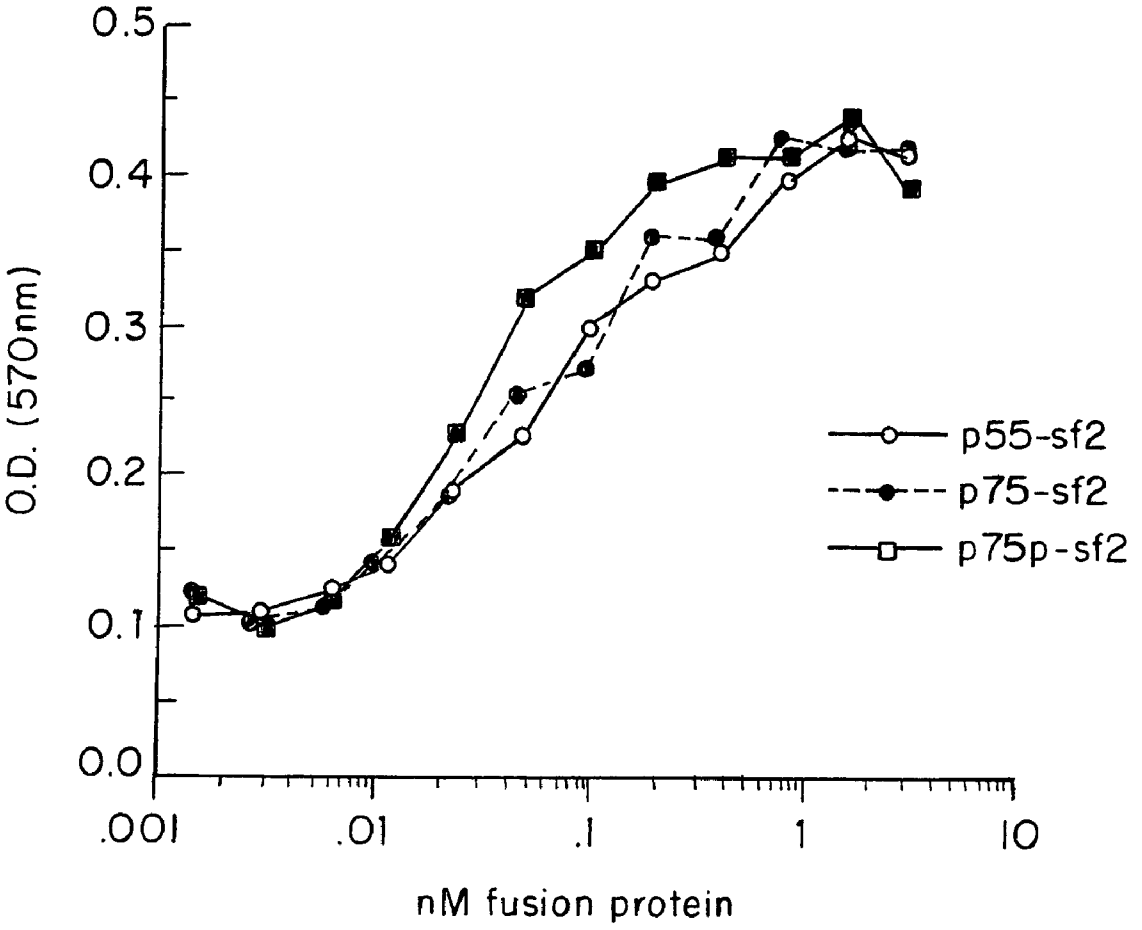
.01
.1
1
10
100
1000
nM fusion protein

FIG. 31C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0.4
0.3
0.2
0.1
0.0
O.D. (570nm)
.001
.01
.1
1
10
nM fusion protein
p55-sf2
p75-sf2
p75p-sf2

FIG. 32

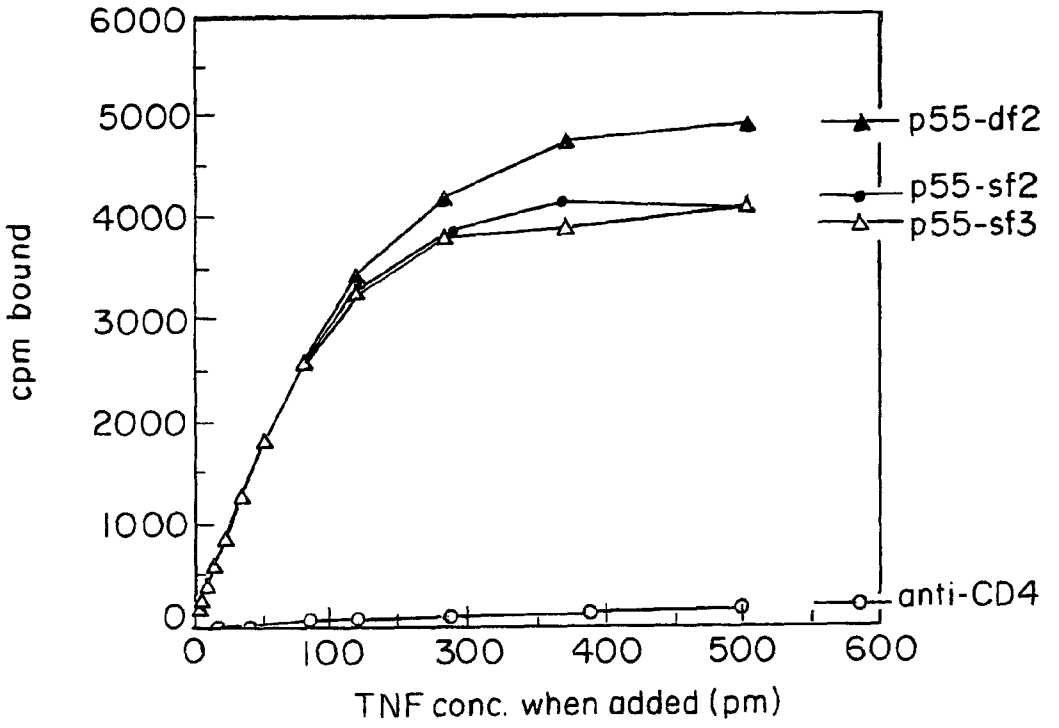


FIG. 33A

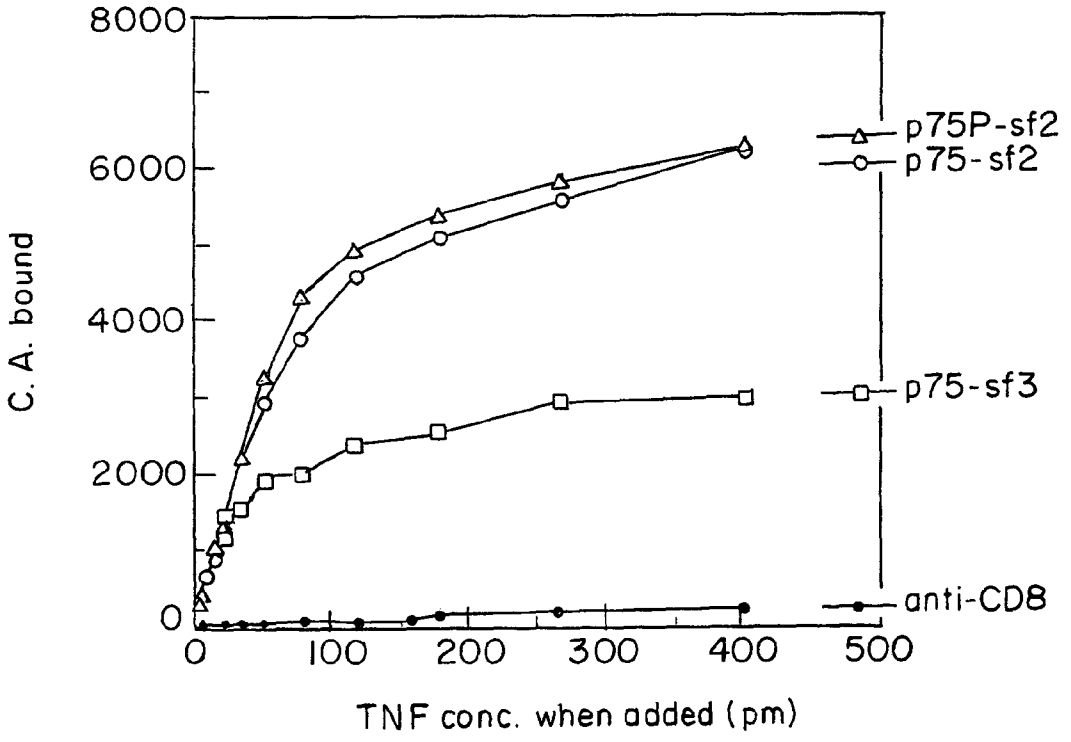


FIG. 33B

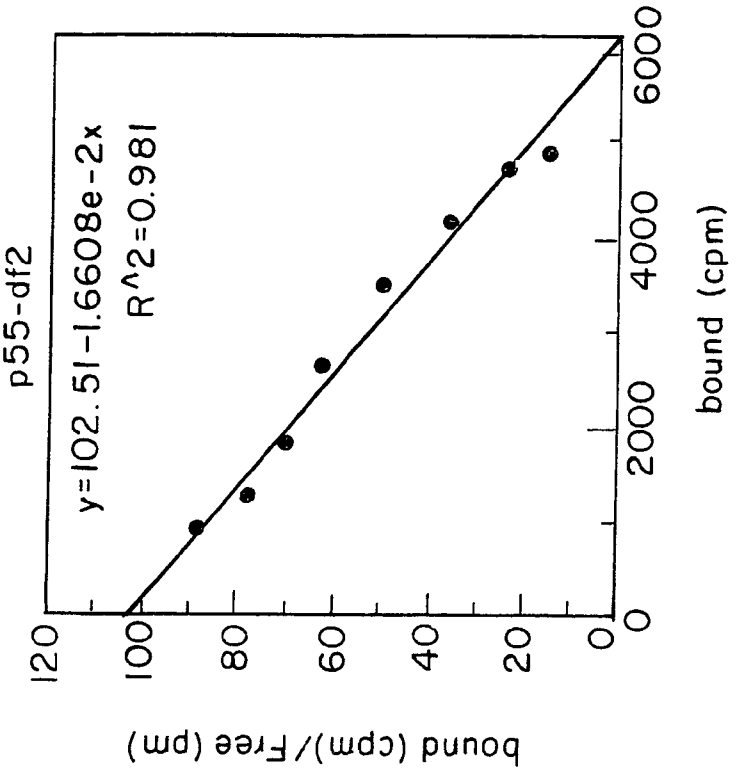


FIG. 33D

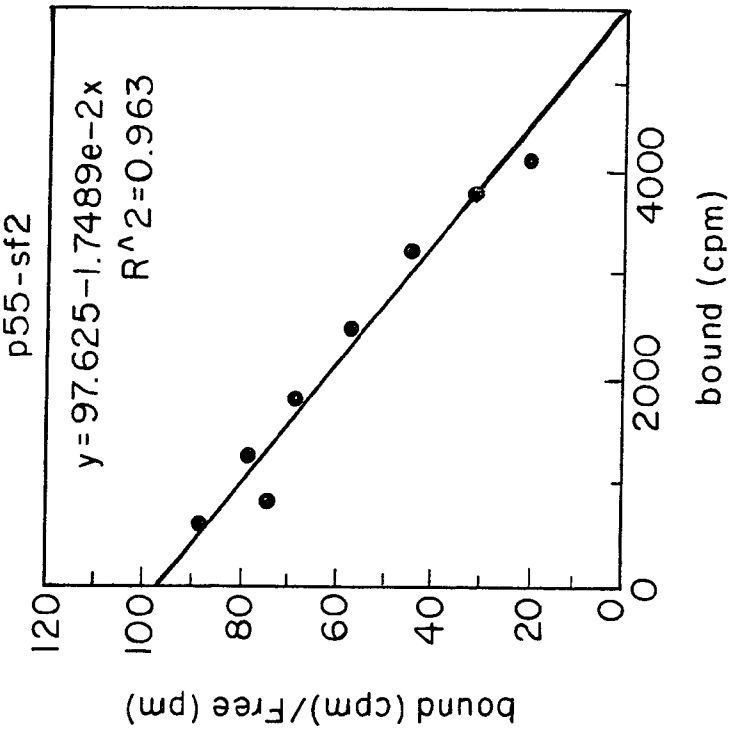


FIG. 33C

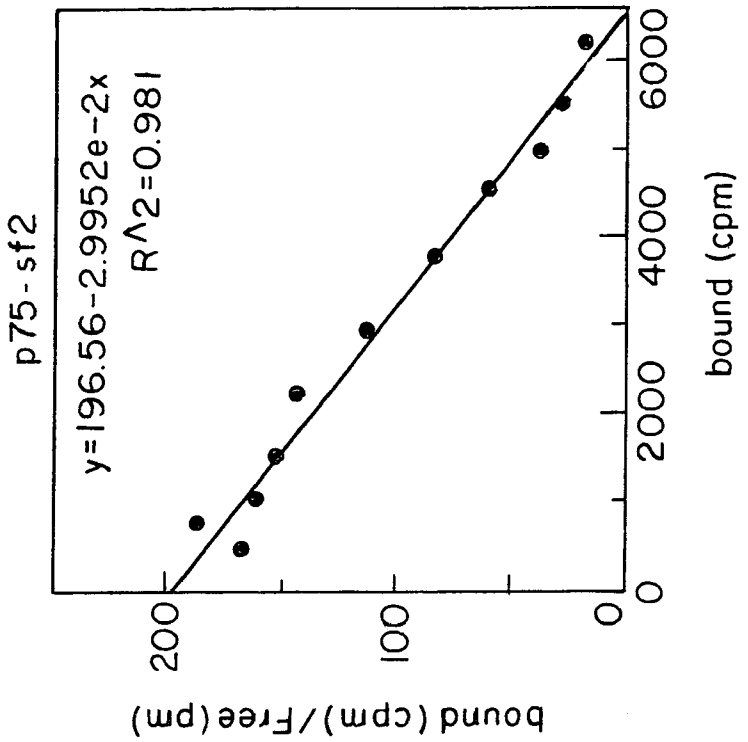


FIG. 33F

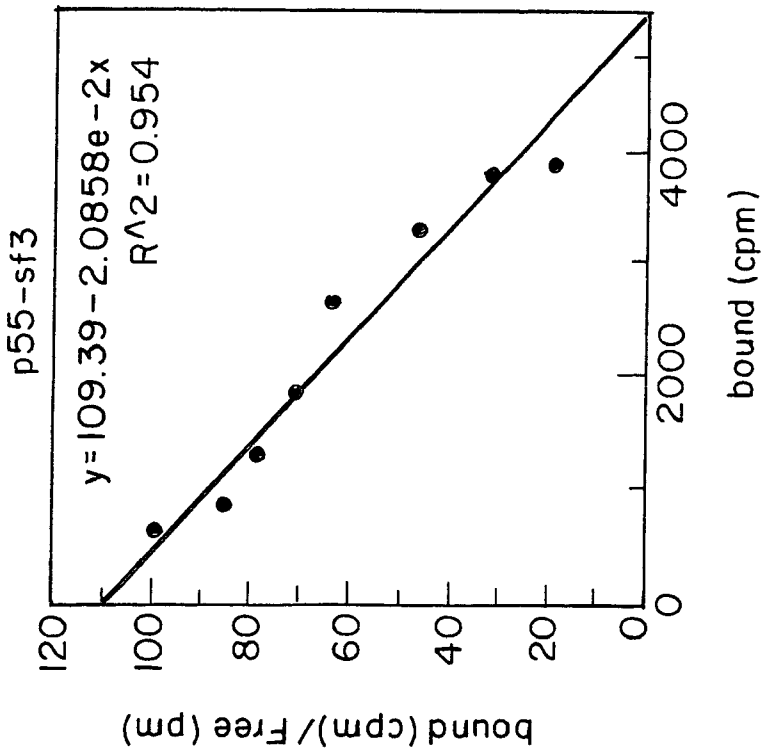


FIG. 33E

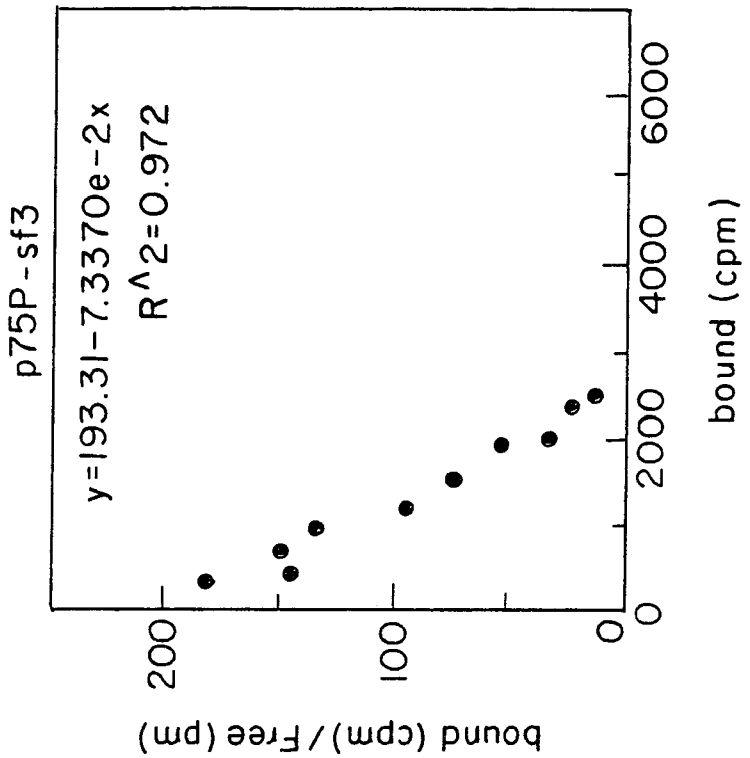


FIG. 33H

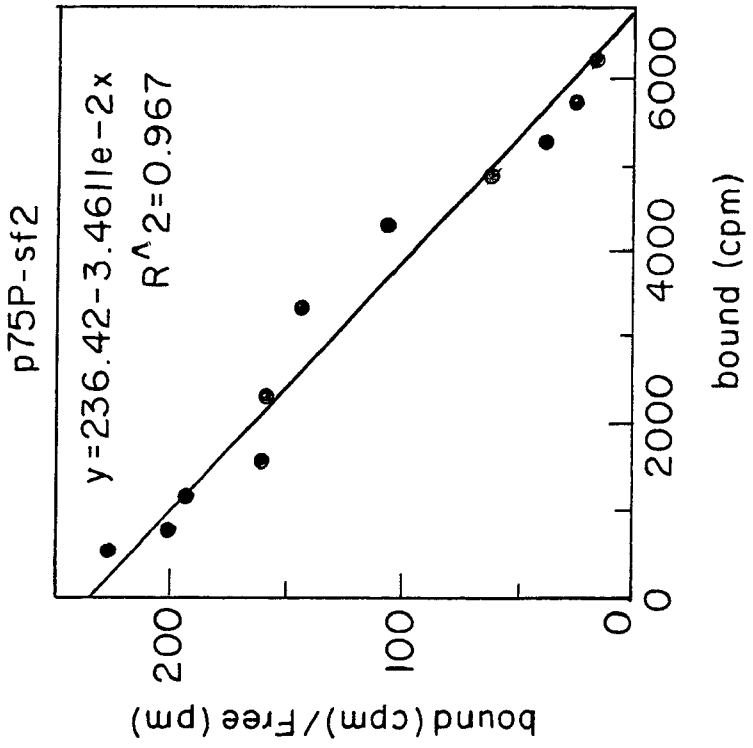
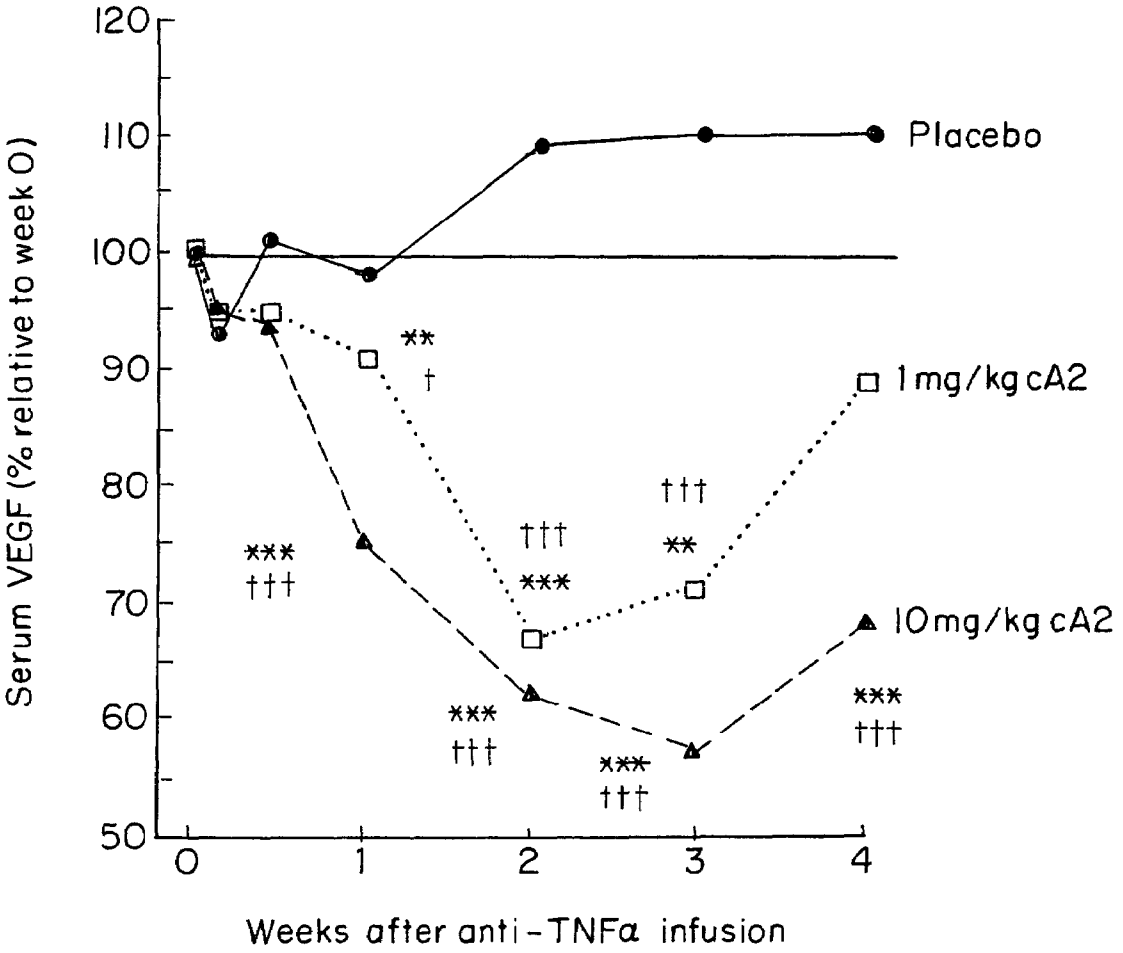


FIG. 33G



* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ *versus* pre-infusion
† $p \leq 0.05$, †† $p \leq 0.01$, ††† $p \leq 0.001$ *versus* change in placebo group

FIG. 34

METHODS OF TREATMENT OF FISTULAS IN CROHN'S DISEASE WITH ANTI-TNF ANTIBODIES

RELATED APPLICATIONS

This application is a continuation of U.S. application Ser. No. 09/756,398, filed Jan. 8, 2001, now U.S. Pat. No. 6,835,823, issued Dec. 28, 2004, which is a divisional of U.S. application Ser. No. 09/133,119, filed Aug. 12, 1998, now U.S. Pat. No. 6,277,969, issued Aug. 21, 2001, which is a divisional of U.S. application Ser. No. 08/570,674, filed Dec. 11, 1995, now abandoned, which is a continuation-in-part of U.S. application Ser. No. 08/324,799, filed Oct. 18, 1994, now U.S. Pat. No. 5,698,195, issued Dec. 16, 1997. Each of the above applications are entirely incorporated herein by reference.

BACKGROUND AND FIELD OF THE INVENTION

The present invention in the field of immunology and medicine relates to anti-tumor necrosis factor (TNF) antibodies, anti-TNF peptides and nucleic acids encoding therefor, and to pharmaceutical and diagnostic compositions and production, diagnostic and therapeutic methods thereof, and to methods for treating human TNF-mediated pathologies.

DESCRIPTION OF THE BACKGROUND ART

Tumor Necrosis Factor

Monocytes and macrophages secrete cytokines known as tumor necrosis factor-α (TNFα) and tumor necrosis factory-β (TNFβ) in response to endotoxin or other stimuli. TNFα is a soluble homotrimer of 17 kD protein subunits (Smith, et al., *J. Biol. Chem.* 262:6951-6954 (1987)). A membrane-bound 26 kD precursor form of TNF also exists (Kriegler, et al., *Cell* 53:45-53 (1988)). For reviews of TNF, see Beutler, et al., *Nature* 320:584 (1986), Old, *Science* 230:630 (1986), and Le, et al., *Lab. Invest.* 56:234.

Cells other than monocytes or macrophages also make TNFα. For example, human non-monocytic tumor cell lines produce TNF (Rubin, et al., *J. Exp. Med.* 164:1350 (1986); Spriggs, et al., *Proc. Natl. Acad. Sci. USA* 84:6563 (1987)). $CD4^+$ and $CD8^+$ peripheral blood T lymphocytes and some cultured T and B cell lines (Cuturi, et al., *J. Exp. Med.* 165:1581 (1987); Sung, et al., *J. Exp. Med.* 168:1539 (1988)) also produce TNFα.

TNF causes pro-inflammatory actions which result in tissue injury, such as inducing procoagulant activity on vascular endothelial cells (Pober, et al., *J. Immunol.* 136: 1680 (1986)), increasing the adherence of neutrophils and lymphocytes (Pober, et al., *J. Immunol.* 138:3319 (1987)), and stimulating the release of platelet activating factor from macrophages, neutrophils and vascular endothelial cells (Camussi, et al., *J. Exp. Med.* 166:1390 (1987)).

Recent evidence associates TNF with infections (Cerami, et al., *Immunol. Today* 9:28 (1988)), immune disorders, neoplastic pathologies (Oliff, et al., *Cell* 50:555 (1987)), autoimmune pathologies and graft-versus host pathologies (Piguet, et al., *J. Exp. Med.* 166:1280 (1987)). The association of TNF with cancer and infectious pathologies is often related to the host's catabolic state. Cancer patients suffer from weight loss, usually associated with anorexia.

The extensive wasting which is associated with cancer, and other diseases, is known as "cachexia" (Kern, et al., (*J. Parent. Enter. Nutr.* 12:286-298 (1988)). Cachexia includes progressive weight loss, anorexia, and persistent erosion of body mass in response to a malignant growth. The fundamental physiological derangement can relate to a decline in food intake relative to energy expenditure. The cachectic state causes most cancer morbidity and mortality. TNF can mediate cachexia in cancer, infectious pathology, and other catabolic states.

TNF also plays a central role in gram-negative sepsis and endotoxic shock (Michie, et al., *Br. J. Surg.* 76:670-671 (1989); Debets, et al., *Second Vienna Shock Forum*, p. 463-466 (1989); Simpson, et al., *Crit. Care Clin.* 5:27-47 (1989)), including fever, malaise, anorexia, and cachexia. Endotoxin strongly, activates monocyte/macrophage production and secretion of TNF and other cytokines (Kombluth, et al., *J. Immunol.* 137:2585-2591 (1986)). TNF and other monocyte-derived cytokines mediate the metabolic and neurohormonal responses to endotoxin (Michie, et al., *New. Engl. J. Med.* 318:1481-1486 (1988)). Endotoxin administration to human volunteers produces acute illness with flu-like symptoms including fever, tachycardia, increased metabolic rate and stress hormone release (Revhaug, et al., *Arch. Surg.* 123:162-170 (1988)). Circulating TNF increases in patients suffering from Gram-negative sepsis (Waage, et al., Lancet 1:355-357 (1987); Hammerle, et al., *Second Vienna Shock Forum* p. 715-718 (1989); Debets, et al., *Crit. Care Med.* 17:489-497 (1989); Calandra, et al., *J. Infect. Dis.* 161:982-987 (1990)).

TNF Antibodies

Polyclonal murine antibodies to TNF are disclosed by Cerami et al. (EPO Patent Publication 0212489, Mar. 4, 1987). Such antibodies were said to be useful in diagnostic immunoassays and in therapy of shock in bacterial infections.

Rubin et al. (EPO Patent Publication 0218868, Apr. 22, 1987) discloses murine monoclonal antibodies to human TNF, the hybridomas secreting such antibodies, methods of producing such murine antibodies, and the use of such murine antibodies in immunoassay of TNF.

Yone et al. (EPO Patent Publication 0288088, Oct. 26, 1988) discloses anti-TNF murine antibodies, including mAbs, and their utility in immunoassay diagnosis of pathologies, in particular Kawasaki's pathology and bacterial infection. The body fluids of patients with Kawasaki's pathology (infantile acute febrile mucocutaneous lymph node syndrome; Kawasaki, *Allergy* 16:178 (1967); Kawasaki, *Shonica* (*Pediatrics*) 26:935 (1985)) were said to contain elevated TNF levels which were related to progress of the pathology (Yone et al., infra).

Other investigators have described rodent or murine mAbs specific for recombinant human TNF which had neutralizing activity in vitro (Liang, et al., (*Biochem. Biophys. Res. Comm.* 137:847-854 (1986); Meager, et al., *Hybridoma* 6:305-311 (1987); Fendly et al., *Hybridoma* 6:359-369 (1987); Bringman, et al., *Hybridoma* 6:489-507 (1987); Hirai, et al., *J. Immunol. Meth.* 96:57-62 (1987); Moller, et al., (*Cytokine* 2:162-169 (1990)). Some of these mAbs were used to map epitopes of human TNF and develop enzyme immunoassays (Fendly et al., infra; Hirai et al., infra; Moller et al., infra) and to assist in the purification of recombinant TNF (Bringman et al., infra). However, these studies do not provide a basis for producing TNF neutralizing antibodies that can be used for in vivo diagnostic or therapeutic uses in humans, due to immunogenicity, lack of specificity and/or pharmaceutical suitability.

Neutralizing antisera or mAbs to TNF have been shown in mammals other than man to abrogate adverse physiological changes and prevent death after lethal challenge in experimental endotoxemia and bacteremia. This effect has been demonstrated, e.g., in rodent lethality assays and in primate pathology model systems (Mathison, et al., *J. Clin. Invest.* 81:1925-1937 (1988); Beutler, et al., *Science* 229: 869-871 (1985); Tracey, et al., *Nature* 330:662-664 (1987); Shimamoto, et al., *Immunol. Lett.* 17:311-318 (1988); Silva, et al., *J. Infect. Dis.* 162:421-427 (1990); Opal, et al., *J. Infect. Dis.* 161:1148-1152 (1990); Hinshaw, et al., *Circ. Shock* 30:279-292 (1990)).

Putative receptor binding loci of hTNF has been disclosed by Eck and Sprang (*J. Biol. Chem.* 264(29), 17595-17605 (1989)), who identified the receptor binding loci of TNF-α as consisting of amino acids 11-13, 37-42, 49-57 and 155-157.

PCT publication WO91/02078 (1991) discloses TNF ligands which can bind to monoclonal antibodies having the following epitopes: at least one of 1-20, 56-77, and 108-127; at least two of 1-20, 56-77, 108-127 and 138-149; all of 1-18, 58-65, 115-125 and 138-149; all of 1-18, and 108-128; all of 56-79, 110-127 and 135- or 136-155; all of 1-30, 117-128 and 141-153; all of 1-26, 117-128 and 141-153; all of 22-40, 49-96 or 49-97, 110-127 and 136-153; all of 12-22, 36-45, 96-105 and 132-157; both of 1-20 and 76-90; all of 22-40, 69-97, 105-128 and 135-155; all of 22-31 and 146-157; all of 22-40 and 49-98; at least one of 22-40, 49-98 and 69-97, both of 22-40 and 70-87.

To date, experience with anti-TNF murine mAb therapy in humans has been limited. In a phase I study, fourteen patients with severe septic shock were administered a murine anti-TNF mAb in a single dose from 0.4-10 mg/kg (Exley, A. R. et al., *Lancet* 335:1275-1277 (1990)). However, seven of the fourteen patients developed a human anti-murine antibody response to the treatment, which treatment suffers from the known problems due to immunogenicity from the use of murine heavy and light chain portions of the antibody. Such immunogenicity causes decreased effectiveness of continued administration and can render treatment ineffective, in patients undergoing diagnostic or therapeutic administration of murine anti-TNF antibodies.

Administration of murine TNF mAb to patients suffering from severe graft versus host pathology has also been reported (Herve, et al., *Lymphoma Res.* 9:591 (1990)).

TNF Receptors

The numerous biological effects of TNFα and the closely related cytokine, TNFβ (lymphotoxin), are mediated by two TNF transmembrane receptors, both of which have been cloned. The p55 receptor (also termed TNF-R55, TNF-RI, or TNFRβ) is a 55 kDa glycoprotein shown to transduce signals resulting in cytotoxic, anti-viral, and proliferative activities of TNFα.

The p75 receptor (also termed TNF-R75, TNF-RII, or TNFRα) is a 75 kDa glycoprotein that has also been shown to transduce cytotoxic and proliferative signals as well as signals resulting in the secretion of GM-CSF. The extracellular domains of the two receptors have 28% homology and have in common a set of four subdomains defined by numerous conserved cysteine residues. The p75 receptor differs, however, by having a region adjacent to the transmembrane domain that is rich in proline residues and contains sites for O-linked glycosylation. Interestingly, the cytoplasmic domains of the two receptors share no apparent homology which is consistent with observations that they can transduce different signals to the interior of the cell.

TNFα inhibiting proteins have been detected in normal human urine and in serum of patients with cancer or endotoxemia. These have since been shown to be the extracellular domains of TNF receptors derived by proteolytic cleavage of the transmembrane forms. Many of the same stimuli that result in TNFα release also result in the release of the soluble receptors, suggesting that these soluble TNFα inhibitors can serve as part of a negative feedback mechanism to control TNFα activity.

Aderka, et al., *Isrl. J. Med. Sci.* 28:126-130 (1992) discloses soluble forms of TNF receptors (sTNF-Rs) which specifically bind TNF and thus can compete with cell surface TNF receptors to bind TNF (Seckinger, et al., *J. Exp. Med.* 167:1511-1516 (1988); Engelmann, et al., *J. Biol. Chem.* 264:11974-11980 (1989)).

Loetscher, et al., *Cell* 61:351-359 (Apr. 20, 1990) discloses the cloning and expression of human 55 kd TNF receptor with the partial amino acid sequence, complete cDNA sequence and predicted amino acid sequence.

Schall et al., *Cell* 61:361-370 (Apr. 20, 1990), discloses molecular cloning and expression of a receptor for human TNF with an isolated cDNA clone including a receptor as a 415 amino acid protein with an apparent molecular weight of 28 kDa, as well as the cDNA sequence and predicted amino acid sequence.

Nophar, et al., *EMBO J.* 9(10):3269-3278 (1990) discloses soluble forms of TNF receptor and that the cDNA for type I TNF-R encodes both the cell surface and soluble forms of the receptor. The cDNA and predicted amino acid sequences are disclosed.

Engelmann, et al., *J. Biol. Chem.* 265(3):1531-1536 (1990), discloses TNF-binding proteins, purified from human urine, both having an approximate molecular weight of 30 kDa and binding TNF-α more effectively than TNF-β. Sequence data is not disclosed. See also Engelmann, et al., *J. Biol. Chem.* 264 (20):11974-11980 (1989).

European Patent publication number 0 433 900 A1, published Jun. 26, 1991, owned by YEDA Research and Development Co., Ltd., Wallach, et al., discloses TNF binding protein I (TBP-I), derivatives and analogs thereof, produced expression of a DNA encoding the entire human type I TNF receptor, or a soluble domain thereof.

PCT publication number WO 92/13095, published Aug. 6, 1992, owned by Synergen, Carmichael et al., discloses methods for treating tumor necrosis factor mediated diseases by administration of a therapeutically effective amount of a TNF inhibitor selected from a 30 kDa TNF inhibitor and a 40 kDa TNF inhibitor selected from the full length 40 kDa TNF inhibitor or modifications thereof.

European Patent Publication number 0 526 905 A2, published Oct. 2, 1993, owned by YEDA Research and Development Company, Ltd., Wallach et al., discloses multimers of the soluble forms of TNF receptors produced by either chemical or recombinant methods which are useful for protecting mammals from the deleterious effects of TNF, which include portions of the hp55 TNF-receptor.

PCT publication WO 92/07076, published Apr. 30, 1992, owned by Charring Cross Sunley Research Center, Feldmann et al., discloses modified human TNFα receptor which consists of the first three cysteine-rich subdomains but lacks the fourth cysteine-rich subdomain of the extracellular binding domain of the 55 kDa or 75 kDa TNF receptor for human TNFα, or an amino acid sequence having a homology of 90% or more with the TNF receptor sequences.

European Patent Publication 0 412 486 A1, published Feb. 13, 1991, owned by YEDA Research and Development Co., Ltd., Wallach et al., discloses antibodies to TNF binding

protein I (TBP-I), and fragments thereof, which can be used as diagnostic assays or pharmaceutical agents, either inhibiting or mimicking the effects of TNF on cells.

European Patent Publication number 0 398 327 A1, published Nov. 22, 1990, owned by YEDA Research and Development Co., Ltd., Wallach et al., discloses TNF binding protein (TBP), isolated and purified, having inhibitory activity on the cytotoxic effect of TNF, as well as TNF binding protein II and salts, functional derivatives, precursors and active fractions thereof, as well as polyclonal and monoclonal antibodies to TNF binding protein II.

European Patent Publication 0 308 378 A2, published Mar. 22, 1989, owned by YEDA Research and Development Co., Ltd., Wallach, et al., discloses TNF inhibitory protein isolated and substantially purified, having activity to inhibit the binding of TNF to TNF receptors and to inhibit the cytotoxicity of TNF. Additionally disclosed are TNF inhibitory protein, salts, functional derivatives and active fractions thereof, used to antagonize the deleterious effects of TNF.

Accordingly, there is a need to provide novel TNF antibodies or peptides which overcome the problems of murine antibody immunogenicity and which provide reduced immunogenicity and increased neutralization activity.

Citation of documents herein is not intended as an admission that any of the documents cited herein is pertinent prior art, or an admission that the cited documents is considered material to the patentability of any of the claims of the present application. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicant and does not constitute any admission as to the correctness of the dates or contents of these documents.

SUMMARY OF THE INVENTION

It is the object of the present invention to overcome one or more deficiencies of the background art.

It is also an object of the present invention to provide methods having utility for in vitro, in situ and/or in vivo diagnosis and/or treatment of animal cells, tissues or pathologies associated with the presence of tumor necrosis factor (TNF), using anti-TNF antibodies and/or anti-TNF peptides.

Anti-TNF antibodies (Abs) are intended to include at least one of monoclonal rodent-human chimeric antibodies, rodent antibodies, human antibodies or any portions thereof, having at least one antigen binding region of an immunoglobulin variable region, which antibody binds TNF.

Anti-TNF peptides are capable of binding TNF under physiological conditions, and can include, but are not limited to, portions of a TNF receptor and/or portions or structural analogs of anti-TNF antibody antigen binding regions or variable regions. Such antibodies or peptides bind TNF with neutralizing and/or inhibiting biological activity.

Anti-TNF antibodies and/or anti-TNF peptides of the present invention can be routinely made and/or used according to methods of the present invention, such as, but not limited to synthetic methods, hybridomas, and/or recombinant cells expressing nucleic acid encoding such anti-TNF antibodies or peptides.

The present invention also provides antigenic polypeptides of hTNF, corresponding to peptides containing neutralizing epitopes or portions of TNF that, when such epitopes on TNF are bound by anti-TNF antibodies or peptides, neutralize or inhibit the biological activity of TNF in vitro, in situ or in vivo.

The present invention also provides anti-TNF antibodies and peptides in the form of pharmaceutical and/or diagnostic compounds and/or compositions, useful for the diagnostic and/or therapeutic methods of the present invention for diagnosing and/or treating TNF-related pathologies.

Anti-TNF Abs or anti-TNF peptides of the present invention are provided for use in diagnostic methods for detecting TNF in patients or animals suspected of suffering from conditions associated with abnormal TNF production, including methods wherein high affinity anti-TNF antibodies or peptides are contacted with a biological sample from a patient and an antigen-antibody reaction detected. Also included in the present invention are kits for detecting TNF in a solution using anti-TNF antibodies or peptides, preferably in detectably labeled form.

The present invention is also directed to an anti-hTNF chimeric antibody comprising two light chains and two heavy chains, each of the chains comprising at least part of a human constant region and at least part of a variable (V) region of non-human origin having specificity to human TNF, said antibody binding with high affinity to a inhibiting and/or neutralizing epitope of human TNF, such as the antibody cA2. The invention also includes a fragments or a derivative such an antibody, such as one or more portions of the antibody chain, such as the heavy chain constant, joining, diversity or variable regions, or the light chain constant, Joining or variable regions.

Methods are also provided for making and using anti-TNF antibodies and peptides for various utilities of the present invention, such as but not limited to, hybridoma, recombinant or chemical synthetic methods for producing anti-TNF antibodies or anti-TNF peptides according to the present invention; detecting TNF in a solution or cell; removing TNF from a solution or cell, inhibiting one or more biological activities of TNF in vitro, in situ or in vitro. Such removal can include treatment methods of the present invention for alleviating symptoms or pathologies involving TNF, such as, by not limited to bacterial, viral or parasitic infections, chronic inflammatory diseases, autoimmune diseases, malignancies, and/or neurodegenerative diseases.

The invention further relates to the unexpected discovery that the inhibition or antagonism of TNF decreases the expression of Vascular Endothelial Growth Factor (VEGF) or Vascular Permeability Factor (VPF). VEGF has been implicated in the angiogenesis in cancer, vascular diseases and rheumatoid arthritis, for example. Thus, a TNF antagonist, such as an anti-TNF antibody, can be administered to a mammal for the treatment to decrease angiogenesis, such as in the treatment of a VEGF-mediated disease.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a graph showing dose dependent binding of mouse mAb A2 to human TNFα.

FIG. **2** is a graph showing lack of recognition of heat-inactivated human TNFα by mAb A2.

FIG. **3** is a graph showing neutralization of in vitro TNF cytotoxicity by murine A2. Control: murine IgG1 anti-lipid A mAb (8A1) with natural human TNF. Average absorbance values for controls were as follows: no TNF added=1.08; natural TNF, no antibody=0.290; and recombinant TNF, no antibody=0.500.

FIG. **4** is a graph showing that mAb A2 and chimeric A2 do not inhibit or neutralize human lymphotoxin (TNFβ).

FIG. **5** is a graph showing that mAbs murine A2 and chimeric CA2 do not inhibit or neutralize murine TNFα.

FIG. **6** and FIG. **7** are graphs showing that mAb A2 inhibits or neutralizes TNF produced by chimpanzee monocytes and rhTNFα.

FIGS. **8**A and **8**B provide schematic diagrams of the plasmids used for expression of the chimeric H (pA2HG1apgpt) and L (pA2HuKapgpt) chains of the chimeric A2 antibody.

FIGS. **9**A and **9**B are graphs showing results of a cross-blocking epitope ELISA with murine A2 (mA2) and chimeric (cA2) antibody competitors.

FIGS. **10**A and **10**B are graphs of a Scatchard analysis of ^{125}I-labelled mAb A2 (mA2) and chimeric A2 (cA2) binding to recombinant human TNFα immobilized on a microtiter plate. Each Ka value was calculated from the average of two independent determinations.

FIG. **11** is a graph showing neutralization of TNF cytotoxicity by chimeric A2. The control is a chimeric mouse/human IgG1 anti-platelet mAb (7E3) reacting with natural human TNF. Average absorbance values for controls were: no TNF added=1.08; natural TNF, no Ab=0.290; and recombinant TNF, no Ab=0.500.

FIG. **12** is a graph showing in vitro neutralization of TNF-induced ELAM-1 expression by chimeric A2. The control is a chimeric mouse/human IgG1 anti-CD4 antibody.

FIG. **13** is an amino acid sequence of human TNF as SEQ ID NO:1.

FIGS. **14**A-**14**B. FIG. **14**A is a graphical representation of epitope mapping of chimeric mAb cA2 indicating relative binding of cA2 to human TNF peptide pins. FIG. **14**B is a graphical representation of epitope mapping of chimeric mAb cA2 indicating relative binding of cA2 to human TNF peptide pins in the presence of human TNF.

FIG. **15** is an amino acid sequence of human TNF showing sequences having portions of epitopes recognized by cA2, corresponding to portions of amino acids 59-80 and/or 87-108 of SEQ ID NO:1.

FIGS. **16**A-**16**B. FIG. **16**A is a nucleic acid sequence (SEQ ID NO:2) and corresponding amino acid sequence (SEQ ID NO:3) of a cloned cA2 light chain variable region. FIG. **16**B is a nucleic acid sequence (SEQ ID NO:4) and corresponding amino acid sequence (SEQ ID NO:5) of a cloned cA2 heavy chain variable region.

FIG. **17** is a graphical representation of the early morning stiffness for the five patients in group I, and the four patients in group II is plotted as the mean percent of the baseline value versus time. Both groups showed an approximately 80 percent decrease or greater in early morning stiffness, which persisted for greater than 40 days.

FIG. **18** is a graphical representation of the assessment of pain using a visual analogue scale for the five patients in group I, and the four patients in group II, is plotted as the mean percent of the baseline value versus time. Both groups showed an approximately 60 to 80 percent decrease in pain score which persisted for greater than 40 days.

FIG. **19** is a graphical representation of the Ritchie Articular Index, (a scale scored of joint tenderness), plotted as the mean percent of the baseline value versus time. Both groups showed an approximately 80 percent decrease in the Ritchie Articular Index, which persisted for greater than 40 days.

FIG. **20** is a graphical representation of the number of swollen joints for the five patients in group I and the four patients in Group II plotted as the mean percent of baseline value versus time. Both groups showed an approximately 70 to 80 percent decrease in swollen joints, which persisted for 30 to 40 days.

FIG. **21** is a graphical representation of the serum C-reactive protein for four to five patients in group I, and three of the four patients in group II, plotted as the mean percent of the baseline value versus time. Both groups showed an approximately 80 percent reduction in CRP which persisted for 30 to 40 days. The values for patient number 1 and patient number 7 were omitted from the computations on which the plots are based, since these patients did not have elevated CRP values at baseline.

FIG. **22** is a graphical representation of the erythrocyte sedimentation rate for the five patients in group I and three of the patients in group II plotted as the mean percent of the baseline value versus time. Both groups showed an approximately 40 percent reduction in ESR which persisted for at least 40 days. The data from patient number 9 is omitted from the computations on which the plots were based, since this patient did not have an elevated ESR at baseline.

FIG. **23** is a graphical representation of the index of Disease Activity, (a composite score of several parameters of disease activity), for the five patients in group I, and the four patients in group II, plotted as the mean percent of the baseline value versus time. Both groups showed a clinically significant reduction in IDA, which persisted for at least 40 days.

FIG. **24** is a graphical representation of swollen joint counts (maximum 28), as recorded by a single observer. Circles represent individual patients and horizontal bars show median values at each time point. The screening time point was within 4 weeks of entry to the study (week 0); data from patient 15 were not included after week 2 (dropout). Significance of the changes, relative to week 0, by Mann Whitney test, adjusted: week 1, $p>0.05$; week 2, $p<0.02$; weeks 3-4, $p<0.002$; weeks 6-8, $p<0.001$.

FIG. **25** is a graphical representation of levels of serum C—reactive protein (CRP)—Serum CRP (normal range 0-10 mg/liter), measured by nephelometry. Circles represent individual patients and horizontal bars show median values at each time point. The screening time point was within 4 weeks of entry to the study (week 0); data from patient 15 were not included after week 2 (dropout). Significance of the changes, relative to week 0, by Mann-Whitney test, adjusted: week 1, $p<0.001$; week 2, $p<0.003$; week 3, $p<0.002$; week 4, $p<0.02$; week 6,8, $p<0.001$.

FIGS. **26**A-**26**B. FIG. **26**A is a schematic illustration of the genes encoding TNF receptor/IgG fusion proteins and the gene encoding the truncated light chain. The gene encoding Ig heavy chain (IgH) fusion proteins had the same basic structure as the naturally occurring, rearranged Ig genes except that the Ig variable region coding sequence was replaced with TNF receptor coding sequence. Except for the TNF receptor coding sequences and a partial human K sequence derived by modifying the murine J region coding sequence in the cM-T412 IgH gene by PCT mutagenesis, the entire genomic fragment shown originated from the cM-T412 chimeric mouse/human IgH gene. Looney et al., *Hum. Antibody Hybrid.* 3:191-200 (1992). The region deleted in the genes encoding p55-sf3 and p75P-sf3 is marked in the figure. The JC_K gene, encoding a truncated Ig Kappa light chain, was constructed by deleting the variable region coding sequence from the cM-T412 chimeric mouse/human Ig Kappa gene (Looney, infra) and using PCR mutagenesis to change the murine J sequence to a partial human J sequence. The p55-light chain fusion in p55-df2 was made by inserting the p55 coding sequence into the EcoRV site in the JC_K gene. Tracey et al., *Nature* 330:662-666 (1987). FIG. **26**B is a schematic illustration of several immunoreceptor molecules of the present invention. The blackened ovals each represent

a domain of the IgG1 constant region. The circles represent the truncated light chain. Small circles adjacent to a p55 or p75 subunit mark the positions of human J sequence. The incomplete circles in p75-sf2 and -s3 are to illustrate that the C-terminal 53 amino acids of the p75 extracellular domain were deleted. Lines between subunits represent disulfide bonds.

FIG. **27** is a schematic illustration of the construction of a cM-T412 heavy chain so that it has a unique cloning site for insertion of foreign genes such as p55 and p75.

FIG. **28** is a schematic illustration of the construction of the vectors used to express the heavy chain of the immunoreceptors.

FIG. **29** is a schematic illustration of the construction of a cM-T412 light chain so that it has a unique cloning site for insertion of foreign genes such as p55 and p75.

FIG. **30** is a schematic illustration of the construction of the vectors used to express the light chain of the immunoreceptors.

FIGS. **31**A-**31**C are graphical representations showing that fusion proteins protected WEHI 164 cells from TNFα cytotoxicity. Cells were first sensitized to TNFα with actinomycin D and then incubated in 2 ng/ml TNFα with varying concentrations of TNFα overnight at 37° C. Cell viability was determined by measuring their uptake of MTT dye. FIG. **31**A shows p55 fusion proteins. FIG. **31**B shows p75 fusion proteins. FIG. **31**C shows comparison of the protective ability of the non-fusion form of p55 (p55-nf) to p55-sf2.

FIG. **32** is a graphical representation of data showing that fusion proteins also effectively protect WEHI 164 cells from TNFβ cytotoxicity.

FIGS. **33**A-**33**H are graphical representations of analyses of binding between the various fusion proteins and TNFα by saturation binding (FIGS. **33**A and **33**B) and Scatchard analysis (FIG. **33**C-**33**H). A microtiter plate was coated with excess goat anti-Fc polyclonal antibody and incubated with 10 ng/ml of fusion protein in TBST buffer (10 mM Tris-HCl, pH 7.8, 150 mM NaCl, 0.05% TWEEN® 20) for 1 hour. Varying amounts of ^{125}I labeled TNFα (specific activity—34.8 μCi/μg) were then incubated with the captured fusion protein in PBS (10 mM Na Phosphate, pH 7.0, 150 mM NaCl) with 1% bovine serum albumin for 2 hours. Unbound TNFα was washed away with four washes in PBS and the cpm bound was quantitated using a y-counter. All samples were analyzed in triplicate. The slope of the lines in (FIGS. **33**C-H) represent the affinity constant, K_a. The dissociation constant (K_d) values (see Table 1) were derived using the equation $K_d=I/K_a$.

FIG. **34** is a graphic illustration depicting VEGF levels in the serum of rheumatoid arthritis patients treated with placebo (circles), 1 mg/kg cA2 antibody (square) or 10 mg/kg (triangle). The figure shows that the administration of an anti-TNF antibody resulted in decreased levels of VEGF.

DETAILED DESCRIPTION OF THE INVENTION

Tumor necrosis factor (TNF) has been discovered to mediate or be involved in many pathologies, such as, but not limited to, bacterial, viral or parasitic infections, chronic inflammatory diseases, autoimmune diseases, malignancies, and/or neurodegenerative diseases. Accordingly, anti-TNF compounds and compositions of the present invention which have neutralizing and/or inhibiting activity against TNF are discovered to provide methods for treating and/or diagnosing such pathologies.

The present invention thus provides anti-TNF compounds and compositions comprising anti-TNF antibodies (Abs) and/or anti-TNF peptides which inhibit and/or neutralize TNF biological activity in vitro, in situ and/or in vivo, as specific for association with neutralizing epitopes of human tumor necrosis factor-alpha (hTNFα) and/or human tumor necrosis factor β (hTNFβ). Such anti-TNF Abs or peptides have utilities for use in research, diagnostic and/or therapeutic methods of the present invention for diagnosing and/or treating animals or humans having pathologies or conditions associated with the presence of a substance reactive with an anti-TNF antibody, such as TNF or metabolic products thereof. Such pathologies can include the generalized or local presence of TNF or related compounds, in amounts and/or concentrations exceeding, or less than, those present in a normal healthy subject, or as related to a pathological condition.

Anti-TNF Antibodies and Methods

The term "antibody" is meant to include polyclonal antibodies, monoclonal antibodies (mAbs), chimeric antibodies, anti-idiotypic (anti-Id) antibodies to antibodies that can be labeled in soluble or bound form, as well as fragments, regions or derivatives thereof, provided by any known technique, such as, but not limited to, enzymatic cleavage, peptide synthesis or recombinant techniques. Such anti-TNF antibodies of the present invention are capable of binding portions of TNF that inhibit the binding of TNF to TNF receptors.

Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen. A monoclonal antibody contains a substantially homogeneous population of antibodies specific to antigens, which population contains substantially similar epitope binding sites. MAbs may be obtained by methods known to those skilled in the art. See, for example Kohler and Milstein, *Nature* 256:495-497 (1975); U.S. Pat. No. 4,376,110; Ausubel et al., eds., *Current Protocols in Molecular Biology*, Greene Publishing Assoc. and Wiley Interscience, N.Y., (1987, 1992); and Harlow and Lane *ANTIBODIES: A Laboratory Manual* Cold Spring Harbor Laboratory (1988); Colligan et al., eds., *Current Protocols in Immunology*, Greene Publishing Assoc. and Wiley Interscience, N.Y., (1992, 1993), the contents of which references are incorporated entirely herein by reference. Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, GILD and any subclass thereof. A hybridoma producing a mAb of the present invention may be cultivated in vitro, in situ or in vivo. Production of high titers of mAbs in vivo or in situ makes this the presently preferred method of production.

Chimeric antibodies are molecules different portions of which are derived from different animal species, such as those having variable region derived from a murine mAb and a human immunoglobulin constant region, which are primarily used to reduce immunogenicity in application and to increase yields in production, for example, where murine mAbs have higher yields from hybridomas but higher immunogenicity in humans, such that human/murine chimeric mAbs are used. Chimeric antibodies and methods for their production are known in the art (Cabilly et al., *Proc. Natl. Acad. Sci. USA* 81:3273-3277 (1984); Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984); Boulianne et al., *Nature* 312:643-646 (1984); Cabilly et al., European Patent Application 125023 (published Nov. 14, 1984); Neuberger et al., *Nature* 314:268-270 (1985); Taniguchi et al., European Patent Application 171496 (published Feb. 19, 1985);

Morrison et al., European Patent Application 173494 (published Mar. 5, 1986); Neuberger et al., PCT Application WO 86/01533, (published Mar. 13, 1986); Kudo et al., European Patent Application 184187 (published Jun. 11, 1986); Morrison et al., European Patent Application 173494 (published Mar. 5, 1986); Sahagan et al., *J. Immunol.* 137:1066-1074 (1986); Robinson et al., International Patent Publication #PCT/US86/02269 (published May 7, 1987); Liu et al., *Proc. Natl. Acad. Sci. USA* 84:3439-3443 (1987); Sun et al., *Proc. Natl. Acad. Sci. USA* 84:214-218 (1987); Better et al., *Science* 240:1041-1043 (1988); and Harlow and Lane *Antibodies: a Laboratory Manual Cold* Spring Harbor Laboratory (1988)). These references are entirely incorporated herein by reference.

An anti-idiotypic (anti-Id) antibody is an antibody which recognizes unique determinants generally associated with the antigen-binding site of an antibody. An Id antibody can be prepared by immunizing an animal of the same species and genetic type (e.g., mouse strain) as the source of the mAb with the mAb to which an anti-Id is being prepared. The immunized animal will recognize and respond to the idiotypic determinants of the immunizing antibody by producing an antibody to these idiotypic determinants (the anti-Id antibody). See, for example, U.S. Pat. No. 4,699,880, which is herein entirely incorporated by reference.

The anti-Id antibody may also be used as an "immunogen" to induce an immune response in yet another animal, producing a so-called anti-anti-Id antibody. The anti-anti-Id may be epitopically identical to the original mAb which induced the anti-Id. Thus, by using antibodies to the idiotypic determinants of a mAb, it is possible to identify other clones expressing antibodies of identical specificity.

Anti-TNF antibodies of the present invention can include at least one of a heavy chain constant region (H_c), a heavy chain variable region (H_v), a light chain variable region (L_v) and a light chain constant region (L_c), wherein a polyclonal Ab, monoclonal Ab, fragment and/or regions thereof include at least one heavy chain variable region (H_v) or light chain variable region (L_v) which binds a portion of a TNF and inhibits and/or neutralizes at least one TNF biological activity.

Preferred antibodies of the present invention are high affinity human-murine chimeric anti-TNF antibodies, and fragments or regions thereof, that have potent inhibiting and/or neutralizing activity in vivo against human TNFα. Such antibodies and chimeric antibodies can include those generated by immunization using purified recombinant hTNFα (SEQ ID NO:1) or peptide fragments thereof. Such fragments can include epitopes of at least 5 amino acids of residues 87-108, or a combination of both of 59-80 and 87-108 of hTNFα (as these corresponding amino acids of SEQ ID NO:1). Additionally, preferred antibodies, fragments and regions of anti-TNF antibodies of the present invention do not recognize amino acids from at least one of amino acids 11-13, 37-42, 49-57 or 155-157 of hTNFα (of SEQ ID NO:1).

Preferred anti-TNF mAbs are also those which will competitively inhibit in vivo the binding to human TNFα of anti-TNFα murine mAb A2, chimeric mAb cA2, or an antibody having substantially the same specific binding characteristics, as well as fragments and regions thereof. Preferred antibodies of the present invention are those that bind epitopes recognized by A2 and cA2, which are included in amino acids 59-80 and/or 87-108 of hTNFα (as these corresponding amino acids of SEQ ID NO:1), such that the epitopes consist of at least 5 amino acids which comprise at least one amino acid from the above portions of human TNFα.

Preferred methods for determining mAb specificity and affinity by competitive inhibition can be found in Harlow, et al., *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1988), Colligan et al., eds., *Current Protocols in Immunology*, Greene Publishing Assoc. and Wiley Interscience, N.Y., (1992, 1993), and Muller, *Meth. Enzymol.* 92:589-601 (1983), which references are entirely incorporated herein by reference.

The techniques to raise antibodies of the present invention to small peptide sequences that recognize and bind to those sequences in the free or conjugated form or when presented as a native sequence in the context of a large protein are well known in the art. Such antibodies include murine, murine-human and human-human antibodies produced by hybridoma or recombinant techniques known in the art.

As used herein, the term "antigen binding region" refers to that portion of an antibody molecule which contains the amino acid residues that interact with an antigen and confer on the antibody its specificity and affinity for the antigen. The antibody region includes the "framework" amino acid residues necessary to maintain the proper conformation of the antigen-binding residues.

Preferably, the antigen binding region will be of murine origin. In other embodiments, the antigen binding region can be derived from other animal species, in particular rodents such as rabbit, rat or hamster.

The antigen binding region of the chimeric antibody of the present invention is preferably derived from a non-human antibody specific for human TNF. Preferred sources for the DNA encoding such a non-human antibody include cell lines which produce antibody, preferably hybrid cell lines commonly known as hybridomas. A preferred hybridoma is the A2 hybridoma cell line.

An "antigen" is a molecule or a portion of a molecule capable of being bound by an antibody which is additionally capable of inducing an animal to produce antibody capable of binding to an epitope of that antigen. An antigen can have one or more than one epitope. The specific reaction referred to above is meant to indicate that the antigen will react, in a highly selective manner, with its corresponding antibody and not with the multitude of other antibodies which can be evoked by other antigens. Preferred antigens that bind antibodies, fragments and regions of anti-TNF antibodies of the present invention include at least 5 amino acids comprising at least one of amino acids residues 87-108 or both residues 59-80 and 87-108 of hTNFα (of SEQ ID NO:1). Preferred antigens that bind antibodies, fragments and regions of anti-TNF antibodies of the present invention do not include amino acids of amino acids 11-13, 37-42, 49-57 or 155-157 of hTNFα (SEQ ID NO:1).

Particular peptides which can be used to generate antibodies of the present invention can include combinations of amino acids selected from at least residues 87-108 or both residues 59-80 and 87-108, which are combined to provide an epitope of TNF that is bound by anti-TNF antibodies, fragments and regions thereof, and which binding provided anti-TNF biological activity. Such epitopes include at least 1-5 amino acids and less than 22 amino acids from residues 87-108 or each of residues 59-80 and 87-108, which in combination with other amino acids of TNF provide epitopes of at least 5 amino acids in length.

TNF residues 87-108 or both residues 59-80 and 87-108 of TNF (of SEQ ID NO:1), fragments or combinations of peptides containing therein are useful as immunogens to

raise antibodies that will recognize peptide sequences presented in the context of the native TNF molecule.

The term "epitope" is meant to refer to that portion of any molecule capable of being recognized by and bound by an antibody at one or more of the Ab's antigen binding regions. Epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and have specific three dimensional structural characteristics as well as specific charge characteristics. By "inhibiting and/or neutralizing epitope" is intended an epitope, which, when bound by an antibody, results in loss of biological activity of the molecule or organism containing the epitope, in vivo, in vitro or in situ, more preferably in vivo, including binding of TNF to a TNF receptor.

Epitopes recognized by antibodies, and fragments and regions thereof, of the present invention can include 5 or more amino acids comprising at least one amino acid of each or both of the following amino acid sequences of TNF, which provide a topographical or three dimensional epitope of TNF which is recognized by, and/or binds with anti-TNF activity, an antibody, and fragments, and variable regions thereof, of the present invention:

59–80: Tyr-Ser-Gln-Val-Leu-Phe-Lys-Gly-Gln-Gly-Cys-Pro-Ser-Thr-His-Val-Leu-Leu-Thr-His-Thr-Ile; and (AA 59–80 of SEQ ID NO:1)

87–108: Tyr-Gln-Thr-Lys-Val-Asn-Leu-Leu-Ser-Ala-Ile-Lys-Ser-Pro-Cys-Gln-Arg-Glu-Thr-Pro-Glu-Gly. (AA 87–108 of SEQ ID NO:1)

Preferred antibodies, fragments and regions of anti-TNF antibodies of the present invention recognize epitopes including 5 amino acids comprising at least one amino acid from amino acids residues 87-108 or both residues 59-80 and 87-108 of hTNFα (of SEQ ID NO:1). Preferred antibodies, fragments and regions of anti-TNF antibodies of the present invention do not recognize epitopes from at least one of amino acids 11-13, 37-42, 49-57 or 155-157 of hTNFα (of SEQ ID NO:1). In a preferred embodiment, the epitope comprises at least 2 amino acids from residues 87-108 or both residues 59-80 and 87-108 of hTNFα (of SEQ ID NO:1). In another preferred embodiment, the epitope comprises at least 3 amino acids from residues 59-80 and 87-108 of hTNFα (of SEQ ID NO:1). In another preferred embodiment, the epitope comprises at least 4 amino acids from residues 87-108 or both residues 59-80 and 87-108 of hTNFα (of SEQ ID NO:1). In another preferred embodiment, the epitope comprises at least 5 amino acids from residues 87-108 or both residues 59-80 and 87-108 of hTNFα (of SEQ ID NO:1). In another preferred embodiment, the epitope comprises at least 6 amino acids from residues 87-108 or both residues 59-80 and 87-108 of hTNFα (of SEQ ID NO:1). In another preferred embodiment, the epitope comprises at least 7 amino acids from residues 87-108 or both residues 59-80 and 87-108 of hTNFα (of SEQ ID NO:1).

As used herein, the term "chimeric antibody" includes monovalent, divalent or polyvalent immunoglobulins. A monovalent chimeric antibody is a dimer (HL) formed by a chimeric H chain associated through disulfide bridges with a chimeric L chain. A divalent chieric antibody is tetramer (H_2L_2) formed by two HL dimers associated through at least one disulfide bridge. A polyvalent chimeric antibody can also be produced, for example, by employing a C_H region that aggregates (e.g., from an IgM H chain, or μ chain).

Murine and chimeric antibodies, fragments and regions of the present invention comprise individual heavy (H) and/or light (L) immunoglobulin chains. A chimeric H chain comprises an antigen binding region derived from the H chain of a non-human antibody specific for TNF, which is linked to at least a portion of a human H chain C region (C_H), such as CH_1 or CH_2.

A chimeric L chain according to the present invention, comprises an antigen binding region derived from the L chain of a non-human antibody specific for TNF, linked to at least a portion of a human L chain C region (C_L).

Antibodies, fragments or derivatives having chimeric H chains and L chains of the same or different variable region binding specificity, can also be prepared by appropriate association of the individual polypeptide chains, according to known method steps, e.g., according to Ausubel infra, Harlow infra, and Colligan infra, the contents of which references are incoporated entirely herein by reference.

With this approach, hosts expressing chimeric H chains (or their derivatives) are separately cultured from hosts expressing chimeric L chains (or their derivatives), and the immunoglobulin chains are separately recovered and then associated. Alternatively, the hosts can be co-cultured and the chains allowed to associate spontaneously in the culture medium, followed by recovery of the assembled immunoglobulin, fragment or derivative.

The hybrid cells are formed by the fusion of a non-human anti-hTNFα antibody-producing cell, typically a spleen cell of an animal immunized against either natural or recombinant human TNF, or a peptide fragment of the human TNFα protein sequence. Alternatively, the non-human anti-TNFα antibody-producing cell can be a B lymphocyte obtained from the blood, spleen, lymph nodes or other tissue of an animal immunized with TNF.

The second fusion partner, which provides the immortalizing function, can be a lymphoblastoid cell or a plasmacytoma or myeloma cell, which is not itself an antibody producing cell, but is malignant. Preferred fusion partner cells include the hybridoma SP2/0-Ag14, abbreviated as SP2/0 (ATCC CRL1581) and the mycloma P3X63Ag8 (ATCC TIB9), or its derivatives. See, e.g, Ausubel infra, Harlow infra, and Colligan infra, the contents of which references are incoporated entirely herein by reference.

Murine hybridomas which produce mAb specific for human TNFα or TNFβ are formed by the fusion of a mouse fusion partner cell, such as SP2/0, and spleen cells from mice immunized against purified hTNFα, recombinant hTNFα, natural or synthetic TNF peptides, including peptides including 5 or more amino acids selected from residues 59-80, and 87-108 of TNF (of SEQ ID NO:1) or other biological preparations containing TNF. To immunize the mice, a variety of different conventional protocols can be followed. For example, mice can receive primary and boosting immunizations of TNF.

The antibody-producing cell contributing the nucleotide sequences encoding the antigen-binding region of the chimeric antibody of the present invention can also be produced

by transformation of a non-human, such as a primate, or a human cell. For example, a B lymphocyte which produces anti-TNF antibody can be infected and transformed with a virus such as Epstein-Barr virus to yield an immortal anti-TNF producing cell (Kozbor et al., *Immunol. Today* 4:72-79 (1983)). Alternatively, the B lymphocyte can be transformed by providing a transforming gene or transforming gene product, as is well-known in the art. See, e.g, Ausubel infra, Harlow infra, and Colligan infra, the contents of which references are incorporated entirely herein by reference.

Antibody Production Using Hybridomas

The cell fusions are accomplished by standard procedures well known to those skilled in the field of immunology. Fusion partner cell lines and methods for fusing and selecting hybridomas and screening for mAbs are well known in the art. See, e.g, Ausubel infra, Harlow infra, and Colligan infra, the contents of which references are incoporated entirely herein by reference.

The hTNFα-specific murine or chimeric mAb of the present invention can be produced in large quantities by injecting hybridoma or transfectoma cells secreting the antibody into the peritoneal cavity of mice and, after appropriate time, harvesting the ascites fluid which contains a high titer of the mAb, and isolating the mAb therefrom. For such in vivo production of the mAb with a non-murine hybridoma (e.g., rat or human), hybridoma cells are preferably grown in irradiated or athymic nude mice. Alternatively, the antibodies can be produced by culturing hybridoma or transfectoma cells in vitro and isolating secreted mAb from the cell culture medium or recombinantly, in eukaryotic or prokaryotic cells.

In a preferred embodiment, the antibody is a MAb which binds amino acids of an epitope of TNF, which antibody is designated A2, rA2 or cA2, which is produced by a hybridoma or by a recombinant host. In another preferred embodiment, the antibody is a chimeric antibody which recognizes an epitope recognized by A2. In a more preferred embodiment, the antibody is a chimeric antibody designated as chimeric A2 (cA2).

As examples of antibodies according to the present invention, murine mAb A2 (ATCC Accesion No. PTA-7045) of the present invention is produced by a cell line designated c134A. Chimeric antibody cA2 is produced by a cell line designated c168A. C134A was deposited purduant to the Budapest Treaty requirements with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Va. 20110-2209, on Sep. 22, 2005. Cell line c134A is deposited as a research cell bank in the Centocor Cell Biology Services Depository, and cell line c168A (RCB) is deposited as a research cell bank in the Centocor Corporate Cell Culture Research and Development Depository, both at Centocor, 200 Great Valley Parkway, Malvern, Pa., 19355. The c168A cell line is also deposited at Centocor BV, Leiden, The Netherlands.

The invention also provides for "derivatives" of the murine or chimeric antibodies, fragments, regions or derivatives thereof, which term includes those proteins encoded by truncated or modified genes to yield molecular species functionally resembling the immunoglobulin fragments. The modifications include, but are not limited to, addition of genetic sequences coding for cytotoxic proteins such as plant and bacterial toxins. The fragments and derivatives can be produced from any of the hosts of this invention. Alternatively, anti-TNF antibodies, fragments and regions can be bound to cytotoxic proteins or compounds in vitro, to provide cytotoxic anti-TNF antibodies which would selectively kill cells having TNF receptors.

Fragments include, for example, Fab, Fab', $F(ab')_2$ and Fv. These fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and can have less non-specific tissue binding than an intact antibody (Wahl et al., *J. Nucl. Med.* 24:316-325 (1983)). These fragments are produced from intact antibodies using methods well known in the art, for example by proteolytic cleavage with enzymes such as papain (to produce Fab fragments) or pepsin (to produce $F(ab')_2$ fragments).

The identification of these antigen binding region and/or epitopes recognized by mAbs of the present invention provides the information necessary to generate additional monoclonal antibodies with similar binding characteristics and therapeutic or diagnostic utility that parallel the embodiments of this application.

In a preferred embodiment, the amino acids of the epitope are not of at least one of amino acids 11-13, 37-42, 49-57 and 155-157 of hTNFα (of SEQ ID NO:1).

Unexpectedly, anti-TNF antibodies or peptides of the present invention can block the action of TNF-α without binding to the putative receptor binding locus such as is presented by Eck and Sprang (*J. Biol. Chem.* 264(29): 17595-17605 (1989), as amino acids 11-13, 37-42, 49-57 and 155-157 of hTNFα (of SEQ ID NO:1).

Recombinant Expression of Anti-TNF Antibodies

Recombinant murine or chimeric murine-human or human-human antibodies that inhibit TNF and bind an epitope included in the amino acid sequences residues 87-108 or both residues 59-80 and 87-108 of hTNFα (of SEQ ID NO:1), can be provided according to the present invention using known techniques based on the teaching provided herein. See, e.g., Ausubel et al., eds. *Current Protocols in Molecular Biology*, Wiley Interscience, N.Y. (1987, 1992, 1993); and Sambrook et al. *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press (1989), the entire contents of which are incorporated herein by reference.

The DNA encoding an anti-TNF antibody of the present invention can be genomic DNA or cDNA which encodes at least one of the heavy chain constant region (H_c), the heavy chain variable region (H_v), the light chain variable region (L_v) and the light chain constant regions (L_c). A convenient alternative to the use of chromosomal gene fragments as the source of DNA encoding the murine V region antigen-binding segment is the use of cDNA for the construction of chimeric immunoglobulin genes, e.g., as reported by Liu et al. (*Proc. Natl. Acad. Sci., USA* 84:3439 (1987) and *J. Immunology* 139:3521 (1987), which references are hereby entirely incorporated herein by reference. The use of cDNA requires that gene expression elements appropriate for the host cell be combined with the gene in order to achieve synthesis of the desired protein. The use of cDNA sequences is advantageous over genomic sequences (which contain introns), in that cDNA sequences can be expressed in bacteria or other hosts which lack appropriate RNA splicing systems.

For example, a cDNA encoding a murine V region antigen-binding segment having anti-TNF activity can be provided using known methods based on the use of the DNA sequence presented in FIG. **16**A (SEQ ID NO:2). Alternatively, a cDNA encoding a murine C region antigen-binding segment having anti-TNF activity can be provided using known methods based on the use of the DNA sequence presented in FIG. **16**B (SEQ ID NO:3). Probes that bind a

portion of the DNA sequence presented in FIG. **16**A or **16**B can be used to isolate DNA from hybridomas expressing TNF antibodies, fragments or regions, as presented herein, according to the present invention, by known methods.

Oligonucleotides representing a portion of the variable region presented in FIG. **16**A or **16**B sequence are useful for screening for the presence of homologous genes and for the cloning of such genes encoding variable or constant regions of an anti-TNF antibody. Such probes preferably bind to portions of sequences according to FIG. **16**A or **16**B which encode light chain or heavy chain variable regions which bind an activity inhibiting epitope of TNF, especially an epitope of at least 5 amino acids of residues 87-108 or a combination of residues 59-80 and 87-108 (of SEQ ID NO:1).

Such techniques for synthesizing such oligonucleotides are well known and disclosed by, for example, Wu, et al., *Prog. Nucl. Acid. Res. Molec. Biol.* 21:101-141 (1978)), and Ausubel et al., eds. *Current Protocols in Molecular Biology*, Wiley Interscience (1987, 1993), the entire contents of which are herein incorporated by reference.

Because the genetic code is degenerate, more than one codon can be used to encode a particular amino acid (Watson, et al., infra). Using the genetic code, one or more different oligonucleotides can be identified, each of which would be capable of encoding the amino acid. The probability that a particular oligonucleotide will, in fact, constitute the actual XXX-encoding sequence can be estimated by considering abnormal base pairing relationships and the frequency with which a particular codon is actually used (to encode a particular amino acid) in eukaryotic or prokaryotic cells expressing an anti-TNF antibody or fragment. Such "codon usage rules" are disclosed by Lathe, et al., *J. Molec. Biol.* 183:1-12 (1985). Using the "codon usage rules" of Lathe, a single oligonucleotide, or a set of oligonucleotides, that contains a theoretical "most probable" nucleotide sequence capable of encoding anti-TNF variable or constant region sequences is identified.

Although occasionally an amino acid sequence can be encoded by only a single oligonucleotide, frequently the amino acid sequence can be encoded by any of a set of similar oligonucleotides. Importantly, whereas all of the members of this set contain oligonucleotides which are capable of encoding the peptide fragment and, thus, potentially contain the same oligonucleotide sequence as the gene which encodes the peptide fragment, only one member of the set contains the nucleotide sequence that is identical to the nucleotide sequence of the gene. Because this member is present within the set, and is capable of hybridizing to DNA even in the presence of the other members of the set, it is possible to employ the unfractionated set of oligonucleotides in the same manner in which one would employ a single oligonucleotide to clone the gene that encodes the protein.

The oligonucleotide, or set of oligonucleotides, containing the theoretical "most probable" sequence capable of encoding an anti-TNF antibody or fragment including a variable or constant region is used to identify the sequence of a complementary oligonucleotide or set of oligonucleotides which is capable of hybridizing to the "most probable" sequence, or set of sequences. An oligonucleotide containing such a complementary sequence can be employed as a probe to identify and isolate the variable or constant region anti-TNF gene (Sambrook et al., infra).

A suitable oligonucleotide, or set of oligonucleotides, which is capable of encoding a fragment of the variable or constant anti-TNF region (or which is complementary to such an oligonucleotide, or set of oligonucleotides) is identified (using the above-described procedure), synthesized, and hybridized by means well known in the art, against a DNA or, more preferably, a cDNA preparation derived from cells which are capable of expressing anti-TNF antibodies or variable or constant regions thereof. Single stranded oligonucleotide molecules complementary to the "most probable" variable or constant anti-TNF region peptide coding sequences can be synthesized using procedures which are well known to those of ordinary skill in the art (Belagaje, et al., *J. Biol. Chem.* 254:5765-5780 (1979); Maniatis, et al., *In: Molecular Mechanisms in the Control of Gene Expression*, Nierlich, et al., Eds., Acad. Press, NY (1976); Wu, et al., *Prog. Nucl. Acid Res. Molec. Biol.* 21:101-141 (1978); Khorana, *Science* 203:614-625 (1979)). Additionally, DNA synthesis can be achieved through the use of automated synthesizers. Techniques of nucleic acid hybridization are disclosed by Sambrook et al. (infra), and by Hayrnes, et al. (In: *Nucleic Acid Hybridization, A Practical Approach*, IRL Press, Washington, D.C. (1985)), which references are herein incorporated by reference. Techniques such as, or similar to, those described above have successfully enabled the cloning of genes for human aldehyde dehydrogenases (Hsu, et al., *Proc. Natl. Acad. Sci. USA* 82:3771-3775 (1985)), fibronectin (Suzuki, et al., *Bur. Mol. Biol. Organ. J.* 4:2519-2524 (1985)), the human estrogen receptor gene (Walter, et al., *Proc. Natl. Acad. Sci. USA* 82:7889-7893 (1985)), tissue-type plasminogen activator (Pennica, et al., *Nature* 301:214-221 (1983)) and human term placental alkaline phosphatase complementary DNA (Keun, et al., *Proc. Natl. Acad. Sci. USA* 82:8715-8719 (1985)).

In an alternative way of cloning a polynucleotide encoding an anti-TNF variable or constant region, a library of expression vectors is prepared by cloning DNA or, more preferably, cDNA (from a cell capable of expressing an anti-TNF antibody or variable or constant region) into an expression vector. The library is then screened for members capable of expressing a protein which competitively inhibits the binding of an anti-TNF antibody, such as A2 or cA2, and which has a nucleotide sequence that is capable of encoding polypeptides that have the same amino acid sequence as anti-TNF antibodies or fragments thereof. In this embodiment, DNA, or more preferably cDNA, is extracted and purified from a cell which is capable of expressing an anti-TNF antibody or fragment. The purified cDNA is fragmentized (by shearing, endonuclease digestion, etc.) to produce a pool of DNA or cDNA fragments. DNA or cDNA fragments from this pool are then cloned into an expression vector in order to produce a genomic library of expression vectors whose members each contain a unique cloned DNA or cDNA fragment such as in a lambda phage library, expression in prokaryotic cell (e.g., bacteria) or eukaryotic cells, (e.g., mammalian, yeast, insect or, fungus). See, e.g., Ausubel, infra, Harlow, infra, Colligan, infra; Nyyssonen et al. *Bio/Technology* 11:591-595 (Can 1993); Marks et al., *Bio/Technology* 11:1145-1149 (October 1993). Once nucleic acid encoding such variable or constant anti-TNF regions is isolated, the nucleic acid can be appropriately expressed in a host cell, along with other constant or variable heavy or light chain encoding nucleic acid, in order to provide recombinant MAbs that bind TNF with inhibitory activity. Such antibodies preferably include a murine or human anti-TNF variable region which contains a framework residue having complimentarity determining residues which are responsible for antigen binding. In a preferred embodiment, an anti-TNF variable light or heavy chain encoded by a nucleic acid as described above binds an epitope of at least 5 amino acids

including residues 87-108 or a combination of residues 59-80 and 87-108 of hTNF (of SEQ ID NO: 1).

Human genes which encode the constant (C) regions of the murine and chimeric antibodies, fragments and regions of the present invention can be derived from a human fetal liver library, by known methods. Human C regions genes can be derived from any human cell including those which express and produce human immunoglobulins. The human C_H region can be derived from any of the known classes or isotypes of human H chains, including gamma, μ, α, δ or ϵ, and subtypes thereof, such as G1, G2, G3 and G4. Since the H chain isotype is responsible for the various effector functions of an antibody, the choice of C_H region will be guided by the desired effector functions, such as complement fixation, or activity in antibody-dependent cellular cytotoxicity (ADCC). Preferably, the C_H region is derived from gamma 1 (IgG1), gamma 3 (IgG3), gamma 4 (IgG4), or μ (IgM).

The human C_L region can be derived from either human L chain isotype, kappa or lambda.

Genes encoding human immunoglobulin C regions are obtained from human cells by standard cloning techniques (Sambrook, et al. (*Molecular Cloning: A Laboratory Manual,* 2nd Edition, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989) and Ausubel et al., eds. *Current Protocols in Molecular Biology* (1987-1993)). Human C region genes are readily available from known clones containing genes representing the two classes of L chains, the five classes of H chains and subclasses thereof. Chimeric antibody fragments, such as $F(ab')_2$ and Fab, can be prepared by designing a chimeric H chain gene which is appropriately truncated. For example, a chimeric gene encoding an H chain portion of an $F(ab')_2$ fragment would include DNA sequences encoding the CH_1 domain and hinge region of the H chain, followed by a translational stop codon to yield the truncated molecule.

Generally, the murine, human or murine and chimeric antibodies, fragments and regions of the present invention are produced by cloning DNA segments encoding the H and L chain antigen-binding regions of a TNF-specific antibody, and joining these DNA segments to DNA segments encoding C_H and C_L regions, respectively, to produce murine, human or chimeric immunoglobulin-encoding genes.

Thus, in a preferred embodiment, a fused chimeric gene is created which comprises a first DNA segment that encodes at least the antigen-binding region of non-human origin, such as a functionally rearranged V region with joining (J) segment, linked to a second DNA segment encoding at least a part of a human C region.

Therefore, cDNA encoding the antibody V and C regions, the method of producing the chimeric antibody according to the present invention involves several steps, outlined below:

1. isolation of messenger RNA (mRNA) from the cell line producing an anti-TNF antibody and from optional additional antibodies supplying heavy and light constant regions; cloning and cDNA production therefrom;
2. preparation of a full length cDNA library from purified mRNA from which the appropriate V and/or C region gene segments of the L and H. chain genes can be: (i) identified with appropriate probes, (ii) sequenced, and (iii) made compatible with a C or V gene segment from another antibody for a chimeric antibody;
3. Construction of complete H or L chain coding sequences by linkage of the cloned specific V region gene segments to cloned C region gene, as described above;
4. Expression and production of L and H chains in selected hosts, including prokaryotic and eukaryotic cells to provide murine-murine, human-murine, human-human or human murine antibodies.

One common feature of all immunoglobulin H and L chain genes and their encoded mRNAs is the J region. H and L chain J regions have different sequences, but a high degree of sequence homology exists (greater than 80%) among each group, especially near the C region. This homology is exploited in this method and consensus sequences of H and L chain J regions can be used to design oligonucleotides for use as primers for introducing useful restriction sites into the J region for subsequent linkage of V region segments to human C region segments.

C region cDNA vectors prepared from human cells can be modified by site-directed mutagenesis to place a restriction site at the analogous position in the human sequence. For example, one can clone the complete human kappa chain C (C_k) region and the complete human gamma-1 C region ($C_{gamma\text{-}1}$). In this case, the alternative method based upon genomic C region clones as the source for C region vectors would not allow these genes to be expressed in bacterial systems where enzymes needed to remove intervening sequences are absent. Cloned V region segments are excised and ligated to L or H chain C region vectors. Alternatively, the human $C_{gamma\text{-}1}$ region can be modified by introducing a termination codon thereby generating a gene sequence which encodes the H chain portion of an Fab molecule. The coding sequences with linked V and C regions are then transferred into appropriate expression vehicles for expression in appropriate hosts, prokaryotic or eukaryotic.

Two coding DNA sequences are said to be "operably linked" if the linkage results in a continuously translatable sequence without alteration or interruption of the triplet reading frame. A DNA coding sequence is operably linked to a gene expression element if the linkage results in the proper function of that gene expression element to result in expression of the coding sequence.

Expression vehicles include plasmids or other vectors. Preferred among these are vehicles carrying a functionally complete human C_H or C_L chain sequence having appropriate restriction sites engineered so that any V_H or V_L chain sequence with appropriate cohesive ends can be easily inserted therein. Human C_H or C_L chain sequence-containing vehicles thus serve as intermediates for the expression of any desired complete H or L chain in any appropriate host.

A chimeric antibody, such as a mouse-human or human-human, will typically be synthesized from genes driven by the chromosomal gene promoters native to the mouse H and L chain V regions used in the constructs; splicing usually occurs between the splice donor site in the mouse J region and the splice acceptor site preceding the human C region and also at the splice regions that occur within the human C region; polyadenylation and transcription termination occur at native chromosomal sites downstream of the human coding regions.

Non-Limiting Exemplary Chimeric A2 (cA2) Anti-TNF Antibody of the Present Invention

Murine MAbs are undesirable for human therapeutic use, due to a short free circulating serum half-life and the stimulation of a human anti-murine antibody (HAMA) response. A murine-human chimeric anti-human TNFα MAb was developed in the present invention with high affinity, epitope specificity and the ability to neutralize the cytotoxic effects of human TNF. Chimeric A2 anti-TNF consists of the antigen binding variable region of the high-

affinity neutralizing mouse antihuman TNF IgG1 antibody, designated A2, and the constant regions of a human IgG1, kappa immunoglobulin. The human IgG1 Fe region is expected to: improve allogeneic antibody effector function; increase the circulating serum half-life; and decrease the immunogenicity of the antibody. A similar murine-human chimeric antibody (chimeric 17-1A) has been shown in clinical studies to have a 6-fold longer in vivo circulation time and to be significantly less immunogenic than its corresponding murine MAb counterpart (LoBuglio et al., *Proc Natl Acad Sci USA* 86: 4220-4224, (1988)).

The avidity and epitope specificity of the chimeric A2 is derived from the variable region of the murine A2. In a solid phase ELISA, cross-competition for TNF was observed between chimeric and murine A2, indicating an identical epitope specificity of cA2 and murine A2. The specificity of cA2 for TNF-α was confirmed by its inability to neutralize the cytotoxic effects of lymphotoxin (TNF-β). Chimeric A2 neutralizes the cytotoxic effect of both natural and recombinant human TNF in a dose dependent manner. From binding assays of cA2 and recombinant human TNF, the affinity constant of cA2 was calculated to be 1.8×10^9 M^1.

ANTI-TNF Immunoreceptor Peptides

Immunoreceptor peptides of this invention can bind to TNFα and/or TNFβ. The immunoreceptor comprises covalently attached to at least a portion of the TNF receptor at least one immunoglobulin heavy or light chain. In certain preferred embodiments, the heavy chain constant region comprises at least a portion of CH_1. Specifically, where a light chain is included with an immunoreceptor peptide, the heavy chain must include the area of CH_1 responsible for binding a light chain constant region.

An immunoreceptor peptide of the present invention can preferably comprise at least one heavy chain constant region and, in certain embodiments, at least one light chain constant region, with a receptor molecule covalently attached to at least one of the immunoglobulin chains. Light chain or heavy chain variable regions are included in certain embodiments. Since the receptor molecule can be linked within the interior of an immunoglobulin chain, a single chain can have a variable region and a fusion to a receptor molecule.

The portion of the TNF receptor linked to the immunoglobulin molecule is capable of binding TNFα and/or TNFβ. Since the extracellular region of the TNF receptor binds TNF, the portion attached to the immunoglobulin molecule of the immunoreceptor consists of at least a portion of the extracellular region of the TNF receptor. In certain preferred embodiments, the entire extracellular region of p55 is included. In other preferred embodiments, the entire extracellular region of p75 is included. In further preferred embodiments, the extracellular region of p75 is truncated to delete at least a portion of a region of O-linked glycosylation and/or a proline-rich region while leaving intact the intramolecular disulfide bridges. Such immunoreceptors comprise at least a portion of a hinge region wherein at least one heavy chain is covalently linked to a truncated p75 extracellular region capable of binding to TNFα or TNFβ or both. Such a truncated molecule includes, for example, sequences 1-178, 1-182 or at least 5 amino acid portions thereof, such as 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, . . . 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290.

Certain embodiments can also include, for example, the C-terminal half of the hinge region to provide a disulfide bridge between heavy chains where both CH_2 and CH_3 chains are present and CH_1 is absent. Alternatively, for example, the N-terminal half of the hinge region can be included to provide a disulfide bridge with a light chain where only the CH_1 region is present.

In certain preferred embodiments of this invention, the non-immunoglobulin molecule is covalently linked to the N-terminus of at least one CH_1 region. In other preferred embodiments, the non-immunoglobulin molecule is covalently linked to an interior section of at least one heavy and/or light chain region. Thus, a portion of the TNF receptor can be, for example, at the end of the immunoglobulin chain or in the middle of the chain.

Where the TNF receptor is attached to the middle of the immunoglobulin, the immunoglobulin chain can be truncated, for example, to compensate for the presence of foreign amino acids, thus resulting in a fusion molecule of approximately the same length as a natural immunoglobulin chain. Alternatively, for example, the immunoglobulin chain can be present substantially in its entirety, thus resulting in a chain that is longer than the corresponding natural immunoglobulin chain. Additionally, the immunoglobulin molecule can be truncated to result in a length intermediate between the size of the entire chain linked to the receptor molecule and the size of the immunoglobulin chain alone.

In certain preferred embodiments, the heavy chain is an IgG class heavy chain. In other preferred embodiments, the heavy chain is an IgM class heavy chain.

In certain preferred embodiments, the heavy chain further comprises at least about 8 amino acids of a J region.

In certain preferred embodiments, at least a portion of the hinge region is attached to the CH_1 region. For example, where CH_1 and CH_2 are present in the molecule, the entire hinge region is also present to provide the disulfide bridges between the two heavy chain molecules and between the heavy and light chains. Where only CH_1 is present, for example, the molecule need only contain the portion of the hinge region corresponding to the disulfide bridge between the light and heavy chains, such as the first 7 amino acids of the hinge.

It will be understood by one skilled in the art, once armed with the present disclosure, that the immunoreceptor peptides of the invention can be, for example, monomeric or dimeric. For example, the molecules can have only one light chain and one heavy chain or two light chains and two heavy chains.

At least one of the non-immunoglobulin molecules linked to an immunoglobin molecule comprises at least a portion of p55 or at least a portion of p75. The portion of the receptor that is included encompasses the TNF binding site.

In certain preferred embodiments, the non-immunoglobulin molecule comprises at least 5 amino acid segments of sequences 2-159 of p55. In other preferred embodiments, the non-immunoglobulin molecule comprises at least 5 amino acid portions of sequences 1-235 of p75. In further preferred embodiments, the non-immunoglobulin molecule comprises at least 5 amino acid portions of sequences 1-182 of p75. The above 5 amino acid portions can be selected from 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290.

In certain preferred embodiments, each of the two heavy chains and each of the two light chains is linked to a portion of the TNF receptor, thus forming a tetravalent molecule. Such a tetravalent molecule can have, for example, four p55 receptor molecules; two on the two heavy chains and two on

the two light chains. Alternatively, a tetravalent molecule can have, for example, a p55 receptor molecule attached to each of the two heavy chains and a p75 receptor molecule attached to each of the two light chains. A tetravalent molecule can also have, for example, p55 receptor attached to the light chains and p75 receptor attached to the heavy chains. Additionally, a tetravalent molecule can have one heavy chain attached to p55, one heavy chain attached to p75, one light chain attached to p75, and one light chain attached to p55. See, for example, the molecules depicted in FIG. **26**B. Further, the molecules can have six receptors attached, for example, two within the heavy chains and four at the ends of the heavy and light chains. Other potential multimers and combinations would also be within the scope of one skilled in the art, once armed with the present disclosure.

In further preferred embodiments, at least one of the heavy chains has a variable region capable of binding to a second target molecule. Such molecules include, for example, CD3, so that one half of a fusion molecule is a monomeric anti-CD3 antibody.

Additionally, in other embodiments of the present invention, the immunoreceptor peptides further include an irrelevant variable region on the light chain and/or heavy chain. Preferably, however, such a region is absent due to the lowered affinity for TNF which can be present due to steric hindrance.

In certain preferred embodiments, the heavy chain is linked to a non-immunoglobulin molecule capable of binding to a second target molecule, such as a cytotoxic protein, thus creating a part immunoreceptor, part immunotoxin that is capable of killing those cells expressing TNF. Such cytotoxic proteins, include, but are not limited to, Ricin-A, *Pseudomonas* toxin, Diphtheria toxin and TNF. Toxins conjugated to ligands are known in the art (see, for example, Olsnes, S. et al., *Immunol. Today* 1989, 10, 291-295). Plant and bacterial toxins typically kill cells by disruption of the protein synthetic machinery.

The immunoreceptors of this invention can be conjugated to additional types of therapeutic moieties including, but not limited to, radionuclides, cytotoxic agents and drugs. Examples of radionuclides include ^{212}Bi, ^{131}I, ^{186}Re, and ^{90}Y, which list is not intended to be exhaustive. The radionuclides exert their cytotoxic effect by locally irradiating the cells, leading to various intracellular lesions, as is known in the art of radiotherapy.

Cytotoxic drugs which can be conjugated to the immunoreceptors and subsequently used for in vivo therapy include, but are not limited to, daunorubicin, doxorubicin, methotrexate, and Mitomycin C. Cytotoxic drugs interfere with critical cellular processes including DNA, RNA, and protein synthesis. For a fuller exposition of these classes of drugs which are known in the art, and their mechanisms of action, see Goodman, A. G., et al., *Goodman and Gilman's The Pharmacological Basis of Therapeutics,* 8th Ed., Macmillan Publishing Col, 1990. Katzung, ed., Basic and Clinical Pharmacology, Fifth Edition, p 768-769, 808-809, 896, Appleton and Lange, Norwalk, Conn.

In preferred embodiments, immunoreceptor molecules of the invention are capable of binding with high affinity to a neutralizing epitope of human TNFα or TNFβ in vivo. Preferably, the binding affinity is at least about 1.6×10^{10} M-1. Additionally, in preferred embodiments, immunoreceptor molecules of the invention are capable of neutralizing TNF at an efficiency of about a concentration of less than 130 pM to neutralize 39.2 pM human TNFα. See, for example, Table 1.

TABLE 1

Summary of affinities of different fusion proteins for TNFα.

Protein	IC_{50}*	Molar ratio fp: TNFα at IC_{50}	K_D(pM)
p55-sf2	70	1.8	57
p55-df2	55	1.4	60
p55-sf3	100	2.6	48
p55-nf	36,000	900	n.d.
p75-sf2	130	3.3	33
p75P-sf2	70	1.8	29
p75P-sf3	130	3.3	15

*IC_{50} = concentration of fusion protein required to inhibit 2 ng/ml (39.2 pM) TNFα by 50%

Once armed with the present disclosure, one skilled in the art would be able to create fragments of the immunoreceptor peptides of the invention. Such fragments are intended to be within the scope of this invention. For example, once the molecules are isolated, they can be cleaved with protease to generate fragments that remain capable of binding TNF.

Once armed with the present disclosure, one skilled in the art would also be able to create derivatives of the immunoreceptor peptides of the invention. Such derivatives are intended to be within the scope of this invention. For example, amino acids in the immunoreceptor that constitute a protease recognition site can be modified to avoid protease cleavage and thus confer greater stability, such as KEX2 sites.

One skilled in the art, once armed with the present disclosure, would be able to synthesize the molecules of the invention. The immunoreceptor peptides can be constructed, for example, by vector-mediated synthesis, as described in Example XXVI. In general, two expression vectors are preferably used; one for the heavy chain, one for the light chain. A vector for expression of an immunoglobulin preferably consists of a promoter linked to the signal sequence, followed by the constant region. The vector additionally preferably contains a gene providing for the selection of transfected cells expressing the construct. In certain preferred embodiments, sequences derived from the J region are also included.

The immunoglobulin gene can be from any vertebrate source, such as murine, but preferably, it encodes an immunoglobulin having a substantial number of sequences that are of the same origin as the eventual recipient of the immunoreceptor peptide. For example, if a human is treated with a molecule of the invention, preferably the immunoglobulin is of human origin.

TNF receptor constructs for linking to the heavy chain can be synthesized, for example, using DNA encoding amino acids present in the cellular domain of the receptor. Putative receptor binding loci of hTNF have been presented by Eck and Sprange, *J. Biol. Chem.* 264(29), 17595-17605 (1989), who identified the receptor binding loci of TNFα as consisting of amino acids 11-13, 37-42, 49-57 and 155-157. PCT application WO91/02078 (priority date of Aug. 7, 1989) discloses TNF ligands which can bind to monoclonal antibodies having the following epitopes of at least one of 1-20, 56-77, and 108-127; at least two of 1-20, 56-77, 108-127 and 138-149; all of 1-18, 58-65, 115-125 and 138-149; all of 1-18, and 108-128; all of 56-79, 110-127 and 135- or 136-155; all of 1-30, 117-128 and 141-153; all of 1-26, 117-128 and 141-153; all of 22-40, 49-96 or -97, 110-127 and 136-153; all of 12-22, 36-45, 96-105 and 132-157; all of both of 1-20 and 76-90; all of 22-40, 69-97,

105-128 and 135-155; all of 22-31 and 146-157; all of 22-40 and 49-98; at least one of 22-40, 9-98 and 69-97, both of 22-40 and 70-87. Thus, one skilled in the art, once armed with the present disclosure, would be able to create TNF receptor fusion proteins using portions of the receptor that are known to bind TNF.

Advantages of using an immunoglobulin fusion protein (immunoreceptor peptide) of the present invention include one or more of (1) possible increased avidity for multivalent ligands due to the resulting bivalency of dimeric fusion proteins, (2) longer serum half-life, (3) the ability to activate effector cells via the Fc domain, (4) ease of purification (for example, by protein A chromatography), (5) affinity for TNFα and TNFβ and (6) the ability to block TNFα or TNFβ cytotoxicity.

TNF receptor/IgG fusion proteins have shown greater affinity for TNFα in vitro than their monovalent, non-fusion counterparts. These types of fusion proteins, which also bind murine TNF with high affinity, have also been shown to protect mice from lipopolysaccharide-induced endotoxemia. Lesslauer et al., *Eur. J. Immunol.* 21, 2883-2886 (1991); and Ashkenazi et al., *Proc. Natl. Acad. Sci. USA* 88:10535-10539 (1991). Unlike the molecules of the present invention, the TNF receptor/IgG fusion proteins reported to date have had the receptor sequence fused directly to the hinge domain of IgGs such that the first constant domain (CH_1) of the heavy chain was omitted. Lesslauer et al., *Eur. J. Immunol.* 1:2883-2886; Ashkenzi et al., *Proc. Natl. Acad. Sci. USA* 88:10535-10539 (1991); and Peppel et al., *J. Exp. Med.* 174:1483-1489 (1991).

While this generally permits secretion of the fusion protein in the absence of an Ig light chain, a major embodiment of the present invention provides for the inclusion of the CH_1 domain, which can confer advantages such as (1) increased distance and/or flexibility between two receptor molecules resulting in greater affinity for TNF, (2) the ability to create a heavy chain fusion protein and a light chain fusion protein that would assemble with each other and dimerize to form a tetravalent (double fusion) receptor molecule, and (3) a tetravalent fusion protein can have increased affinity and/or neutralizing capability for TNF compared to a bivalent (single fusion) molecule.

Unlike other TNF receptor/IgG fusion proteins that have been reported, the fusion proteins of a major embodiment of the present invention include the first constant domain (CH_1) of the heavy chain. The CH_1 domain is largely responsible for interactions with light chains. The light chain, in turn, provides a vehicle for attaching a second set of TNF receptor molecules to the immunoreceptor peptide.

It was discovered using the molecules of the present invention that the p55/light chain fusion proteins and p55/heavy chain fusion proteins would assemble with each other and dimerize to form an antibody-like molecule that is tetravalent with respect to p55. The resulting tetravalent p55 molecules can confer more protection against, and have greater affinity for, TNFα or TNFβ than the bivalent p55 molecules. Despite the presumed close proximity of the two light chain p55 domains to the heavy chain p55 domains, they do not appear sterically hinder or reduce the affinity for TNF.

Inclusion of the CH_1 domain also meant that secretion of the fusion protein was likely to be inefficient in the absence of light chain. This has been shown to be due to a ubiquitous immunoglobulin binding protein (BiP) that binds to the CH_1 domain of heavy chains that are not assembled with a light chain and sequesters them in the endoplasmic reticulum. Karlsson et al., *J. Immunol. Methods* 145:229-240 (1991).

In initial experiments, an irrelevant light chain was co-transfected with the p55-heavy chain construct and subsequent analyses showed that the two chains did assemble and that the resulting fusion protein protected WEHI cells from TNFα. However, it was considered likely that the variable region of the irrelevant light chain would sterically hinder interactions between the p55 subunits and TNFα. For this reason, a mouse-human chimeric antibody light chain gene was engineered by (1) deleting the variable region coding sequence, and (2) replacing the murine J coding sequence with human J coding sequence. Use of this truncated light chain, which was shown to assemble and disulfide bond with heavy chains, increased the efficiency of TNF inhibition by approximately 30-fold compared to use of a complete irrelevant light chain.

Comparison of the abilities of p75-sf2 and p75P-sf2 to inhibit TNF cytotoxicity indicated that the C-terminal 53 amino acids of the extracellular domain of p75, which defines a region that is rich in proline residues and contains the only sites of O-linked glycosylation, are not necessary for high-affinity binding to TNFα or TNFβ. In fact, the p75P-sf2 construct repeatedly showed higher affinity binding to TNFβ than p75-sf2. Surprisingly, there was no difference observed between the two constructs in their affinity for TNFα.

It is possible that a cell-surface version of p75-P would also bind TNFβ with higher affinity than the complete p75 extracellular domain. A similar region is found adjacent to the transmembrane domain in the low affinity nerve growth factor receptor whose extracellular domain shows the same degree of similarity to p75 as p55 does. Mallerr et al., *Immunol. Today* 12:220-223 (1991).

Two groups have reported that in cell cytotoxicity assays, their p55 fusion protein could be present at a 3-fold (Lesslauer et al., *Eur. J. Immunol.* 21:2883-2886 (1991)) or 6-8 fold (Ashkenazi et al., *Proc. Natl. Acad. Sci. USA* 88:10535-10539 (1991)) lower concentration than their monovalent p55 and still get the same degree of protection, while another group (Peppel et al., *J. Exp. Med.* 174:1483-1489 (1991)) showed that their p55 fusion protein could be present at a 1000-fold lower concentration than monomeric p55. Thus, the prior art has shown unpredictability in the great variability in the efficiency of different fusion proteins.

The molecules of the present invention have demonstrated the same degree of protection against TNF in a 5000-fold lower molar concentration than monomeric p55. (See Table 1.) It is believed that the presence of the CH_1 chain in the molecules of a major embodiment of the present invention can confer greater flexibility to the molecule and avoid steric hindrance with the binding of the TNF receptor.

Recombinant Expression of Anti-TNF Antibodies and Anti-TNF Peptides

A nucleic acid sequence encoding at least one anti-TNF peptide or Ab fragment of the present invention may be recombined with vector DNA in accordance with conventional techniques, including blunt-ended or staggered-ended termini for ligation, restriction enzyme digestion to provide appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and ligation with appropriate ligases. Techniques for such manipulations are disclosed, e.g., by Ausubel, infra, Sambrook, infra, entirely incorporated herein by reference, and are well known in the art.

A nucleic acid molecule, such as DNA, is said to be "capable of expressing" a polypeptide if it contains nucleotide sequences which contain transcriptional and transla-

tional regulatory information and such sequences are "operably linked" to nucleotide sequences which encode the polypeptide. An operable linkage is a linkage in which the regulatory DNA sequences and the DNA sequence sought to be expressed are connected in such a way as to permit gene expression as anti-TNF peptides or Ab fragments in recoverable amounts. The precise nature of the regulatory regions needed for gene expression may vary from organism to organism, as is well known in the analogous art. See, e.g., Sambrook, supra and Ausubel supra.

The present invention accordingly encompasses the expression of an anti-TNF peptide or Ab fragment, in either prokaryotic or eukaryotic cells, although eukaryotic expression is preferred.

Preferred hosts are bacterial or eukaryotic hosts including bacteria, yeast, insects, fungi, bird and mammalian cells either in vivo, or in situ, or host cells of mammalian, insect, bird or yeast origin. It is preferred that the mammalian cell or tissue is of human, primate, hamster, rabbit, rodent, cow, pig, sheep, horse, goat, dog or cat origin, but any other mammalian cell may be used.

Further, by use of, for example, the yeast ubiquitin hydrolase system, in vivo synthesis of ubiquitin-transmembrane polypeptide fusion proteins may be accomplished. The fusion proteins so produced may be processed in vivo or purified and processed in vitro, allowing synthesis of an anti-TNF peptide or Ab fragment of the present invention with a specified amino terminus sequence. Moreover, problems associated with retention of initiation codon-derived methionine residues in direct yeast (or bacterial) expression maybe avoided. Sabin et al., *Bio/Technol.* 7(7): 705-709 (1989); Miller et al., *Bio/Technol.* 7(7):698-704 (1989).

Any of a series of yeast gene expression systems incorporating promoter and termination elements from the actively expressed genes coding for glycolytic enzymes produced in large quantities when yeast are grown in mediums rich in glucose can be utilized to obtain anti-TNF peptides or Ab fragments of the present invention. Known glycolytic genes can also provide very efficient transcriptional control signals. For example, the promoter and terminator signals of the phosphoglycerate kinase gene can be utilized.

Production of anti-TNF peptides or Ab fragments or functional derivatives thereof in insects can be achieved, for example, by infecting the insect host with a baculovirus engineered to express a transmembrane polypeptide by methods known to those of skill. See Ausubel et al., eds. *Current Protocols in Molecular Biology* Wiley Interscience, §§16.8-16.11 (1987, 1993).

In a preferred embodiment, the introduced nucleotide sequence will be incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. Any of a wide variety of vectors maybe employed for this purpose. See, e.g., Ausubel et al., infra, §§ 1.5, 1.10, 7.1, 7.3, 8.1, 9.6, 9.7, 13.4, 16.2, 16.6, and 16.8-16.11. Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species.

Preferred prokaryotic vectors known in the art include plasmids such as those capable of replication in *E. coli* (such as, for example, pBR322, ColE1, pSC101, pACYC 184, πVX). Such plasmids are, for example, disclosed by Maniatis, T., et al. (*Molecular Cloning, A Laboratory Manual*, Second Edition, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989); Ausubel, infra. *Bacillus* plasmids include pC194, pC221, pT127, etc. Such plasmids are disclosed by Gryczan, T. (In: *The Molecular Biology of the Bacilli*, Academic Press, NY (1982), pp. 307-329). Suitable *Streptomyces* plasmids include pIJ101 (Kendall, K. J., et al., *J. Bacteriol.* 169:4177-4183 (1987)), and streptomyces bacteriophages such as φC31 (Chater, K. F., et al., In: *Sixth International Symposium on Actinomycetales Biology*, Akademiai Kaido, Budapest, Hungary (1986), pp. 45-54). *Pseudomonas* plasmids are reviewed by John, J. F., et al. (*Rev. Infect. Dis.* 8:693-704 (1986)), and Izaki, K. (*Jpn. J. Bacteriol.* 33:729-742 (1978); and Ausubel et al., supra).

Alternatively, gene expression elements useful for the expression of cDNA encoding anti-TNF antibodies or peptides include, but are not limited to (a) viral transcription promoters and their enhancer elements, such as the SV40 early promoter (Okayama, et al., *Mol. Cell. Biol.* 3:280 (1983)), Rous sarcoma virus LTR (Gorman, et al., *Proc. Natl. Acad. Sci., USA* 79:6777 (1982)), and Moloney murine leukemia virus LTR (Grosschedl, et al., *Cell* 41:885 (1985)); (b) splice regions and polyadenylation sites such as those derived from the SV40 late region (Okayarea et al., infra); and (c) polyadenylation sites such as in SV40 (Okayama et al., infra).

Immunoglobulin cDNA genes can be expressed as described by Liu et al., infra, and Weidle et al., *Gene* 51:21 (1987), using as expression elements the SV40 early promoter and its enhancer, the mouse immunoglobulin H chain promoter enhancers, SV40 late region mRNA splicing, rabbit S-globin intervening sequence, immunoglobulin and rabbit S-globin polyadenylation sites, and SV40 polyadenylation elements.

For immunoglobulin genes comprised of part cDNA, part genomic DNA (Whittle et al., *Protein Engineering* 1:499 (1987)), the transcriptional promoter can be human cytomegalovirus, the promoter enhancers can be cytomegalovirus and mouse/human immunoglobulin, and mRNA splicing and polyadenylation regions can be the native chromosomal immunoglobulin sequences.

In one embodiment, for expression of cDNA genes in rodent cells, the transcriptional promoter is a viral LTR sequence, the transcriptional promoter enhancers are either or both the mouse immunoglobulin heavy chain enhancer and the viral LTR enhancer, the splice region contains an intron of greater than 31 bp, and the polyadenylation and transcription termination regions are derived from the native chromosomal sequence corresponding to the immunoglobulin chain being synthesized. In other embodiments, cDNA sequences encoding other proteins are combined with the above-recited expression elements to achieve expression of the proteins in mammalian cells.

Each fused gene is assembled in, or inserted into, an expression vector. Recipient cells capable of expressing the chimeric immunoglobulin chain gene product are then transfected singly with an anti-TNF peptide or chimeric H or chimeric L chain-encoding gene, or are co-transfected with a chimeric H and a chimeric L chain gene. The transfected recipient cells are cultured under conditions that permit expression of the incorporated genes and the expressed immunoglobulin chains or intact antibodies or fragments are recovered from the culture.

In one embodiment, the fused genes encoding the anti-TNF peptide or chimeric H and L chains, or portions thereof, are assembled in separate expression vectors that are then used to co-transfect a recipient cell.

Each vector can contain two selectable genes, a first selectable gene designed for selection in a bacterial system and a second selectable gene designed for selection in a eukaryotic system, wherein each vector has a different pair of genes. This strategy results in vectors which first direct the production, and permit amplification, of the fused genes in a bacterial system. The genes so produced and amplified in a bacterial host are subsequently used to co-transfect a eukaryotic cell, and allow selection of a co-transfected cell carrying the desired transfected genes.

Examples of selectable genes for use in a bacterial system are the gene that confers resistance to ampicillin and the gene that confers resistance to chloramphenicol. Preferred selectable genes for use in eukaryotic transfectants include the xanthine guanine phosphoribosyl transferase gene (designated gpt) and the phosphotransferase gens from Tn5 (designated neo).

Selection of cells expressing gpt is based on the fact that the enzyme encoded by this gene utilizes xanthine as a substrate for purine nucleotide synthesis, whereas the analogous endogenous enzyme cannot. In a medium containing (1) mycophenolic acid, which blocks the conversion of inosine monophosphate to xanthine monophosphate, and (2) xanthine, only cells expressing the gpt gene can survive. The product of the neo blocks the inhibition of protein synthesis by the antibiotic G418 and other antibiotics of the neomycin class.

The two selection procedures can be used simultaneously or sequentially to select for the expression of immunoglobulin chain genes introduced on two different DNA vectors into a eukaryotic cell. It is not necessary to include different selectable markers for eukaryotic cells; an H and an L chain vector, each containing the same selectable marker can be co-transfected. After selection of the appropriately resistant cells, the majority of the clones will contain integrated copies of both H and L chain vectors and/or anti-TNF peptides.

Alternatively, the fused genes encoding the chimeric H and L chains can be assembled on the same expression vector.

For transfection of the expression vectors and production of the chimeric antibody, the preferred recipient cell line is a myeloma cell. Myeloma cells can synthesize, assemble and secrete immunoglobulins encoded by transfected immunoglobulin genes and possess the mechanism for glycosylation of the immunoglobulin. A particularly preferred recipient cell is the recombinant Ig-producing myeloma cell SP2/0 (ATCC #CRL 8287). SP2/0 cells produce only immunoglobulin encoded by the transfected genes. Myeloma cells can be grown in culture or in the peritoneal cavity of a mouse, where secreted immunoglobulin can be obtained from ascites fluid. Other suitable recipient cells include lymphoid cells such as B lymphocytes of human or non-human origin, hybridoma cells of human or non-human origin, or interspecies heterohybridoma cells.

The expression vector carrying a chimeric antibody construct or anti-TNF peptide of the present invention can be introduced into an appropriate host cell by any of a variety of suitable means, including such biochemical means as transformation, transfection, conjugation, protoplast fusion, calcium phosphate-precipitation, and application with polycations such as diethylaminoethyl (DEAE) dextran, and such mechanical means as electroporation, direct microinjection, and microprojectile bombardment (Johnston et al., *Science* 240:1538 (1988)). A preferred way of introducing DNA into lymphoid cells is by electroporation (Potter et al., *Proc. Natl. Acad. Sci. USA* 81:7161 (1984); Yoshikawa, et al., *Jpn. J. Cancer Res.* 77:1122-1133). In this procedure, recipient cells are subjected to an electric pulse in the presence of the DNA to be incorporated. Typically, after transfection, cells are allowed to recover in complete medium for about 24 hours, and are then seeded in 96-well culture plates in the presence of the selective medium. G418 selection is performed using about 0.4 to 0.8 mg/ml G418. Mycophenolic acid selection utilizes about 6 μg/ml plus about 0.25 mg/ml xanthine. The electroporation technique is expected to yield transfection frequencies of about 10^{-5} to about 10^{-4} for Sp2/0 cells. In the protoplast fusion method, lysozyme is used to strip cell walls from catarrhal harboring the recombinant plasmid containing the chimeric antibody gene. The resulting spheroplasts are fused with myeloma cells with polyethylene glycol.

The immunoglobulin genes of the present invention can also be expressed in nonlymphoid mammalian cells or in other eukaryotic cells, such as yeast, or in prokaryotic cells, in particular bacteria.

Yeast provides substantial advantages over bacteria for the production of immunoglobulin H and L chains. Yeasts carry out post-translational peptide modifications including glycosylation. A number of recombinant DNA strategies now exist which utilize strong promoter sequences and high copy number plasmids which can be used for production of the desired proteins in yeast. Yeast recognizes leader sequences of cloned mammalian gene products and secretes peptides bearing leader sequences (i.e., pre-peptides) (Hitzman, et al., 11*th International Conference on Yeast, Genetics and Molecular Biology*, Montpelier, France, Sep. 13-17, 1982).

Yeast gene expression systems can be routinely evaluated for the levels of production, secretion and the stability of anti-TNF peptides, antibody and assembled murine and chimeric antibodies, fragments and regions thereof. Any of a series of yeast gene expression systems incorporating promoter and termination elements from the actively expressed genes coding for glycolytic enzymes produced in large quantities when yeasts are grown in media rich in glucose can be utilized. Known glycolytic genes can also provide very efficient transcription control signals. For example, the promoter and terminator signals of the phosphoglycerate kinase (PGK) gene can be utilized. A number of approaches can be taken for evaluating optimal expression plasmids for the expression of cloned immunoglobulin cDNAs in yeast (see Glover, ed., *DNA Cloning, Vol. II*, pp45-66, IRL Press, 1985).

Bacterial strains can also be utilized as hosts for the production of antibody molecules or peptides described by this invention, *E. coli* K12 strains such as *E. coli* W3110 (ATCC 27325), and other enterobacteria such as *Salmonella typhimurium* or *Serratia marcescens*, and various *Pseudomonas* species can be used.

Plasmid vectors containing replicon and control sequences which are derived from species compatible with a host cell are used in connection with these bacterial hosts. The vector carries a replication site, as well as specific genes which are capable of providing phenotypic selection in transformed cells. A number of approaches can be taken for evaluating the expression plasmids for the production of murine and chimeric antibodies, fragments and regions or antibody chains encoded by the cloned immunoglobulin cDNAs in bacteria (see Glover, ed., *DNA Cloning, Vol. I,* IRL Press, 1985, Ausubel, infra, Sambrook, infra, Colligan, infra).

Preferred hosts are mammalian cells, grown in vitro or in vivo. Mammalian cells provide post-translational modifica-

tions to immunoglobulin protein molecules including leader peptide removal, folding and assembly of H and L chains, glycosylation of the antibody molecules, and secretion of functional antibody protein.

Mammalian cells which can be useful as hosts for the production of antibody proteins, in addition to the cells of lymphoid origin described above, include cells of fibroblast origin, such as Vero (ATCC CRL 81) or CHO-K1 (ATCC CRL 61).

Many vector systems are available for the expression of cloned anti-TNF peptides H and L chain genes in mammalian cells (see Glover, ed., *DNA Cloning, Vol. II*, pp143-238, IRL Press, 1985). Different approaches can be followed to obtain complete H_2L_2 antibodies. As discussed above, it is possible to co-express H and L chains in the same cells to achieve intracellular association and linkage of H and L chains into complete tetrameric H_2L_2 antibodies and/or anti-TNF peptides. The co-expression can occur by using either the same or different plasmids in the same host. Genes for both H and L chains and/or anti-TNF peptides can be placed into the same plasmid, which is then transfected into cells, thereby selecting directly for cells that express both chains. Alternatively, cells can be transfected first with a plasmid encoding one chain, for example the L chain, followed by transfection of the resulting cell line with an H chain plasmid containing a second selectable marker. Cell lines producing anti-TNF peptides and/or H_2L_2 molecules via either route could be transfected with plasmids encoding additional copies of peptides, H, L, or H plus L chains in conjunction with additional selectable markers to generate cell lines with enhanced properties, such as higher production of assembled H_2L_2 antibody molecules or enhanced stability of the transfected cell lines.

Anti-Idiotype Abs

In addition to monoclonal or chimeric anti-TNF antibodies, the present invention is also directed to an anti-idiotypic (anti-Id) antibody specific for the anti-TNF antibody of the invention. An anti-Id antibody is an antibody which recognizes unique determinants generally associated with the antigen-binding region of another antibody. The antibody specific for TNF is termed the idiotypic or Id antibody. The anti-Id can be prepared by immunizing an animal of the same species and genetic type (e.g. mouse strain) as the source of the Id antibody with the Id antibody or the antigen-binding region thereof. The immunized animal will recognize and respond to the idiotypic determinants of the immunizing antibody and produce an anti-Id antibody. The anti-Id antibody can also be used as an "immunogen" to induce an immune response in yet another animal, producing a so-called anti-anti-Id antibody. The anti-anti-Id can be epitopically identical to the original antibody which induced the anti-Id. Thus, by using antibodies to the idiotypic determinants of a mAb, it is possible to identify other clones expressing antibodies of identical specificity.

Accordingly, mAbs generated against TNF according to the present invention can be used to induce anti-Id antibodies in suitable animals, such as BALB/c mice. Spleen cells from such immunized mice can be used to produce anti-Id hybridomas secreting anti-Id mAbs. Further, the anti-Id mAbs can be coupled to a carrier such as keyhole limpet hemocyanin (KLH) and used to immunize additional BALB/c mice. Sera from these mice will contain anti-anti-Id antibodies that have the binding properties of the original mAb specific for a TNF epitope.

Screening Methods for determining tissue necrosis factor neutralizing and/or inhibiting activity are also provided in the present invention. In the context of the present invention, TNF neutralizing activity or TNF inhibiting activity refers to the ability of a TNF neutralizing compound to block at least one biological activity of TNF, such as preventing TNF from binding to a TNF receptor, blocking production of TNF by intracellular processing, such as transcription, translation or post-translational modification, expression on the cell surface, secretion or assembly of the bioactive trimer of TNF. Additionally, TNF neutralizing compounds can act by inducing regulation of metabolic pathways such as those involving the up or down regulation of TNF production. Alternatively TNF neutralizing compounds can modulate cellular sensitivity to TNF by decreasing such sensitivity. TNF neutralizing compounds can be selected from the group consisting of antibodies, or fragments or portions thereof, peptides, peptido mimetic compounds or organo mimetic compounds that neutralizes TNF activity in vitro, in situ or in vivo is considered a TNF neutralizing compound if used according to the present invention. Screening methods which can be used to determine TNF neutralizing activity of a TNF neutralizing compound can include in vitro or in vivo assays. Such in vitro assays can include a TNF cytotoxicity assay, such as a radioimmuno assay, which determines a decrease in cell death by contact with TNF, such as chimpanzee or human TNF in isolated or recombinant form, wherein the concurrent presence of a TNF neutralizing compound reduces the degree or rate of cell death. The cell death can be determined using ID50 values which represent the concentration of a TNF neutralizing compound which decreases the cell death rate by 50%. For example, MAb's A2 and cA2 are found to have ID50 about 17 mg/ml+/−3 mg/ml, such as 14-20 mg/ml, or any range or value therein. Such a TNF cytotoxicity assay is presented in Example II.

Alternatively or additionally, another in vitro assay which can be used to determine neutralizing activity of a TNF neutralizing compound is an assay which measures the neutralization of TNF induced procoagulant activity, such as presented in Example XI.

Alternatively or additionally, TNF neutralizing activity of a TNF neutralizing compound can be measured by an assay for the neutralization of TNF induced IL-6 secretion, such as using cultured human umbilical vein endothelial cells (HUVEC), for example. Also presented in Example XI.

Alternatively or additionally, in vivo testing of TNF neutralizing activity of TNF neutralizing compounds can be tested using survival of a mouse given lethal doses of Rh TNF with controlled and varied concentrations of a TNF neutralizing compound, such as TNF antibodies. Preferably galactosamine sensitive mice are used. For example, using a chimeric human anti-TNF antibody as a TNF neutralizing compound, a concentration of 0.4 milligrams per kilogram TNF antibody resulted in a 70-100% increase in survival and a 2.0 mg/kg dose of TNF antibody resulted in a 90-100% increase in survival rate using such an assay, for example, as presented in Example XVI.

Additionally, after TNF neutralizing compounds are tested for safety in animal models such as chimpanzees, for example as presented in Example XVII, TNF neutralizing compounds can be used to treat various TNF related pathologies, as described herein, and as presented in Examples XVIII-XXV.

Accordingly, any suitable TNF neutralizing compound can be used in methods according to the present invention. Examples of such TNF neutralizing compound can be selected from the group consisting of antibodies or portions thereof specific to neutralizing epitopes of TNF, p55 receptors, p75 receptors, or complexes thereof, portions of TNF

receptors which bind TNF, peptides which bind TNF, any peptido mimetic drugs which bind TNF and any organo mimetic drugs that block TNF.

Such TNF neutralizing compounds can be determined by routine experimentation based on the teachings and guidance presented herein, by those skilled in the relevant arts.

Structural Analogs of Anti-TNF Antibodies and Anti-TNF Peptides

Structural analogs of anti-TNF Abs and peptides of the present invention are provided by known method steps based on the teaching and guidance presented herein.

Knowledge of the three-dimensional structures of proteins is crucial in understanding how they function. The three-dimensional structures of more than 400 proteins are currently available in protein structure databases (in contrast to around 15,000 known protein sequences in sequence databases). Analysis of these structures shows that they fall into recognizable classes of motifs. It is thus possible to model a three-dimensional structure of a protein based on the protein's homology to a related protein of known structure. Many examples are known where two proteins that have relatively low sequence homology, can have very similar three dimensional structures or motifs.

In recent years it has become possible to determine the three dimensional structures of proteins of up to about 15 kDa by nuclear magnetic resonance (NMR). The technique only requires a concentrated solution of pure protein. No crystals or isomorphous derivatives are needed. The structures of a number of proteins have been determined by this method. The details of NMR structure determination are well-known in the art (See, e.g., Wuthrich, *NMR of Proteins and Nucleic Acids*, Wiley, New York, 1986; Wuthrich, K. *Science* 243:45-50 (1989); Clore et al., *Crit. Rev. Bioch. Molec. Biol.* 24:479-564 (1989); Cooke et al., *Bioassays* 8: 52-56 (1988), which references are hereby incorporated herein by reference).

In applying this approach, a variety of ^{1}H NMR 2D data sets are collected for anti-TNF Abs and/or anti-TNF peptides of the present invention. These are of two main types. One type, COSY (Correlated Spectroscopy) identifies proton resonances that are linked by chemical bonds. These spectra provide information on protons that are linked by three or less covalent bonds. NOESY (nuclear Overhauser enhancement spectroscopy) identifies protons which are close in space (less than 0.5 nm). Following assignment of the complete spin system, the secondary structure is defined by NOESY. Cross peaks (nuclear Overhauser effects or NOE's) are found between residues that are adjacent in the primary sequence of the peptide and can be seen for protons less than 0.5 nm apart. The data gathered from sequential NOE's combined with amide proton coupling constants and NOE's from non-adjacent amino acids that are adjacent to the secondary structure, are used to characterize the secondary structure of the polypeptides. Aside from predicting secondary structure, NOE's indicate the distance that protons are in space in both the primary amino acid sequence and the secondary structures. Tertiary structure predictions are determined, after all the data are considered, by a "best fit" extrapolation.

Types of amino acid are first identified using through-bond connectivities. The second step is to assign specific amino acids using through-space connectivities to neighboring residues, together with the known amino acid sequence. Structural information is then tabulated and is of three main kinds: The NOE identifies pairs of protons which are close in space, coupling constants give information on dihedral angles and slowly exchanging amide protons give information on the position of hydrogen bonds. The restraints are used to compute the structure using a distance geometry type of calculation followed by refinement using restrained molecular dynamics. The output of these computer programs is a family of structures which are compatible with the experimental data (i.e. the set of pairwise <0.5 nm distance restraints). The better that the structure is defined by the data, the better the family of structures can be superimposed, (i.e., the better the resolution of the structure). In the better defined structures using NMR, the position of much of the backbone (i.e. the amide, Cα and carbonyl atoms) and the side chains of those amino acids that lie buried in the core of the molecule can be defined as clearly as in structures obtained by crystallography. The side chains of amino acid residues exposed on the surface are frequently less well defined, however. This probably reflects the fact that these surface residues are more mobile and can have no fixed position. (In a crystal structure this might be seen as diffuse electron density).

Thus, according to the present invention, use of NMR spectroscopic data is combined with computer modeling to arrive at structural analogs of at least portions of anti-TNF Abs and peptides based on a structural understanding of the topography. Using this information, one of ordinary skill in the art will know how to achieve structural analogs of anti-TNF Abs and/or peptides, such as by rationally-based amino acid substitutions allowing the production of peptides in which the TNF binding affinity is modulated in accordance with the requirements of the expected therapeutic or diagnostic use of the molecule, preferably, the achievement of greater specificity for TNF binding.

Alternatively, compounds having the structural and chemical features suitable as anti-TNF therapeutics and diagnostics provide structural analogs with selective TNF affinity. Molecular modeling studies of TNF binding compounds, such as TNF receptors, anti-TNF antibodies, or other TNF binding molecules, using a program such as MACROMODEL®, INSIGHT®, and DISCOVER® provide such spatial requirements and orientation of the anti-TNF Abs and/or peptides according to the present invention. Such structural analogs of the present invention thus provide selective qualitative and quantitative anti-TNF activity in vitro, in situ and/or in vivo.

Therapeutic Methods for Treating TNF-Related Pathologies

The anti-TNF peptides, antibodies, fragments and/or derivatives of the present invention are useful for treating a subject having a pathology or condition associated with abnormal levels of a substance reactive with an anti-TNF antibody, in particular TNF, such as TNFα or TNFβ, in excess of levels present in a normal healthy subject, where such excess levels occur in a systemic, localized or particular tissue type or location in the body. Such tissue types can include, but are not limited to, blood, lymph, CNS, liver, kidney, spleen, heart muscle or blood vessels, brain or spinal cord white matter or grey matter, cartilage, ligaments, tendons, lung, pancreas, ovary, testes, prostate. Increased TNF concentrations relative to normal levels can also be localized to specific regions or cells in the body, such as joints, nerve blood vessel junctions, bones, specific tendons or ligaments, or sites of infection, such as bacterial or viral infections.

TNF related pathologies include, but are not limited to, the following:

(A) acute and chronic immune and autoimmune pathologies, such as systemic lupus erythematosus (SLE), rheuma-

toid arthritis, thyroidosis, graft versus host disease, scleroderma, diabetes mellitus, Graves' disease, Beschet's disease, and the like;

(B) infections, including, but not limited to, sepsis syndrome, cachexia, circulatory collapse and shock resulting from acute or chronic bacterial infection, acute and chronic parasitic and/or infectious diseases, bacterial, viral or fungal, such as a HIV, AIDS (including symptoms of cachexia, autoimmune disorders, AIDS dementia complex and infections);

(C) inflammatory diseases, such as chronic inflammatory pathologies and vascular inflammatory pathologies, including chronic inflammatory pathologies such as sarcoidosis, chronic inflammatory bowel disease, ulcerative colitis, and Crohn's pathology and vascular inflammatory pathologies, such as, but not limited to, disseminated intravascular coagulation, atherosclerosis, and Kawasaki's pathology:

(D) neurodegenerative diseases, including, but are not limited to, demyelinating diseases, such as multiple sclerosis and acute transverse myelitis; extrapyramidal and cerebellar disorders such as lesions of the corticospinal system; disorders of the basal ganglia or cerebellar disorders; hyperkinetic movement disorders such as Huntington's Chorea and senile chorea; drug-induced movement disorders, such as those induced by drugs which block CNS dopamine receptors; hypokinetic movement disorders, such as Parkinson's disease; Progressive supranucleo palsy; Cerebellar and Spinocerebellar Disorders, such as astructural lesions of the cerebellum; spinocerebellar degenerations (spinal ataxia, Friedreich's ataxia, cerebellar cortical degenerations, multiple systems degenerations (Mencel, Dejerine-Thomas, Shi-Drager, and MachadoJoseph)); and systemic disorders (Refsum's disease, abetalipoprotemia, ataxia, telangiectasia, and mitochondrial multi-system disorder); demyelinating core disorders, such as multiple sclerosis, acute transverse myelitis; disorders of the motor unit, such as neurogenic muscular atrophies (anterior horn cell degeneration, such as amyotrophic lateral sclerosis, infantile spinal muscular atrophy and juvenile spinal muscular atrophy); Alzheimer's disease; Down's Syndrome in middle age; Diffuse Lewy body disease; Senile Dementia of Lewy body type; Wemicke-Korsakoff syndrome; chronic alcoholism; Creutzfeldt-Jakob disease; Subacute sclerosing panencephalitis, Hallerrorden-Spatz disease; and Dementia pugilistica, or any subset thereof;

(E) malignant pathologies involving TNF-secreting tumors or other malignancies involving TNF, such as, but not limited to leukemias (acute, chronic myelocytic, chronic lymphocytic and/or myelodyspastic syndrome); lymphomas (Hodgkin's and non-Hodgkin's lymphomas, such as malignant lymphomas (Burkitt's lymphoma or Mycosis fungoides)); carcinomas (such as colon carcinoma) and metastases thereof; cancer-related angiogenesis; infantile haemangiomas;

(F) alcohol-induced hepatitis; and

(G) other diseases related to angiogenesis or VEGF/VPF, such as ocular neovascularization, psoriasis, duodenal ulcers, angiogenesis of the female reproductive tract.

See, e.g., Berkow et al., eds., *The Merck Manual,* 16th edition, chapter 11, pp 1380-1529, Merck and Co., Rahway, N.J., 1992, which reference, and references cited therein, are entirely incorporated herein by reference. See also Folkman, *Nature Medicine*, Volume 1, No. 1 (1995).

Such treatment comprises parenterally administering a single or multiple doses of the antibody, fragment or derivative. Preferred for human pharmaceutical use are high affinity potent hTNFα-inhibiting and/or neutralizing murine and chimeric antibodies, fragments and regions of this invention.

Anti-TNF peptides or MAbs of the present invention can be administered by any means that enables the active agent to reach the agent's site of action in the body of a mammal. In the case of the antibodies of this invention, the primary focus is the ability to reach and bind with TNF released by monocytes and macrophages or other TNF producing cells. Because proteins are subject to being digested when administered orally, parenteral administration, i.e., intravenous, subcutaneous, intramuscular, would ordinarily be used to optimize absorption.

Therapeutic Administration

Anti-TNF peptides and/or MAbs of the present invention can be administered either as individual therapeutic agents or in combination with other therapeutic agents. They can be administered alone, but are generally administered with a pharmaceutical carrier selected on the basis of the chosen route of administration and standard pharmaceutical practice.

The dosage administered will, of course, vary depending upon known factors such as the pharmacodynamic characteristics of the particular agent, and its mode and route of administration; age, health, and weight of the recipient; nature and extent of symptoms, kind of concurrent treatment, frequency of treatment, and the effect desired. Usually a daily dosage of active ingredient can be about 0.01 to 100 milligrams per kilogram of body weight. Ordinarily 1.0 to 5, and preferably 1 to 10 milligrams per kilogram per day given in divided doses 1 to 6 times a day or in sustained release form is effective to obtain desired results.

As a non-limiting example, treatment of TNF-related pathologies humans or animals can be provided as a daily dosage of anti-TNF peptides, monoclonal chimeric and/or murine antibodies of the present invention 0.1 to 100 mg/kg, such as 0.5, 0.9, 1.0, 1.1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 40, 45, 50, 60, 70, 80, 90 or 100 mg/kg, per day, on at least one of day 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40, or alternatively, at least one of week 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20, or any combination thereof, using single or divided doses of every 24, 12, 8, 6, 4, or 2 hours, or any combination thereof.

Since circulating concentrations of TNF tend to be extremely low, in the range of about 10 pg/ml in non-septic individuals, and reaching about 50 pg/ml in septic patients and above 100 pg/ml in the sepsis syndrome (Hammerle, A. F. et al., 1989, infra) or can be only be detectable at sites of TNF-mediated pathology, it is preferred to use high affinity and/or potent in vivo TNF-inhibiting and/or neutralizing antibodies, fragments or regions thereof, for both TNF immunoassays and therapy of TNP-mediated pathology. Such antibodies, fragments, or regions, will preferably have an affinity for hTNFα, expressed as Ka, of at least 10^8 M^{-1}, more preferably, at least 10^9 M^{-1}, such as 10^8-$10^{10}M^{-1}$, $5\times10^8M^{-1}$, $8\times10^8M^{-1}$, $2\times10^9M^{-1}$, $4\times10^9M^{-1}$, $6\times10^9M^{-1}$, $8\times10^9M^{-1}$, or any range or value therein.

Preferred for human therapeutic use are high affinity murine and chimeric antibodies, and fragments, regions and derivatives having potent in vivo TNFα-inhibiting and/or neutralizing activity, according to the present invention, that block TNF-induced IL-6 secretion. Also preferred for human therapeutic uses are such high affinity murine and chimeric anti-TNFα antibodies, and fragments, regions and

derivatives thereof, that block TNF-induced procoagulant activity, including blocking of TNF-induced expression of cell adhesion molecules such as ELAM-I and ICAM-I and blocking of TNF mitogenic activity, in vivo, in situ, and in vitro.

Dosage forms (composition) suitable for internal administration generally contain from about 0.1 milligram to about 500 milligrams of active ingredient per unit. In these pharmaceutical compositions the active ingredient will ordinarily be present in an amount of about 0.5-95% by weight based on the total weight of the composition.

For parenteral administration, anti-TNF peptides or antibodies can be formulated as a solution, suspension, emulsion or lyophilized powder in association with a pharmaceutically acceptable parenteral vehicle. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Liposomes and nonaqueous vehicles such as fixed oils can also be used. The vehicle or lyophilized powder can contain additives that maintain isotonicity (e.g., sodium chloride, mannitol) and chemical stability (e.g., buffers and preservatives). The formulation is sterilized by commonly used techniques.

Suitable pharmaceutical carriers are described in the most recent edition of Remington's Pharmaceutical Sciences, A. Osol, a standard reference text in this field of art.

For example, a parenteral composition suitable for administration by injection is prepared by dissolving 1.5% by weight of active ingredient in 0.9% sodium chloride solution.

Anti-TNF peptides and/or antibodies of this invention can be adapted for therapeutic efficacy by virtue of their ability to mediate antibody-dependent cellular cytotoxicity (ADCC) and/or complement-dependent cytotoxicity (CDC) against cells having TNF associated with their surface. For these activities, either an endogenous source or an exogenous source of effector cells (for ADCC) or complement components (for CDC) can be utilized. The murine and chimeric antibodies, fragments and regions of this invention, their fragments, and derivatives can be used therapeutically as immunoconjugates (see for review: Dillman, R. O., *Ann. Int. Med.* 111:592-603 (1989)). Such peptides or Abs can be coupled to cytotoxic proteins, including, but not limited to ricin-A, Pseudomonas toxin and Diphtheria toxin. Toxins conjugated to antibodies or other ligands or peptides are well known in the art (see, for example, Olsnes, S. et al., *Immunol. Today* 10:291-295 (1989)). Plant and bacterial toxins typically kill cells by disrupting the protein synthetic machinery.

Anti-TNF peptides and/or antibodies of this invention can be conjugated to additional types of therapeutic moieties including, but not limited to, radionuclides, therapeutic agents, cytotoxic agents and drugs. Examples of radionuclides which can be coupled to antibodies and delivered in vivo to sites of antigen include ^{212}Bi, ^{131}I, ^{186}Re, and ^{90}Y, which list is not intended to be exhaustive. The radionuclides exert their cytotoxic effect by locally irradiating the cells, leading to various intracellular lesions, as is known in the art of radiotherapy.

Cytotoxic drugs which can be conjugated to anti-TNF peptides and/or antibodies and subsequently used for in vivo therapy include, but are not limited to, daunorubicin, doxorubicin, methotrexate, and Mitomycin C. Cytotoxic drugs interfere with critical cellular processes including DNA, RNA, and protein synthesis. For a description of these classes of drugs which are well known in the art, and their mechanisms of action, see Goodman et al., *Goodman and Gilman's THE PHARAMACOLOGICAL BASIS OF THERAPEUTICS,* 8th Ed., Macmillan Publishing Co., 1990.

Anti-TNF peptides and/or antibodies of this invention can be advantageously utilized in combination with other monoclonal or murine and chimeric antibodies, fragments and regions, or with lymphokines or hemopoietic growth factors, etc., which serve to increase the number or activity of effector cells which interact with the antibodies.

Anti-TNF peptides and/or antibodies, fragments or derivatives of this invention can also be used in combination with TNF therapy to block undesired side effects of TNF. Recent approaches to cancer therapy have included direct administration of TNF to cancer patients or immunotherapy of cancer patients with lymphokine activated killer (LAK) cells (Rosenberg et al., *New Eng. J. Med.* 313:1485-1492 (1985)) or tumor infiltrating lymphocytes (TIL) (Kumick et al. (*Clin. Immunol. Immunopath.* 38:367-380 (1986); Kradin et al., *Cancer Immunol. Immunother.* 24:76-85 (1987); Kradin et al., *Transplant. Proc.* 20:336-338 (1988)). Trials are currently underway using modified LAK cells or TIL which have been transfected with the TNF gene to produce large amounts of TNF. Such therapeutic approaches are likely to be associated with a number of undesired side effects caused by the pleiotropic actions of TNF as described herein and known in the related arts. According to the present invention, these side effects can be reduced by concurrent treatment of a subject receiving TNF or cells producing large amounts of TIL with the antibodies, fragments or derivatives of the present invention. Effective doses are as described above. The dose level will require adjustment according to the dose of TNF or TNF-producing cells administered, in order to block side effects without blocking the main antitumor effect of TNF. One of ordinary skill in the art will know how to determine such doses without undue experimentation.

Treatment of Arthritis

In rheumatoid arthritis, the main presenting symptoms are pain, stiffness, swelling, and loss of function (Bennett J C. *The etiology of rheumatoid arthritis*. In Textbook of Rheumatology (Kelley W N, Harris E D, Ruddy S, Sledge C B, eds.) W B Saunders, Philadelphia pp 879-886, 1985). The multitude of drugs used in controlling such symptoms seems largely to reflect the fact that none is ideal. Although there have been many years of intense research into the biochemical, genetic, microbiological, and immunological aspects of rheumatoid arthritis, its pathogenesis is not completely understood, and none of the treatments clearly stop progression of joint destruction (Harris E D. *Rheumatoid Arthritis: The clinical spectrum*. In Textbook of Rheumatology (Kelley, et al., eds.) W B Saunders, Philadelphia pp 915-990, 1985).

TNFα is of major importance in the pathogenesis of rheumatoid arthritis. TNFα is present in rheumatoid arthritis joint tissues and synovial fluid at the protein and mRNA level (Buchan G, et al., *Clin. Exp. Immunol* 73: 449-455, 1988), indicating local synthesis. However detecting TNFα in rheumatoid arthritis joints even in quantities sufficient for bioactivation does not necessarily indicate that it is important in the pathogenesis of rheumatoid arthritis, nor that it is a good candidate therapeutic target. In order to address these questions, the effects of anti-TNF antibody and peptides (rabbit or monoclonal) on rheumatoid joint cell cultures, and for comparison, osteoarthritic cell cultures, have been studied. IL-1 production was abolished, showing TNFα as a suitable therapeutic target for the therapy of rheumatoid arthritis, since anti-TNFα blocks both TNF and IL-1, the

two cytokines known to be involved in cartilage and bone destruction (Breiman et al., *Lancet* 11:244-247, 1989).

Subsequent studies in rheumatoid arthritis tissues have supported this hypothesis. Anti-TNF Abs abrogated the production of another proinflammatory cytokine, GM-CSF (Haworth et al., *Bur. J. Immunol.* 21:2575-2579, 1991). This observation has been independently confirmed (Alvaro-Gracia et al, 1991). It has also been demonstrated that anti-TNF diminishes cell adhesion and HLA class II expression in rheumatoid arthritis joint cell cultures.

The administration of the antibody also established that VEGF/VPF serum levels were significantly decreased (FIG. **34**) in rheumatoid arthritis (RA) patients. A prominant feature of rheumatoid arthritis lesions is an infiltrate of inflammatory cells from the blood, together with invading pannus which is associated with prominent new blood formation, thus perpetuating the ingress of nutrients and cells, and the inflammatory reactions which culminate in bone and cartilage destruction. VEGF is a potent inducer to angiogenesis and has been implicated in the formation of blood vessels and activation of microvascular endothelium in RA.

VEGF serum levels are significantly increased in patients with active RA, relative to serum levels in a population of age-matched controls. Furthermore, a time- and dose-dependent decrease in serum VEGF levels in RA patients after infusion with anti-TNFα, cA2, was observed. In patients who received 1 mg/kg cA2 (n=24), the maximal effect (33% decrease, $p<0.001$ versus change in placebo group and versus pre-infusion levels) was detected 2 weeks post-infusion, but subsequently VEGF levels returned to pre-infusion levels. In contrast, patients who received 10 mg/kg cA2 (n=21), the reduction was maintained even 4 weeks post-infusion (32% decrease, $p<0.001$), although serum levels were still higher than in normal individuals. The data indicate that treatment of RA or other VEGF-mediated diseases with TNF antagonists decreases VEGF levels in vivo, thereby leading to reduction in the vascularity of the pannus. Also, since VEGF is a survival factor for newly formed blood vessels and endothelial cell apoptosis, as a consequence of suppression of VEGF secretion, leads to vessel regression, TNF antagonists can exert a beneficial effect by causing regression of existing blood vessels in the arthritic pannus.

Diagnostic Methods

The present invention also provides the above anti-TNF peptides and antibodies, detectably labeled, as described below, for use in diagnostic methods for detecting TNFα in patients known to be or suspected of having a TNFα-mediated condition.

Anti-TNF peptides and/or antibodies of the present invention are useful for immunoassays which detect or quantitate TNF, or anti-TNF antibodies, in a sample. An immunoassay for TNF typically comprises incubating a biological sample in the presence of a detectably labeled high affinity anti-TNF peptide and/or antibody of the present invention capable of selectively binding to TNF, and detecting the labeled peptide or antibody which is bound in a sample. Various clinical assay procedures are well known in the art, e.g., as described in *Immunoassays for the* 80*'s, A*. Voller et al., eds., University Park, 1981.

Thus, an anti-TNF peptide or antibody can be added to nitrocellulose, or another solid support which is capable of immobilizing cells, cell particles or soluble proteins. The support can then be washed with suitable buffers followed by treatment with the detectably labeled TNF-specific peptide or antibody. The solid phase support can then be washed with the buffer a second time to remove unbound peptide or antibody. The amount of bound label on the solid support can then be detected by known method steps.

By "solid phase support" or "carrier" is intended any support capable of binding peptide, antigen or antibody. Well-known supports or carriers, include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material can have virtually any possible structural configuration so long as the coupled molecule is capable of binding to TNF or an anti-TNF antibody. Thus, the support configuration can be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface can be flat, such as a sheet, culture dish, test strip, etc. Preferred supports include polystyrene beads. Those skilled in the art will know many other suitable carriers for binding antibody, peptide or antigen, or can ascertain the same by routine experimentation.

Well known method steps can determine binding activity of a given lot of anti-TNF peptide and/or antibody. Those skilled in the art can determine operative and optimal assay conditions by routine experimentation.

Detectably labeling a TNF-specific peptide and/or antibody can be accomplished by linking to an enzyme for use in an enzyme immunoassay (EIA), or enzyme-linked immunosorbent assay (ELISA). The linked enzyme reacts with the exposed substrate to generate a chemical moiety which can be detected, for example, by spectrophotometric, fluorometric or by visual means. Enzymes which can be used to detectably label the TNF-specific antibodies of the present invention include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase.

By radioactively labeling the TNF-specific antibodies, it is possible to detect TNF through the use of a radioimmunoassay (RIA) (see, for example, Work, et al., *Laboratory Techniques and Biochemistry in Molecular Biology*, North Holland Publishing Company, N.Y. (1978)). The radioactive isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography. Isotopes which are particularly useful for the purpose of the present invention are: ^{3}H, ^{125}I, ^{131}I, ^{35}S, ^{14}C, and, preferably, ^{125}I.

It is also possible to label the TNF-specific antibodies with a fluorescent compound. When the fluorescent labeled antibody is exposed to light of the proper wave length, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labelling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

The TNF-specific antibodies can also be detectably labeled using fluorescence-emitting metals such as ^{125}Eu, or others of the lanthamide series. These metals can be attached to the TNF-specific antibody using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) or ethylenediamine-tetraacetic acid (EDTA).

The TNF-specific antibodies also can be detectably labeled by coupling to a chemiluminescent compound. The presence of the chemiluminescently labeled antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound can be used to label the TNF-specific antibody, fragment or derivative of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

Detection of the TNF-specific antibody, fragment or derivative can be accomplished by a scintillation counter, for example, if the detectable label is a radioactive gamma emitter, or by a fluorometer, for example, if the label is a fluorescent material. In the case of an enzyme label, the detection can be accomplished by colorometric methods which employ a substrate for the enzyme. Detection can also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

For the purposes of the present invention, the TNF which is detected by the above assays can be present in a biological sample. Any sample containing TNF can be used. Preferably, the sample is a biological fluid such as, for example, blood, serum, lymph, urine, inflammatory exudate, cerebrospinal fluid, amniotic fluid, a tissue extract or homogenate, and the like. However, the invention is not limited to assays using only these samples, it being possible for one of ordinary skill in the art to determine suitable conditions which allow the use of other samples.

In situ detection can be accomplished by removing a histological specimen from a patient, and providing the combination of labeled antibodies of the present invention to such a specimen. The antibody (or fragment) is preferably provided by applying or by overlaying the labeled antibody (or fragment) to a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of TNF but also the distribution of TNF in the examined tissue. Using the present invention, those of ordinary skill will readily perceive that any of a wide variety of histological methods (such as staining procedures) can be modified in order to achieve such in situ detection.

The antibody, fragment or derivative of the present invention can be adapted for utilization in an immunometric assay, also known as a "two-site" or "sandwich" assay. In a typical immunometric assay, a quantity of unlabeled antibody (or fragment of antibody) is bound to a solid support that is insoluble in the fluid being tested and a quantity of detectably labeled soluble antibody is added to permit detection and/or quantitation of the ternary complex formed between solid-phase antibody, antigen, and labeled antibody.

Typical, and preferred, immunometric assays include "forward" assays in which the antibody bound to the solid phase is first contacted with the sample being tested to extract the TNF from the sample by formation of a binary solid phase antibody-TNF complex. After a suitable incubation period, the solid support is washed to remove the residue of the fluid sample, including unreacted TNF, if any, and then contacted with the solution containing a known quantity of labeled antibody (which functions as a "reporter molecule"). After a second incubation period to permit the labeled antibody to complex with the TNF bound to the solid support through the unlabeled antibody, the solid support is washed a second time to remove the unreacted labeled antibody. This type of forward sandwich assay can be a simple "yes/no" assay to determine whether TNF is present or can be made quantitative by comparing the measure of labeled antibody with that obtained for a standard sample containing known quantities of TNF. Such "two-site" or "sandwich" assays are described by Wide (*Radioimmune Assay Method*, Kirkham, ed., Livingstone, Edinburgh, 1970, pp. 199-206).

Other type of "sandwich" assays, which can also be useful with TNF, are the so-called "simultaneous" and "reverse" assays. A simultaneous assay involves a single incubation step wherein the antibody bound to the solid support and labeled antibody are both added to the sample being tested at the same time. After the incubation is completed, the solid support is washed to remove the residue of fluid sample and uncomplexed labeled antibody. The presence of labeled antibody associated with the solid support is then determined as it would be in a conventional "forward" sandwich assay.

In the "reverse" assay, stepwise addition first of a solution of labeled antibody to the fluid sample followed by the addition of unlabeled antibody bound to a solid support after a suitable incubation period, is utilized. After a second incubation, the solid phase is washed in conventional fashion to free it of the residue of the sample being tested and the solution of unreacted labeled antibody. The determination of labeled antibody associated with a solid support is then determined as in the "simultaneous" and "forward" assays. In one embodiment, a combination of antibodies of the present invention specific for separate epitopes can be used to construct a sensitive three-site immunoradiometric assay.

TNF Removal from Solutions

The murine and chimeric antibodies, fragments and regions, fragments, or derivatives of this invention, attached to a solid support, can be used to remove TNF from fluids or tissue or cell extracts. In a preferred embodiment, they are used to remove TNF from blood or blood plasma products. In another preferred embodiment, the murine and chimeric antibodies, fragments and regions are advantageously used in extracorporeal immunoadsorbent devices, which are known in the art (see, for example, *Seminars in Hematology,* 26 (2 *Suppl.* 1) (1989)). Patient blood or other body fluid is exposed to the attached antibody, resulting in partial or complete removal of circulating TNF (free or in immune complexes), following which the fluid is returned to the body. This immunoadsorption can be implemented in a continuous flow arrangement, with or without interposing a cell centrifugation step. See, for example, Terman, et al., *J. Immunol.* 117:1971-1975 (1976).

Having now generally described the invention, the same will be further understood by reference to certain specific examples which are included herein for purposes of illustration only and are not intended to be limiting unless otherwise specified.

EXAMPLE I

Production a Mouse Anti-Human TNF mAb

To facilitate clinical study of TNF mAb, a high-affinity potent inhibiting and/or neutralizing mouse anti-human TNF IgG1 mAb designated A2 was produced.

Female BALB/c mice, 10 weeks old, were obtained from the Jackson Laboratory (Bar Harbor, Me.). Forty μg of purified *E. coli*-derived recombinant human TNF (rhTNF) emulsified with an equal volume of complete Freund's adjuvant (obtained from Difco Laboratories) in 0.4 ml was injected subcutaneously and intraperitoneally (i.p.) into a mouse. One week later, an injection of 5 μg of rhTNF in incomplete Freund's adjuvant was given i.p. followed by four consecutive i.p. injections of 10 μg of TNF without adjuvant. Eight weeks after the last injection, the mouse was boosted i.p. with 10 μg of TNF.

Four days later, the mouse was sacrificed, the spleen was obtained and a spleen cell suspension was prepared. Spleen cells were fused with cells of the nonsecreting hybridoma, Sp2/0 (ATCC CRL1581), at a 4:1 ratio of spleen cells to Sp2/0 cells, in the presence of 0.3 ml of 30% polyethylene glycol, PEG 1450. After incubation at 37° C. for 6 hours, the fused cells were distributed in 0.2 ml aliquots into 96-well plates at concentrations of 2×10^4 SP2/0 cells per well. Feeder cells, in the form of 5×10^4 normal BALB/c spleen cells, were added to each well.

The growth medium used consisted of RPM1-1640 medium, 10% heat-inactivated fetal bovine serum (FBS) (HYCLONE), 0.1 mM minimum essential medium (MEM) nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (GIBCO Laboratories) and, for selection, hypoxanthine-aminopterin-thymidine (HAT) (Boehringer Mannheim). A solid-phase radioimmunoassay (RIA) was employed for screening supernatants for the presence of mAbs specific for rhTNFα. This assay is described in Example II, below. The background binding in this assay was about 500 cpm. A supernatant was considered positive if it yielded binding of 2000 cpm or higher.

Of 322 supernatants screened, 25 were positive by RIA. Of these 25, the one with the highest binding (4800 cpm) was designated A2. Positive wells were subcloned at limiting dilution on mouse feeder cells. Upon further analysis of the supernatants in neutralization assays, A2 was found to be the only positive clone showing potent inhibiting and/or neutralizing activity. Thus, the hybridoma line A2 was selected. This line was maintained in RPM1-1640 medium with 10% FBS (GIBCO), 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

Alternatively, anti-TNF antibodies which inhibit TNF biological activity can be screened by binding to peptide including at least 5 amino acids of residues 87-108 or both residues 59-80 and 87-108 of TNF (of SEQ ID NO: 1) or combinations of peptides contained therein, which are used in place of the rTNF protein, as described above.

EXAMPLE II

Characterization of an Anti-TNF Antibody of the Present Invention Radioimmunoassays

E. coli-derived rhTNF was diluted to 1 μg/ml in BCB buffer, pH 9.6, and 0.1 ml of the solution was added to each assay well. After incubation at 4° C. overnight, the wells were washed briefly with BCB, then sealed with 1% bovine incubated with 40 pg/ml of natural (GENZYME, Boston, Mass.) or recombinant (SUNTORY, Osaka, Japan) human TNFα with varying concentrations of mAb A2 in the presence of 20 μg/ml cycloheximide at 39° C. overnight. Controls included medium alone or medium+TNF in each well. Cell death was measured by staining with naphthol blue-black, and the results read spectrophotometrically at 630 nm. Absorbance at this wave length correlates with the number of live cells present.

It was found that A2 inhibited or neutralized the cytotoxic effect of both natural and rhTNF in a dose-dependent manner (FIG. **3**).

In another experiment, the specificity of this inhibiting and/or neutralizing activity was tested. A673/6 cells were seeded at 3×10^4 cells/well 20 hr before the TNF bioassay. Two-fold serial dilutions of rhTNF, *E. coli*-derived recombinant human lymphotoxin (TNFβ), and *E. coli*-derived recombinant murine TNF were prepared. The A2 hybridoma supernatant was added to an equal volume of the diluted TNF preparations, and the mixtures were incubated at room temperature for 30 min. Aliquots of 0.1 ml were transferred to the wells containing A673/6 cells, 20 μg/ml of cycloheximide was added, and the cells were incubated at 39° C. overnight. The cells were then fixed and stained for evaluation of cytotoxicity. The results indicate that mAb A2 specifically inhibited or neutralized the cytotoxicity of rhTNFα, whereas it had no effect on human lymphotoxin (TNFβ) (FIG. **4**) or murine TNF (FIG. **5**).

Experiments were next performed to analyze the cross-reactivity of mAb A2 with TNF derived from non-human primates. Monocytes isolated from B514 (baboon), J91 (cynomolgus) and RH383 (rhesus) blood by Ficoll gradient centrifugation and adherence, were incubated at 1×10^5 cells/well in RPMi 1640 medium with 5% FBS and 2 μg/ml of *E. coli* LPS for 3 or 16 hr at 37° C. to induce TNF production. Supernatants from duplicate wells were pooled and stored at 4° C. for less than 20 hr until the TNF bioassay was performed, as described above, using A673/6 cells. Two-fold dilutions of the culture supernatants were mixed with either medium or purified mAb A2 at a final concentration of 1 μg/ml, incubated at room temperature for 30 min. and aliquots transferred to the indicator cells. The results showed that mAb A2 failed to significantly inhibit or neutralize the cytotoxic activity of TNF produced by baboon, cynomolgus and rhesus monkey monocytes.

A further experiment was conducted with chimpanzee TNF. Monocytes isolated from CH563 (chimpanzee) blood were incubated as described above to generate TNF-containing supernatants. The ability of 10 μg/ml of mAb A2 to inhibit or neutralize the bioactivity of these supernatants was assayed as above. Human TNF was used as a positive control. Results, shown in FIG. **6**, indicate that mAb A2 had potent inhibiting and/or neutralizing activity for chimpanzee TNF, similar to that for human TNF (FIG. **7**).

The inhibiting and/or neutralizing activity of mAb A2 was compared with three other murine mAbs specific for human TNF, termed TNF-1, TNF-2 and TNF-3, and a control mAb. Two-fold serial dilutions of purified mAbs were mixed with rhTNF (40 pg/ml), incubated at room temperature for 30 min, and aliquots tested for TNF bioactivity as above. It was found that mAbs TNF-1, TNF-2 and TNF-3 each had a similar moderate degree of inhibiting and/or neutralizing activity. In contrast, mAb A2 had much more potent inhibiting and/or neutralizing activity.

EXAMPLE III

General Strategy for Cloning Antibody V and C Genes

The strategy for cloning the V regions for the H and L chain genes from the hybridoma A2, which secretes the anti-TNF antibody described above, was based upon the

linkage in the genome between the V region and the corresponding J (joining) region for functionally rearranged (and expressed) Ig genes. J region DNA probes can be used to screen genomic libraries to isolate DNA linked to the J regions. Although DNA in the germline configuration (i.e., unrearranged) would also hybridize to J probes, this DNA would not be linked to a Ig V region sequence and can be identified by restriction enzyme analysis of the isolated clones.

The cloning utilized herein was to isolate V regions from rearranged H and L chain genes using J_H and J_K probes. These clones were tested to see if their sequences were expressed in the A2 hybridoma by Northern analysis. Those clones that contained expressed sequence were cloned into expression vectors containing human C regions and transfected into mouse myeloma cells to determine if an antibody was produced. The antibody from producing cells was then tested for binding specificity and functionally compared to the A2 murine antibody.

EXAMPLE IV

Construction of a L Chain Genomic Library

To isolate the L chain V region gene from the A2 hybridoma, a size-selected genomic library was constructed using the phage lambda vector charon 27. High molecular weight DNA was isolated from A2 hybridoma cells and digested to completion with restriction endonuclease HindIII. The DNA was then fractionated on a 0.8% agarose gel and the DNA fragments of three different size ranges of approximately 3 kb, 4 kb and 6 kb were isolated from the gel by electroelution. The size ranges for library construction were chosen based upon the size of HindIII fragments that hybridized on a southern blot with the J_K probe. After phenol/chloroform extraction and ethanol precipitation, the DNA fragments from each size class were ligated with lambda charon 27 arms and packaged into phage particles in vitro using Gigapack Gold from Stratagene (LaJolla, Calif.).

These libraries were screened directly at a density of approximately 20,000 plaques per 150 mm petri dish using a ^{32}P-labeled J_K probe. The mouse L chain J_K probe was a 2.7 kb HindIII fragment containing all five J_K segments. The probe was labeled with ^{32}P by random priming using a kit obtained from Boehringer Mannheim. Free nucleotides were removed by centrifugation through a Sephadex G-SO column. The specific activities of the probe was approximately 10^9 cpm/μg.

Plaque hybridizations were carried out in 5×SSC, 50% formamide, 2× Denhardt's reagent, and 200 μg/ml denatured salmon sperm DNA at 42° C. for 18-20 hours. Final washes were in 0.5×SSC, 0.1% SDS at 65° C. Positive clones were identified after autoradiography.

EXAMPLE V

Construction of H Chain Genomic Library

To isolate the V region gene for the A2H chain, a genomic library was constructed in the lambda gt10 vector system. High molecular weight DNA was digested to completion with restriction endonuclease EcoRI and fragments of approximately 7.5 kb were isolated after agarose gel electrophoresis. These fragments were ligated with lambda gt10 arms and packaged into phage particles in vitro using Gigapack Gold.

This library was screened at a density of 20,000 plaques per 150 mm plate using a J_H probe. The J_H probe was a 2 kb BamHI/EcoRI fragment containing both J3 and J4 segments. The probe was labeled as in Example III and had a similar specific radioactivity. Hybridization and wash conditions were identical to those used in Example III.

EXAMPLE VI

Cloning of the TNF-Specific V Gene Regions

Several positive clones were isolated from the H and L chain libraries after screening approximately 10^6 plaques from each library using the J_H and J_K probes, respectively. Following plaque purification, bacteriophage DNA was isolated for each positive clone, digested with either EcoRI (H chain clones) or HindIII (L chain clones) and fractionated on 1% agarose gels. The DNA was transferred to nitrocellulose and the blots were hybridized with the J_H or the J_K probe.

Several H chain clones were obtained that contained 7.5 k/D EcoRI DNA encoding fragments of MAbs to the J_H probe. For the light chain libraries, several clones from each of the three size-selected libraries were isolated that contained HindIII fragments that hybridize to the J_K probe. For the L chain, several independently derived HindIII fragments of 2.9 kb from the 2 kb library hybridized with a 1250 bp mRNA from A2, but not with SP2/0 mRNA (see Example VII). In addition, several HindIII fragments derived from the 4 kb library hybridized both to the A2 mRNA and the fusion partner mRNA. A 5.7 kb HindIII fragment from the 6 kb library did not hybridize to either RNA.

The observed lengths of hybridizing A2 mRNA were the correct sizes for H and L chain mRNA, respectively. Because the RNA expression was restricted to the A2 hybridoma, it was assumed that the 7.5 kb H chain fragments and the 2.9 kb L chain fragments contained the correct V region sequences from A2. One example of each type was chosen for further study. The important functional test is the demonstration that these V regions sequences, when combined with appropriate C region sequences, are capable of directing the synthesis of an antibody with a specificity and affinity similar to that of the murine A2 antibody.

The 7.5 kb H chain fragment and the 2.9 kb L chain fragment were subcloned into plasmid vectors that allow expression of the chimeric mouse/human proteins in murine myeloma cells (see Examples VIII and IX). These plasmids were co-transfected into SP2/0 cells to ascertain if intact antibody molecules were secreted, and if so, if they were of the correct specificity and affinity. Control transfections were also performed pairing the putative anti-TNF H chain with an irrelevant, but expressed, L chain; the putative anti-TNF L chain was also paired with an irrelevant, but expressed, H chain. The results indicated that the 7.5 kb H chain fragment could be expressed, whereas the 2.9 kb L chain fragment could not. This was confirmed by DNA sequence analysis that suggested portions of the coding region were not in the proper amino acid reading frame when compared to other known L chain amino acid sequences.

Because the 2.9 kb HindIII fragment appeared not to contain a functional V gene, the 4.0 kb and 5.7 kb HindIII fragments isolated from L chain libraries were cloned into expression vectors and tested for expression of chimeric antibody after co-transfection with the 7.5 kb H chain. The 5.7 kb HindIII fragment was incapable of supporting antibody expression, whereas the 4.0 kb HindIII fragment did support antibody expression. The antibody resulting from the co-transfection of the 7.5 kb putative H chain V region and the 4.0 kb L chain V region was purified, tested in solid phase TNF binding assay, and found to be inactive. It was concluded that the V region contained on the 4.0 kb HindIII fragment was not the correct anti-TNF V region, but was contributed to the hybridoma by the fusion partner. This was

subsequently confirmed by sequence analysis of cDNA derived from the A2 hybridoma and from the fusion partner.

Other independently derived L chain clones containing 2.9 kb HindIII fragments that hybridized with A2 mRNA were characterized in more detail. Although the restriction maps were similar, the clones fell into two classes with respect to the presence or absence of an AccI enzyme site. The original (non-functional) 2.9 kb fragment (designated clone 8.3) was missing an AccI site present in some other clones (represented by clone 4.3). The DNA sequence of clone 4.3 was extremely similar to clone 8.3, but contained a single amino acid reading frame with close homology to known L chains, unlike clone 8.3. The 2.9 kb HindIII fragment from clone 4.3 was subcloned into the L chain expression vector and co-transfected with the putative anti-TNF H chain into SP2/0 cells. An antibody was synthesized, purified and tested in the solid phase TNF binding assay. This antibody bound to TNF, and therefore, the clone 4.3 L chain V region was assumed to be the correct one.

The A2 murine hybridoma has been shown to contain at least four rearranged L chain V region genes. At least two of these are expressed as proteins: clone 4.3 (the correct anti-TNF L chain gene) and the gene contained in the 4.0 kb HindIII fragment (contributed by the fusion partner). The expression of two L chains implies that the resulting antibody secreted from the murine hybridoma is actually a mixture of antibodies, some using the correct L chain, some using the incorrect L chain, and some using one of each. The presence of two different L chains in the murine A2 antibody has been confirmed by SDS gel and N-terminal protein sequence analysis of the purified antibody. Because construction of the chimeric A2 antibody involves cloning the individual H and L chain genes and expressing them in a non-producing cell line, the resulting antibody will have only the correct L chain and therefore should be a more potent antibody (see Examples X, XI and XII).

EXAMPLE VII

Northern Analysis of Cloned DNA

Cloned DNA corresponding to the authentic H and L chain V regions from the A2 hybridoma would be expected to hybridize to A2 mRNA. Non-functional DNA rearrangements at either the H or L chain genetic loci should not be expressed.

Ten μg total cellular RNA was subjected to electrophoresis on 1% agarose/formaldehyde gels (Sambrook et al., infra) and transferred to nitrocellulose. Blots were hybridized with random primed DNA probes in 50% formamide, 2× Denhardt's solution, 5×SSC, and 200 μg/ml denatured salmon sperm DNA at 42° C. for 10 hours. Final wash conditions were 0.5×SSC, 0.1% SDS at 65° C.

The subcloned DNA fragments were labeled with ^{32}P by random priming and hybridized to Northern blots containing total RNA derived from A2 cells or from cells of SP2/0, the fusion partner parent of A2. The 7.5 kb EcoRI H chain fragment hybridized with a 2 kb mRNA from A2, but not with SP2/0 mRNA. Similarly, the 2.9 kb L chain HindIII fragment (clone 4.3) hybridized with a 1250 bp mRNA from A2, but not with SP2/0 mRNA. The observed lengths of A2 mRNA hybridizing were the correct sizes for H and L chain mRNA, respectively, confirming that the V region sequences on these DNA fragments are expressed in A2 hybridoma cells.

EXAMPLE VIII

Construction of Expression Vectors

The putative L (clone 4.3) and H chain V genes described above were joined to human kappa and gamma1 constant region genes in expression vectors. The 7.5 kb EcoRI fragment corresponding to the putative V_H region gene from A2 was cloned into an expression vector containing the human C_{gamma1} gene and the Ecogpt gene to yield the plasmid designated pA2HGlapgpt (see FIG. **8**).

The 2.9 kb putative VL fragment from clone 4.3 was cloned into a vector containing the human kappa C_k gene and the Ecogpt gene to allow selection in mammalian cells. The resulting plasmid was designated pA2HuKapgpt (See FIG. **8**).

EXAMPLE IX

Expression of Chimeric Antibody Genes

To express the chimeric H and L chain genes, the expression plasmids were transfected into cells of the non-producing mouse myeloma cell line, SP2/0. Plasmid DNA to be transfected was purified by centrifuging to equilibrium in ethidium bromide/cesium chloride gradients twice. Plasmid DNA (10-50 μg) was added to 10^7 SP2/0 cells in medium containing Hank's salts, and the mixture was placed in a BIORAD electroporation apparatus. Electroporation was performed at 20 volts, following which the cells were plated in 96 well microtiter plates.

Mycophenolic acid selection was applied after 24 hours and drug resistant colonies were identified after 1-2 weeks. Resistant colonies were expanded to stable cell lines and tissue culture supernatant from these cell lines was tested for antibody using an ELISA assay with goat anti-human IgG Fc antibody and goat anti-human H+L conjugated with alkaline phosphatase (obtained from Jackson Laboratories).

The chimeric A2 antibody was purified from tissue culture supernatant by Protein A-Sepharose chromatography. The supernatant was adjusted to 0.1M Tris, 0.002M EDTA, pH 8.0 and loaded on a Protein A-Sepharose column equilibrated in the same buffer. The IgG was eluted with 0.1M citrate, pH 3.5, inhibited or neutralized with 1M Tris, and dialyzed into phosphate buffered saline (PBS).

The purified chimeric antibody was evaluated for its binding and inhibiting and/or neutralizing activity.

EXAMPLE X

Specificity of an Anti-TNF Chimeric Antibody

Since the antigen binding domain of cA2 was derived from murine A2, these mAbs would be expected to compete for the same binding site on TNF. Fixed concentrations of chimeric A2 and murine mAb A2 were incubated with increasing concentrations of murine and chimeric A2 competitor, respectively, in a 96-well microtiter plate coated with rhTNF (Dainippon, Osaka, Japan). Alkaline-phosphatase conjugated anti-human immunoglobulin and anti-mouse immunoglobulin second antibodies were used to detect the level of binding of chimeric and murine A2, respectively. Cross-competition for TNF antigen was observed in this solid-phase ELISA format (FIGS. **9**A and **9**B). This finding is consistent with the expected identical epitope specificity of cA2 and murine A2.

The affinity constant for binding of mouse mAb A2 and cA2 to rhTNFα was determined by Scatchard analysis (see, for example, Scatchard, *Ann. N.Y. Acad. Sci.* 51:660 (1949)).

The results are shown in FIG. **10**. This analysis involved measuring the direct binding of ^{125}I labelled cA2 to immobilized rhTNFα in a 96-well plate. The antibodies were each labelled to a specific activity of about 9.7 μCi/μg by the iodogen method. An affinity constant (Ka) of 0.5×10^9 liters/mole was calculated for the mouse mAb A2. Unexpectedly, the chimeric A2 antibody had a higher affinity, with a Ka of 1.8×10^9 liters/mole. Thus, the chimeric anti-TNFα antibody of the present invention was shown to exhibit a significantly higher affinity of binding to human TNFα than did the parental murine A2 mAb. This finding was surprising, since murine and chimeric antibodies, fragments and regions would be expected to have affinities that are equal to or less than that of the parent mAb.

Such high affinity anti-TNF antibodies, having affinities of binding to TNFα of at least 1×10^8 M^{-1}, more preferably at least 1×10^9 M^{-1} (expressed as Ka) are preferred for immunoassays which detect very low levels of TNF in biological fluids. In addition, anti-TNF antibodies having such high affinities are preferred for therapy of TNF-α-mediated conditions or pathology states.

The specificity of cA2 for TNF was confirmed by testing for cross-neutralization of human lymphotoxin (TNF-β). Lymphotoxin shares some sequence homology and certain biological activities, for example, tumor cell cytotoxicity, with TNF (Pennica, et al., *Nature* 312:724-729 (1984)). Cultured human A673 cells were incubated with increasing concentrations of human lymphotoxin (GENENTECH, San Francisco, Calif.) with or without 4 μg/ml chimeric A2 in the presence of 20 μg/ml cycloheximide at 39 C overnight. Cell death was measured by vital staining with naphthol blue-black, as above. The results indicated that cA2 was ineffective at inhibiting and/or neutralizing human lymphotoxin, confirming the TNFα-specificity of the chimeric antibody.

The ability of A2 or cA2 to react with TNF from different animal species was also evaluated. As mentioned earlier, there are multiple epitopes on human TNF to which inhibiting and/or neutralizing mAbs will bind (Moller, et al., infra). Human TNF has bioactivity in a wide range of host animal species. However, certain inhibiting and/or neutralizing epitopes on human TNF are conserved amongst different animal species and others appear to be restricted to humans and chimpanzees.

Neutralization experiments utilized endotoxin-activated cell supernatants from freshly isolated human, chimpanzee, rhesus and cynomolgus monkey, baboon, pig, dog, rabbit, or rat monocytes as the TNF source. As discussed above, murine mAb A2 inhibited or neutralized activity of only human and chimpanzee TNF, and had no effect on TNF derived from other primates and lower animals. A2 also did not inhibit or neutralize the cytotoxic effect of recombinant mouse TNF.

Thus, the epitope recognized by A2 is one shared by human and chimpanzee TNFα. Chimeric A2 was also tested in this manner for cross-reactivity with monocyte-derived TNF from rat, rabbit, dog and pig, as well as with purified recombinant mouse TNFα, and natural and recombinant human TNFα. Chimeric A2 only inhibited or neutralized natural and recombinant human TNFα. Therefore, cA2 appears to share species specificity with murine A2.

EXAMPLE XI

In Vitro Activity and Neutralization Efficacy of a Chimeric Anti-TNF Antibody

Both the murine and chimeric anti-TNFα antibodies, A2 and cA2 were determined to have potent TNF-inhibiting and/or neutralizing activity. In the TNF cytotoxicity assay described above, murine A2, at a concentration of about 125 ng/ml completely inhibited or neutralized the biological activity of a 40 pg/ml challenge of rhTNFα. Two separate determinations of inhibiting and/or neutralizing potency, expressed as the 50% Inhibitory Dose (ID50) were determined to be 15.9±1.01 and 17.9±1.6 ng/ml (Mean+Std error). Thus the mAb A2 has an ID50 of about 17 ng/ml.

In this same experimental system, three other murine anti-TNFα antibodies (termed TNF-1, TNF-2 and TNF-3) of comparable binding affinity to TNF were found to have ID50 values of 1-2 orders of magnitude greater, and thus were significantly less potent in neutralization than A2.

The ability of cA2 to inhibit or neutralize human TNFα bioactivity in vitro was tested using the bioassay system described above. Cultured A673 cells were incubated with 40 pg/ml natural (Genzyme, Boston, Mass.) or recombinant (Suntory, Osaka, Japan) human TNF with or without antibody overnight as above, and cell death was measured by vital staining. As expected based upon the above results with the A2 mouse mAb, cA2 also inhibited or neutralized both natural and rhTNF in a dose-dependent manner in the cytotoxicity assay (FIG. **11**). In this assay format, levels of cA2 as low as 125 ng/ml completely abolished the toxic activity of TNF. Upon repeated analysis, the cA2 exhibited greater TNF-inhibiting and/or neutralizing activity than did the parent murine A2 mAb. Such inhibiting and/or neutralizing potency, at antibody levels below 1 μg/ml, can easily be attained in the blood of a subject to whom the antibody is administered. Accordingly, such highly potent inhibiting and/or neutralizing anti-TNF antibodies, in particular the chimeric antibody, are preferred for therapeutic use in TNFα-mediated pathologies or conditions.

As mentioned above, TNF induces cellular secretion of IL-6. Furthermore, there is evidence that IL-6 is involved in the pathophysiology of sepsis, although the precise role of IL-6 in that syndrome is unclear (Fong, et al., *J. Exp. Med.* 170:1627-1633 (1989); Starnes Jr., et al., *J. Immunol.* 145: 4185-4191 (1990)). The ability of cA2 to inhibit or neutralize TNF-induced IL-6 secretion was evaluated using cultured human diploid FS-4 fibroblasts. The results in Table 2 show that cA2 was effective in blocking IL-6 secretion in cells that had been incubated overnight with TNF. TNF-induced IL-6 secretion was not inhibited in the absence of a mAb or in the presence of a control mAb specific for an irrelevant antigen.

TABLE 2

In Vitro Neutralization of TNF-Induced IL-6 Secretion

	TNF Concentration (ng/ml)			
Antibody	0	0.3	1.5	7.5
None	<0.20	1.36	2.00	2.56
Control mAb	<0.20	1.60	1.96	2.16
cA2	<0.20	<0.20	<0.20	0.30

Values represent mean concentrations of IL-6 of duplicate wells, in ng/ml. RhTNF (Suntory, Osaka, Japan), with or without 4 μg/ml antibody, was added to cultures of FS-4 fibroblasts and after 18 h, the supernatant was assayed for IL-6 using the QUANTIKINE ® Human IL-6 Immunoassay (from R&D Systems, Minneapolis, MN).
Control mAb = chimeric mouse/human IgG1 anti-platelet mAb (7E3).

The ability of TNF to activate procoagulant and adhesion molecule activities of endothelial cells (EC) is thought to be an important component of pathology pathophysiology. In particular, this can be associated with the vascular damage, disseminated intravascular coagulation, and severe hypotension that is associated with the sepsis syndrome. Therefore,

the ability of cA2 to block TNF-induced activation of cultured human umbilical vein endothelial cells (HUVEC) was evaluated. TNF stimulation of procoagulant activity was determined by exposing intact cultured HUVEC cells to TNF (with or without antibody) for 4 hours and analyzing a cell lysate in a human plasma clotting assay. The results in Table 3 show the expected upregulation by TNF of HUVEC procoagulant activity (reflected by a decreased clotting time). Chimeric antibody cA2 effectively inhibited or neutralized this TNF activity in a dose-dependent manner.

TABLE 3

In Vitro Neutralization of TNF-Induced Procoagulant Activity

		TNF Concentration (ng/ml)		
Antibody	μg/ml	250	25	0
None	—	64 ± 4*	63 ± 1	133 ± 13
Control Ab	10.00	74 ± 6	N.D.	178 ± 55
cA2	10.00	114 ± 5	185 ± 61	141 ± 18
cA2	3.30	113 ± 2	147 ± 3	N.D.
cA2	1.10	106 ± 1	145 ± 8	N.D.
A2	0.37	73 ± 17	153 ± 4	N.D.
cA2	0.12	64 ± 1	118 ± 13	N.D.

*Values represent mean plasma clotting time, in seconds (±S.D.). Clotting time was determined in normal human plasma after addition of the rhTNF (Dainippon, Osaka, Japan) with or without antibody-treated HUVEC lysate and Ca^{++} at 37° C.
N.D. = not done.
Control Ab is a chimeric mouse/human IgG1 anti-CD4 antibody.

In addition to stimulating procoagulant activity, TNF also induces surface expression of endothelial cell adhesion molecules such as ELAM-1 and ICAM-1. The ability of cA2 to inhibit or neutralize this activity of TNF was measured using an ELAM-1 specific detection radioimmunoassay. Cultured HUVEC were stimulated with 250 ng/ml rhTNF (Dainippon, Osaka, Japan) with or without antibody at 37° C. overnight in a 96-well plate format. Surface expression of ELAM-1 was determined by sequential addition of a mouse anti-human ELAM-1 mAb and ^{125}I-labelled rabbit anti-mouse immunoglobulin second antibody directly to culture plates at 4° C.

As shown in FIG. **12**, TNF induced the expression of ELAM-1 on the surface of cultured HUVEC cells, and this activity was again effectively blocked in a dose-related manner by cA2.

Finally, TNF is known to stimulate mitogenic activity in cultured fibroblasts. Chimeric A2 inhibited or neutralized TNF-induced mitogenesis of human diploid FS-4 fibroblasts cultures, confirming the potent inhibiting and/or neutralizing capability of cA2 against a broad spectrum of in vitro TNF biological activities.

EXAMPLE XII

Determination of Amino Acid Sequences (Epitope) on Human TNF-α Recognized by cA2 mAb Reagents

The following reagents are readily available from commercial sources. FMOC-L-Ala-OPfp, FMOC-L-Cys(Trt)-OPfp, FMOC-L-Asp(OtBu)-OPfp, FMOC-L-Giu (OtBu)-OPfp, FMOC-L-Phe-OPfp, FMOC-Gly-OPfp, FMOC-L-His (Boc)-OPfp, FMOC-L-Ile-OPfp, FMOC-L-Lys(Boc)-OPfp, FMOC-L-Leu-OPfp, FMOC-L-Asn-OPfp, FMOC-L-Pro-OPfp, FMOC-L-Gin-OPfp, FMOC-L-Arg(Mtr)-OPfp, FMOC-L-Ser(tBu)-ODhbt, FMOC-L-Thr(tBu)-ODhbt, FMOC-L-Val-OPfp, FMOC-L-Trp-OPfp, FMOC-L-Try (tBu)-OPfp, and 1-hydrox-fbenotriazol (HOBT) were obtained from Cambridge Research Biochemicals. Piperidine and was obtained from Applied Biosystems, Inc. 1-Methyl-2-Pyrrolidinone (NMP) was obtained from EM Science; Methanol from JT Baker; Acetic Anhydride from Applied Biosystems, Inc., Trifluoroaccetic acid (TFA) from Applied Biosystems, Inc.; Diisopropylamne (DIEA), Triethylamine, Dithiothreitol (DTT) and Anisole from Aldrich and Hydrochloric Acid (HCl) from J T Baker.

Abbreviations: FMOC, 9-fluorenylmethoxycarbonyl; tBu t-butyl ether; OrB, t-butyl ester; Boc, t-butyloxycarbonyl; Mtr, 4-methoxy-2,3,6-trimethyl-benzenesulfonyl; Trt, trityl; OPfp, pentafluorophenylester; ODnbt. oxo-benzotriazone ester.

A chimeric antibody of the present invention, designated cA2, was used to determine which portions of the TNF amino acid sequence were involved in inhibitory binding by the antibody by epitope mapping, whereby the amino acid sequences of TNF-α recognized by cA2 have been identified.

The complete primary sequence of human TNFα, according to Pennica et al., *Nature* 312:724-729 (1984) is shown in FIG. **13** (SEQ ID NO:1). Overlapping decapeptides beginning with every second amino acid and covering the entire amino acid sequence of human TNF-α were synthesized on polyethylene pins using the method of Geysen (Geysen et al., *Peptides: Chemistry and Biological*, Proceedings of the Twelfth American Peptide Symposium, p. 519-523, Ed, G. R. Marshall, Escom, Leiden, 1988). Sets of peptide pins bearing free N-terminal amino groups and acetylated N-terminal amino groups were individually prepared. Both sets of peptide pins were incubated in solutions containing the anti-TNF mAb cA2 to determine the amino acid sequences that make up the cA2 epitope on human TNF-α, as described below. FIG. **14**A shows the results of binding to the overlapping decapeptides that comprise the entire sequence of human TNFα. The O.D. (optional density) correlates directly with the increased degree of cA2 binding. FIG. **14**B shows the results of binding of cA2 to the same set of peptide pins in the presence of human TNFα. This competitive binding study delineates peptides which can show non-specific binding to cA2.

There are at least two non-contiguous peptide sequences of TNF-α recognized by cA2. Using the conventional protein numbering system wherein the N-terminal amino acid is number 1, the cA2 mAb recognizes an epitope composed at least in part of amino acids located within residues 87-108 or both residues 59-80 and 87-108 of TNF (SEQ ID NO:1). FIG. **15** presents these non-contiguous sequences within the TNF sequence.

Unexpectedly, the mAb cA2 blocks the action of TNF-α without binding to the putative receptor binding locus, which can include one or more of, e.g., 11-13, 37-42, 49-57 or 155-157 of hTNFα (of SEQ ID NO:1). Preferred anti-TNF mAbs are those that inhibit this binding of human TNF-α to its receptors by virtue of their ability to bind to one or more of these peptide sequences. These antibodies can block the activity of TNF by virtue of binding to the cA2 epitope, such binding demonstrated to inhibit TNF activity. The identification of those peptide sequences recognized by cA2 provides the information necessary to generate additional MAbs with binding characteristics and therapeutic utility that parallel the embodiments of this application.

Peptide Pin Synthesis

Using an epitope mapping kit purchased from Cambridge Research Biochemicals, Inc. (CRB), dodecapeptides corresponding to the entire sequence of human TNF-α were synthesized on polyethylene pins.

A synthesis schedule was generated using the CRB epitope mapping software. Prior to the first amino acid coupling, the pins were deprotected with a 20% piperidine in NMP solution for 30 minutes at room temperature. After deprotection, the pins were washed with NMP for five minutes at room temperature, followed by three methanol washes. Following the wash steps, the pins were allowed to air dry for at least 10 minutes.

The following procedure was performed for each coupling cycle:

1) The amino acid derivatives and the HOBT were weighted out according to the weights required in the synthesis schedule.
2) The HOBT was dissolved in the appropriate amount of NMP according to the synthesis schedule.
3) The amino acid derivatives were dissolved in the recommended amount of HOBT solution and 150 microliters were pipeted into the appropriate wells as directed by the well position sheet of the synthesis schedule.
4) The blocks containing the pins were placed into the wells, and the "sandwich" units stored in plastic bags in a 30° C. water bath for 18 hours.
5) The pins were removed from the wells and washed once (for 5 minutes) with NMP, three times (for two minutes) with methanol and air dried for 10 minutes.
6) The pins were deprotected as described above and the procedure repeated.

To acetylate the peptides on one block of pins, the peptide pins were washed, deprotected and treated with 150 microliters of a solution containing NMP; acetic anhydride:triethylamine (5:2:1) for 90 minutes at 30° C., followed by the washing procedure outlined above. The second set of peptide pins was deprotected by not acetylated to give free N-terminal amino groups.

The final deprotection of the peptides to remove the side chain protecting groups was done using a mixture of TFA: anisole:dithiothreitol, 95:2.5:2.5 (v/v/w) for four hours at ambient temperature. After deprotection, the pins were air dried for 10 minutes, followed by a 15 minute sonication in a solution of 0.1% HCl in methanol/distilled water (1:1). The pins dried over night and were then ready for testing.

ELISA Assay for cA2 Binding to TNF-α Peptide PINs

Reagents: Disruption Buffer

Sodium dihydrogen phosphate (31.2 g, Sigma cat # S-0751 or equivalent) and sodium dodecylsulfate (20.0 g, Sigma cat # L-3771 or equivalent) were dissolved in 2.0 L of MILLI-Q® water. The pH was adjusted to 7.2±0.1 with 50% w/w sodium hydroxide (VWR cat # VW6730-3 or equivalent).

Blocking Buffer

Sodium dihydrogen phosphate (0.39 g, Sigma cat #S-0751 or equivalent) disodium hydrogen phosphate (1.07 g, Baker cat # 3828-1 or equivalent) and sodium chloride (8.50 g, Baker cat # 3624-5 or equivalent) were dissolved in 1.0 L of MILLI-Q® water. The pH was adjusted to 7.2±0.1 with 50% w/w sodium hydroxide (VWR cat VW6730-3 or equivalent). Chicken egg albumin (10.0 g, Sigma cat #A-5503 or equivalent) and bovine serum albumin (10.0 g, Sigma, cat #A-3294 or equivalent) were dissolved at room temperature with gentle stirring. The solution was filtered, and to the solution was added TWEEN® 20 (2.0 ml, Sigma cat #P-13.79 or equivalent). The solution was stirred gently at room temperature for 30 min, filtered and stored at 40°.

PBS/TWEEN® 20

A 10× concentrate was prepared by dissolving sodium dihydrogen phosphate (3.90 g, Sigma cat # S-0751 or equivalent), disodium hydrogen phosphate (10.70 g, Baker cat #3828-1 or equivalent) and sodium chloride (85.0 g, Baker cat #3624-5 or equivalent) in 1.0 L of MILLI-Q® water. The pH was adjusted to 7.2±0.1 with 50% w/w sodium hydroxide (VWR cat #VW 6730 or equivalent). To the solution was added TWEEN® 20 (5.0 mL, Sigma cat #P-1379 or equivalent), and the mixture stirred gently. Just prior to use 100 mL of this solution was diluted to 1.0 L with MILLI-Q® water.

Substrate Solution

Substrate buffer was prepared by dissolving citric acid (4.20 g, Malinckrodt cat #0627 or equivalent) and disodium hydrogen phosphate (7.10 g, Baker cat #3828-1 or equivalent) in 1.0 L of MILLI-Q® water. The pH was adjusted to 5.00 with 50% w/w sodium hydroxide (VWR cat #VW6730-3 or equivalent). Immediately prior to use an OPD substrate tablet (30 mg, Sigma cat #P-8412 or equivalent and 30% (v/v) hydrogen peroxide (40 μL, Sigma cat #P-1379 or equivalent) were added to the substrate buffer 25.0 mL). The solution was wrapped in foil and mixed thoroughly.

4 NH_2S04

Sulfuric acid (53 mL, EM Science cat #SX1244-5 or equivalent) was slowly added to MILLI-Q® water (447 mL) and cooled to room temperature prior to use.

Equipment

Molecular Devices Model nu-max plate reader or equivalent. Scientific Products Model R4140 Oscillating table shaker and equivalent. BRANSON Model 5200 ultra-sonic bath or equivalent. FINNPIPETTE Model 4172317 multichannel pipeter or equivalent. CORNING Model 2580196 well disposable polystyrene Elisa Plates.

Prior to use and after each subsequent use the peptide pins were cleaned using the following procedure. Disruption buffer (2.0 L) was heated to 60° and placed in an ultra-sonic bath in a fume hood. To the disruption buffer was added dithiolthreitol (2.5 g, Sigma cat #D-0632 or equivalent). The peptide pins were sonicated in this medium for 30 min, washed thoroughly with MILLI-Q® water, suspended in a boiling ethanol bath for 2 min, and air-dried.

Blocking buffer (200 μL) was added to a 96 well disposable polystyrene Elisa plate and the peptide pins suspended in the wells. The peptide pins and plate were incubated for 2 hours at room temperature on an oscillating table shaker. The plates and peptide pins were washed with PBS/TWEEN® 20 (four times). To each well was added a 20 μg/ml concentration of cA2 antibody (diluted with blocking buffer, 175 μL/well). TNF competition was done by incubation of TNFα (40 μg/ml) and cA2 (20 μg/ml) in BSA/ovalbumin/BBS for three hours at room temperature. The peptide pins were suspended in the plate and incubated at 4° overnight. The peptide pins and plate were washed with PBS/TWEEN® 20 (four times). To each well was added anti-human goat antibody conjugated to horseradish peroxidase (diluted with blocking buffer to ½000, 175 μL/well, Jackson IMMUNORESEARCH Labs). The peptide pins were suspended in the plate, and incubated for 1 hour at room temperature on a oscillating table shaker. The plates

and peptide pins were washed with PBS/TWEEN® 20 (four times). To each well was added freshly prepared substrate solution (150 μL/well), the peptide pins were suspended in the plate and incubated for 1 hour at room temperature on an oscillating table shaker. The peptide pins were removed and to each well is added 4N H_2SO_4 (50 μL). The plates were read in a Molecular Devices plate reader (490 nm, subtracting 650 nm as a blank), and the results are shown in FIGS. **14**A and **14**B, as described above.

EXAMPLE XIII

Production of Mouse Anti-Human TNF mAb Using TNF Peptide Fragments

Female BALB/c mice, as in Example I above, are injected subcutaneously and intraperitoneally (i.p.) with forty μg of purified *E. coli*-derived recombinant human TNF (rhTNF) fragments comprising anti-TNF epitopes of at least 5 amino acids located within the non-contiguous sequence 59-80, 87-108 or both residues 59-80 and 87-108 of TNF (of SEQ ID NO:1), as presented above, emulsified with an equal volume of complete Freund's adjuvant (obtained from Difco Laboratories) in 0.4 ml is into a mouse. One week later, a booster injection of 5 μg of these rhTNF fragments in incomplete Freund's adjuvant is given i.p. followed by four consecutive i.p. injections of 10 μg of TNF fragments including anti-TNF epitopes including amino acids from residues 59-80, 87-108 or both 59-80 and 87-108 of hTNFα (of SEQ ID NO:1) without adjuvant. Eight weeks after the last injection, the mouse is boosted i.p. with 10 μg of TNF.

Four days later, the mouse is sacrificed, the spleen is obtained and a spleen cell suspension is prepared. Spleen cells are fused with cells of the nonsecreting hybridoma, Sp2/0 (ATCC CRL1581), at a 4:1 ratio of spleen cells to Sp2/0 cells, in the presence of 0.3 ml of 30% polyethylene glycol, PEG 1450. After incubation at 37° C. for 6 hours, the fused cells are distributed in 0.2 ml aliquots into 96-well plates at concentrations of 2×10^4 SP2/0 cells per well. Feeder cells, in the form of 5×10^4 normal BALB/c spleen cells, are added to each well.

The growth medium used consisted of RPM1-1640 medium, 10% heat-inactivated fetal bovine serum (FBS) (Hyclone), 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (GIBCO Laboratories) and, for selection, hypoxanthine-aminopterin-thymidine (HAT) (Boehringer Mannheim). A solid-phase radioimmunoassay (RIA) is employed for screening supernatants for the presence of mAbs specific for rhTNFα fragments including portions of residues 59-80, 87-108 or both 59-80 and 87-108 of hTNFα (of SEQ ID NO:1). This assay is described in Example II, above. The background binding in this assay is about 500 cpm. A supernatant is considered positive if it yielded binding of 2000 cpm or higher.

Of the supernatants screened, one or more positive supernatants are routinely identified by RIA. Of these positive supernatants, the highest binding (as shown by the higher cpm values) are subcloned at limiting dilution on mouse feeder cells. Upon further analysis of the supernatants in neutralization assays, routinely one or more antibodies are found to have potent inhibiting and/or neutralizing activity. These positive and inhibiting and/or neutralizing hybridoma lines are then selected and maintained in RPM1-1640 medium with 10% FBS (GIBCO), 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

EXAMPLE XIV

Production of Murine and Chimeric Antibodies, Fragments and Regions from TNF Peptides

Murine and chimeric antibodies, fragments and regions are obtained by construction of chimeric expression vectors encoding the mouse variable region of antibodies obtained in Example XIII and human constant regions, as presented in Examples IV-IX above.

The resulting chimeric A2 antibody is purified from tissue culture supernatant by Protein A-Sepharose chromatography. The supernatant is adjusted to 0.1M Tris, 0.002M EDTA, pH 8.0 and loaded on a Protein A-Sepharose column equilibrated in the same buffer. The IgG is then eluted with 0.1M citrate, pH 3.5, neutralized with 1M Tris, and dialyzed into phosphate buffered saline (PBS).

The purified murine and chimeric antibodies, fragments and regions are evaluated for its binding and inhibiting and/or neutralizing activity.

EXAMPLE XV

In Vitro Activity and Neutralization Efficacy of a Chimeric Anti-TNF Antibody

Both the murine and chimeric anti-TNFα antibodies of the present invention, as obtained according to Examples XIII and XIV, are determined to have potent TNF-inhibiting and/or neutralizing activity, as shown for example, in the TNF cytotoxicity assay described above, expressed as the 50% Inhibitory Dose (ID50).

In this same experimental system, three other murine anti-TNFα antibodies (termed TNF-1, TNF-2 and TNF-3) of comparable binding affinity to TNF are found to have ID50 values of 1-2 orders of magnitude greater, and thus have significantly less potency in neutralization, than both the murine and chimeric anti-TNFα antibodies of the present invention.

The ability of both the murine and chimeric anti-TNFα antibodies of the present invention, as obtained according to Examples XIII and XIV, to inhibit or neutralize human TNFα bioactivity in vitro is tested using the bioassay system described above. Cultured cells producing the murine or chimeric anti-TNFα antibodies of the present invention, as obtained according to Examples XIII and XIV, are incubated with 40 pg/ml natural (Genzyme, Boston, Mass.) or recombinant (Suntory, Osaka, Japan) human TNF with or without antibody overnight as above, and cell death is measured by vital staining. As expected, both the murine and chimeric anti-TNFα antibodies of the present invention, as obtained according to Examples XIII and XIV, inhibited or neutralized both natural and rhTNF in a dose-dependent manner in the cytotoxicity assay. Such inhibiting and/or neutralizing potency, at antibody levels below 1 μg/ml, can easily be attained in the blood of a subject to whom the antibody is administered. Accordingly, such highly potent inhibiting and/or neutralizing anti-TNF antibodies, in particular the chimeric antibody, are preferred for therapeutic use in TNFα-mediated pathologies or conditions.

The ability of cA2 to inhibit or neutralize TNF-induced IL-6 secretion is evaluated using cultured human diploid FS-4 fibroblasts. The results are expected to show that both murine and chimeric anti-TNFα antibodies of the present invention, as obtained according to Examples XIII and XIV, are effective in blocking IL-6 secretion in cells that had been incubated overnight with TNF. TNF-induced IL-6 secretion

is not inhibited in the absence of a mAb or in the presence of a control mAb specific for an irrelevant antigen.

The ability of TNF to activate procoagulant and adhesion molecule activities of endothelial cells (EC) is thought to be an important component of pathology pathophysiology. In particular, this can be associated with the vascular damage, disseminated intravascular coagulation, and severe hypotension that is associated with the sepsis syndrome. Therefore, the ability of both the murine and chimeric anti-TNFα antibodies of the present invention, as obtained according to Examples XIII and XIV, to block TNF-induced activation of cultured human umbilical vein endothelial cells (HUVEC) is evaluated. TNF stimulation of procoagulant activity is determined by exposing intact cultured HUVEC cells to TNF (with or without antibody) for 4 hours and analyzing a cell lysate in a human plasma clotting assay. The results are expected to show the expected upregulation by TNF of HUVEC procoagulant activity (reflected by a decreased clotting time). Both the murine and chimeric anti-TNFα antibodies of the present invention, as obtained according to Examples XIII and XIV, are expected to effectively inhibit or neutralize this TNF activity in a dose-dependent manner.

In addition to stimulating procoagulant activity, TNF also induces surface expression of endothelial cell adhesion molecules such as ELAM-1 and ICAM-1. Both the murine and chimeric anti-TNFα antibodies of the present invention, as obtained according to Examples XIII and XIV, are expected to inhibit or neutralize this activity of TNF is measured using an ELAM-1 specific detection radioimmunoassay. Cultured HUVEC are stimulated with 250 ng/ml rhTNF (Dainippon, Osaka, Japan) with or without antibody at 37° C. overnight in a 96-well plate format. Surface expression of ELAM-1 is determined by sequential addition of a mouse anti-human ELAM-1 mAb and ^{125}I-labelled rabbit anti-mouse immunoglobulin second antibody directly to culture plates at 4° C.

TNF is expected to induce the expression of ELAM-1 on the surface of cultured HUVEC cells, and this activity is again expected to be effectively blocked in a dose-related manner by both the murine and chimeric anti-TNFα antibodies of the present invention, as obtained according to Examples XIII and XIV.

Finally, TNF is known to stimulate mitogenic activity in cultured fibroblasts. Both the murine and chimeric anti-TNFα antibodies of the present invention, as obtained according to Examples XIII and XIV, are expected to inhibit or neutralize TNF-induced mitogenesis of human diploid FS-4 fibroblasts cultures, confirming the potent inhibiting and/or neutralizing capability of both the murine and chimeric anti-TNFα antibodies of the present invention, as obtained according to Examples XIII and XIV against a broad spectrum of in vitro TNF biological activities.

EXAMPLE XVI

In Vivo Activity and Efficacy of cA2 Antibody

Evidence that the potent in vitro inhibiting and/or neutralizing capability of cA2 is manifest in vivo was obtained. Earlier animal studies showed that administration of TNF to experimental animals mimics the pathology state obtained with either Gram-negative bacterial infection or direct endotoxin administration (Tracey et al., infra (1986); Tracey et al., infra (1987); Lehmann et al. infra).

An in vivo model wherein lethal doses of human TNF are administered to galactosamine-sensitized mice (Lehmann, V. et al., infra) is substantially modified for testing the capability of both the murine and chimeric anti-TNFα antibodies of the present invention, as obtained according to Examples XIII and XIV above, to inhibit or neutralize TNF in vivo. An i.p. challenge with 5 μg (0.25 mg/kg) of rhTNF resulted in 80-90 percent mortality in untreated control animals and in animals treated i.v. 15-30 minutes later with either saline or 2 mg/kg control antibody (a chimeric IgG1 derived from murine 7E3 anti-platelet mAb). In contrast, treatment with both the murine and chimeric anti-TNFα antibodies of the present invention, as obtained according to Examples XIII and XIV, is expected to reduce mortality to 0-30 percent with 0.4 mg/kg of antibody, and to 0-10 percent with 20 mg/kgs. These expected results indicate that both the murine and chimeric anti-TNFα antibodies of the present invention, as obtained according to Examples XIII and XIV, are capable of inhibiting and/or neutralizing the biological activity of TNF in vivo as well as in vitro.

TABLE 4

Prevention of Human TNF-Induced Lethality by Chimeric A2

	Outcome (Survivors/Total)	
Antibody	Experiment #1	Experiment #2
None	1/10	N.D.
Control Ab, 2 mg/kg	2/10	1/10
cA2 (2 mg/kg) (p = 0.0001)	9/10 (p = 0.0055)	10/10
cA2 (0.4 mg/kg) (p = 0.0001)	7/10 (p = 0.07)	10/10

Female C3H/HeN mice were administered 5 μg rhTNF (Dainippon, Osaka, Japan) + 18 mg galactosamine i.p. and antibody was administered 15–30 minutes later i.v. Deaths were recorded 48 hours post-challenge.
Control MAb = chimeric mouse/human IgG1 anti-platelet MAb (7E3).
N.D. = not done.
p values refer to comparison with the control Ab.

EXAMPLE XVII

cA2 MAb Safety in Chimpanzees

The epitope specificity of A2 can be for an epitope which predominates in humans and chimpanzees. Therefore, the chimpanzee was chosen as a relevant mammalian species to determine the toxicological potential and provide safety information for cA2. Chimpanzees were dosed at levels of 15 mg/kg for four to five consecutive days and 30 mg/kg once or for three consecutive days. No adverse clinical signs, and no changes considered to be cA2 treatment related were observed in the monitored parameters including routine hematology and blood chemistry. Thus, doses of up to 30 mg/kg for three consecutive days were well tolerated in chimpanzees.

EXAMPLE XVIII

Clinical Activity and Efficacy of cA2 Antibody

Chimeric IgG1 anti-human TNF MAb cA2 was administered to healthy male human volunteers as patients. One hour after receiving 4 ng/kg of an NIH reference endotoxin, the volunteers were administered either saline, as a control, or 0.01, 0.10 or 10 mg/kg of cA2 in a pharmaceutically acceptable form. TNF levels in serum were measured over time and were found to show a dose dependent decrease in peak TNF levels with no TNF being detected in volunteers receiving a 10 mg/kg dose of cA2. Accordingly, therapy with

an anti-TNF antibody of the present invention is expected to inhibit TNF-mediated effects in humans.

Patients receiving endotoxin developed pronounced leukopenia thought to be due to margination of white blood cells. As the white blood cells become activated, they can attach to endothelial receptors with resultant endothelial damage. At doses of 1.0 to 10.0 mg/kg, this leukopenia is prevented, whereas, at 0.01 and 0.1 mg/kg dosages, a drop in white cell count was observed. The drop was most pronounced among the polymorph cell line. In all patients there was a subsequent leukocytosis, which was unchanged by treatment with anti-TNF antibody cA2. This blocking effect on white blood cell margination is expected to represent a protective effect against the endothelial damage associated with TNF. It is expected in the art that this TNF-related endothelial damage plays a significant role in the morbidity and mortality associated with sepsis, and it is therefore expected that the anti-TNF antibodies of the present invention will provide a protective effect against these damaging effects, as presented herein.

EXAMPLE XIX

Treatment of Sepsis in Humans Using a Chimeric Anti-TNF Antibody

The chimeric anti-TNF MAb cA2 has been used in two phase I/II studies. In a phase I/II study in septic patients, 20 patients with the sepsis syndrome received a single dose of either 0.1, 1.0, 5.0 or 10 milligrams of cA2 per kilogram bodyweight. Another 60 patients received 100 milligrams of HA-1A, a human anti-lipid A Mab currently under evaluation for gram negative sepsis, followed with either placebo or 1.0, 5.0, or 10 milligrams cA2 per kilogram bodyweight. The cA2 was administered as a single, intravenous infusion over a 60 minute period. Clinical assessment, vital signs, and laboratory parameters were measured before, during and periodically for 28 days after the infusion. In this study, cA2 was well tolerated. No adverse events were reported as "probably" or "definitely" related to cA2. All deaths were reported as "definitely not" related to cA2.

Accordingly, human treatment of rheumatoid arthritis in human patients was expected, and found, to provide a suitable treatment, as described herein.

EXAMPLE XX

Clinical Treatment of Rheumatoid Arthritis by a Anti-TNF Antibody or Peptide of the Present Invention

A Phase I open label study was conducted for methods and compositions of the present invention using a chimeric anti-TNF MAb for the treatment of patients with severe refractory rheumatoid arthritis. Nine patients were enrolled in the study. The first five patients were treated with chimeric anti-TNF antibody (cA2), 10 mg/kg as a single dose infused over a period of two hours. These patients were subsequently retreated with a second infusion of 10 mg/kg on day 14 of the study. The second group of five patients received an infusion of 5 mg/kg on the first day of the study. They were then treated with additional infusions of 5 mg/kg on days 5, 9, and 13. Four of the planned five patients in this second group have been treated to date.

Preparation, Administration, and Storage of Test Material

The chimeric monoclonal anti-TNF antibody was supplied in single-use glass vials containing 20 mL with 100 mg of anti-TNF (5 mg/mL). The anti-TNF antibody was stored at 2-8° C. Prior to infusion, the antibody was withdrawn from the vials and filtered through a low-protein-binding 0.22 μm filter. This filtered antibody was then diluted to a final volume of 300 mL with normal saline. The 300 mL antibody preparation was then infused via an in-line filter over a period of not less than two hours.

Prior to each repeat infusion of study medication a test dose of 0.1 mL of the infusion was diluted in 10 mL of normal saline and administered by slow IV push over 5 minutes. The patient was observed for 15 minutes for signs or symptoms of an immediate hypersensitivity reaction. If no reaction was observed in this time period, the full dose was administered as described above.

Administration Protocol

Group 1 (patients 1-5): a total of 2 infusions, on day 1 and day 15 of the trial; dosage 10 mg/kg on each occasion;

Group 2 (patients 6-9): a total of 4 infusions, on days 1, 5, 9 and 13 of the trial; dosage 5 mg/kg on each occasion.

All infusions were administered iv over 2 hours in a total volume of cA2+saline of 300 ml. Infusions subsequent to the first in any patient were preceded by a small test dose administered as an iv push. All patients had at least three years of disease activity with rheumatoid arthritis. The patients ranged in age from 23 to 63. All patients had failed therapy with at least three different DMARD (Disease Modifying Anti-Rheumatic Drug). Six of the nine patients had serum rheumatoid factors, and all nine patients had erosions present on X-rays.

Clinical Monitoring

Patients were monitored during and for 24 hours after infusions for hemodynamic change, fever or other adverse events. Clinical and laboratory monitoring for possible adverse events was undertaken on each follow-up assessment day. Clinical response parameters were performed at the time-points as specified in the flow charts present in Tables 9A and 9B. These evaluations were performed prior to receiving any infusions.

Clinical response studies will be comprised of the following parameters:

1. Number of tender joints and assessment of pain/tenderness
   The following scoring will be used:
   0=No pain/tenderness
   1=Mild pain. The patient says it is tender upon questioning.
   2=Moderate pain. The patient says it is tender and winces.
   3=Severe pain. The patient says it is tender and winces and withdraws.
2. Number of swollen joints
   Both tenderness and swelling will be evaluated for each joint separately. MCP's, PIP's etc. will not be considered as one joint for the evaluation.
3. Duration of morning stiffness (in minutes)
4. Grip strength
5. Visual analog pain scale (0-10 cm)
6. Patients and blinded evaluators will be asked to assess the clinical response to the drug. Clinical response will be assessed using a subjective scoring system as follows:
   5=Excellent response (best possible anticipated response)
   4=Good response (less than best possible anticipated response)
   3=Fair response (definite improvement but could be better)
   2=No response (no effect)
   1=Worsening (disease worse)

Measurement of index of disease activity is scored according to the following Table 5.

TABLE 5

Clinical Characteristics of Patients 1–5

Patient Number	Age/ Sex	Disease Duration (years)	Rheumat. Factor	Erosions/ Nodules	Previous Treatment (DMARDs only)	Concomitant Anti-rheumatic Therapy
01	48/F	7	+ve	−ve/+ve	*Sal, DP, Myo, Aur, MTX, Aza, Chl	**Pred 5 mg
02	63/F	7	−ve	+ve/−ve	Sal, Myo, DP	Para 1–2 g
03	59/M	3	+ve	+ve/−ve	Aur, Chl, Myo, MTX, Sal	Pred 10 mg; Ind 225 mg
04	56/M	10	+ve	+ve/−ve	Myo, DP, Aza, Sal	Pred 12.5 mg, Ibu 2 g, Para 1–2 g
05	28/F	3	+ve	+ve/−ve	Myo, Sal, DP, Aza	Pred 8 mg, Para 1–2 g, Cod 16 mg

*Sal = Sulphasalazine;
DP = D-penicillamine;
Myo = Myocrisin;
Aur = auranofin;
MTX = methotrexate;
Aza = azathioprine;
Chl = hydroxychloroquine.
**Pred = prednisolone (dosage/day);
Para = paracetamol;
Ind = indomethacin;
Ibu = ibuprofen;
Cod = codeine phosphate.

TABLE 6

Clinical Characteristics of Patients 6–9

Patient Number	Age/ Sex	Disease Duration (years)	Rheumat. Factor	Erosions/ Nodules	Previous Treatment (DMARDs only)	Concomitant Anti-rheumatic Therapy
06	40/M	3	+ve	+ve/−ve	*Sal, Chl, Aur	**Nap 1 g
07	54/F	7	−ve	+ve/−ve	DP, Myo, Sal, Aza, MTX	Para 1–2 g Cod 16–32 mg
08	23/F	11	+ve	+ve/−ve	Chl, Myo, Sal, MTX, Aza	Pred 7.5 mg, Dicl 100 mg, Para 1–2 g, Dext 100–200 mg
09	51/F	15	−ve	+ve/+ve	Myo, Chl, DP, MTX	Pred 7.5 mg, Dicl 125 mg, Para 1–3 g

*Sal = Sulphasalazine;
Chl = chloroquine or hydroxychloroquine;
Aur = auranofin;
DP = D-penicillamine;
Myo = Myocrisin;
Aza = azathioprine;
MTX = methotrexate.
**Nap = naprosyn (dosage/day);
Para = paracetamol;
Cod = codeine phosphate;
Pred = prednisolone;
Dicl = diclofenac;
Dext = dextropropoxyphene.

TABLE 7

Disease Activity at Entry for Patients 1–5

Patient Number	Morning Stiffness (mins)	Pain (10—10 cm on VAS)	Number Swollen Joints (0–28)	Ritchie Articular Index (0–69)	Grip Strength L/R (mm/Hg; max 300)	ESR (mm/hr normal ranges: F < 15; M < 10)	CRP (mg/l; normal range < 10)	IDA (range 1–4)
01	60	3.9	19	30	108/107	35	5	2.67
02	20	2.7	25	31	67/66	18	14	2.0
03	90	4.9	14	16	230/238	48	44	2.5
04	30	6.9	17	12	204/223	24	35	2.33
05	90	5.7	28	41	52/89	87	107	3.0

TABLE 8

Disease Activity at Entry for Patients 6–9

Patient Number	Morning Stiffness (mins)	Pain (0–10 cm on VAS)	Number Swollen Joints (0–28)	Ritchie Articular Index (0–69)	Grip Strength L/R (mm/Hg; max 300)	ESR (mm/hr normal ranges: F < 15; M < 10)	CRP (mg/l; normal range < 10)	IDA (range 1–4)
06	120	5.0	3	4	260/280	23	33	2.33
07	105	7.4	27	31	59/80	25	10	2.83
08	270	9.3	17	37	73/125	35	31	3.17
09	180	4.5	20	26	53/75	15	33	2.5

All patients have tolerated the infusions of chimeric anti-CD4 and no serious adverse reactions have been observed. Specifically, no episodes of hemodynamic instability, fevers, or allergic reactions were observed in association with the infusions. Patients have not experienced any infections.

Although this is a non-blinded study, all patients experienced improvement in their clinical assessments of disease status, as well in biochemical parameters of inflammation measured in their serum.

Clinical assessments, including the duration of early morning stiffness; the assessment of pain on a visual analogue scale; total count of swollen joints; Ritchie articular index (a scaled score which assesses the total number of tender joints and the degree of joint tenderness); and Index of Disease Activity (a scaled score which incorporates several clinical and laboratory parameters), showed impressive improvements compared to-controls. These improvements were typically in the range of an 80% drop from the baseline score; a degree of improvement which is well beyond the amount of improvement that can be attributed to placebo response. In addition, the duration of these improvements was for six to eight weeks in most cases, a duration of response far longer than would be anticipated from a placebo.

The improvements in clinical assessments were corroborated by improvements in biochemical inflammatory parameters measured in serum. The patients showed rapid drops of serum C-reactive protein, usually in the range of 80% from the baseline. Reductions in the erythrocyte sedimentation rate, usually in the range of 40%, were also observed. Circulating soluble TNF receptors were also decreased following therapy. The durations of the biochemical responses were similar to the duration of the clinical responses.

Preliminary assessment of immune responses to the chimeric anti-TNF antibody has shown no antibody response in the first four patients.

In summary, the preliminary evaluation of the results of this Phase I trial indicate that treatment of patients with advanced rheumatoid arthritis with anti-TNF MAb of the present invention is well tolerated and anti-TNF treatment is associated with rapid and marked improvement in clinical parameters of disease activity, including early morning stiffness, pain, and a number of tender and swollen joints; and is accompanied by improvement of biochemical parameters of inflammation.

Although this was an open label study, the magnitude of the clinical improvements is well beyond the degree of improvement that would be anticipated from a placebo response, such that the present invention is shown to have significant clinical efficacy for treating rheumatoid arthritis.

TABLE 9A

Flowchart for Chimeric Anti-TNF Study C0168TRA Group I (10 mg/kg at day 1 and day 14)

	Pre-Scr	Screening	Wk 0 D 1	Wk 0 D 2	Wk 1	Wk 2 D 14	Wk 3	Wk 4	Wk 6	Wk 8
Consent	X									
Demography		X								
Physical Examination		X								X
Pregnancy Test		X								
Weight		X	X			X				X
Vital Signs		X	X*	X	X	X*	X	X	X	X
Anti-TNF Infusion			X			X				
Labs, see Chart		X	X'	X	X	X'	X	X	X	X
Clinical (Safety)				X	X	X'	X	X	X	X
Clinical (Response)		X	X'		X	X'	X	X	X	X
Synovial Biopsy		X			X7					
Response Evaluation										X
Hematology + ESR		X	X'		X	X'	X	X	X	X
Biochemistry		X	X'		X	X'	X	X	X	X
Urinalysis			X'		X	X'	X	X	X	X
CRP + RF			X'		X	X'	X	X	X	X
Serum Cytokines			X'		X	X'	X	X	X	X
PBL			X	X	X					
Pharmacokinetic			X#	X#		X$				
HACA Response			X'		X	X'	X	X	X	X

X* = Vital signs will be obtained prior to infusion, every 30 minutes during the infusion and every 30 minutes for 2 hours after the infusion;
X' = Needs to be done prior to the infusion;
X# = Serum samples will be obtained prior to the infusion and at 1, 2, 4, 8, 12, and 24 hours after the end of the infusion;
X$ = Serum samples will be obtained to the infusion and at 2 hours after the end of the infusion.

TABLE 9B

Flowchart for Chimeric Anti-TNF Study C0168TRA Group 2 (5 mg/kg at days 1, 5, 9 and 13, 4 times total)

	Pre-Scr	Screening	Wk 0 D 1	Wk 0 D 2	Wk 0 D 5	Wk 0 D 9	Wk 1 D 13	Wk 2	Wk 3	Wk 4	Wk 6	Wk 8
Consent	X											
Demography		X										
Physical Examination		X										X
Pregnancy Test		X										
Weight		X	X		X	X	X					X
Vital Signs		X	X*	X	X*	X*	X*	X	X	X	X	X
Anti-TNF Infusion			X		X	X	X					
Labs, see Chart		X	X'	X	X'	X'	X'	X	X	X	X	X
Clinical (Safety)				X	X'	X'	X'	X	X	X	X	X
Clinical (Response)		X	X'			X'		X	X	X	X	X
Synovial Biopsy		X					X7					
Response Evaluation												X
Hematology + ESR		X	X'			X'		X	X	X	X	X
Biochemistry		X	X'			X'		X	X	X	X	X
Urinalysis			X'			X'		X	X	X	X	X
CRP + RF			X'			X'		X	X	X	X	X

TABLE 9B-continued

Flowchart for Chimeric Anti-TNF Study C0168TRA Group 2 (5 mg/kg at days 1, 5, 9 and 13, 4 times total)

	Pre-Scr	Screening	Wk 0 D 1	Wk 0 D 2	Wk 0 D 5	Wk 0 D 9	Wk 1 D 13	Wk 2	Wk 3	Wk 4	Wk 6	Wk 8
Serum Cytokines			X'			X'		X	X	X	X	X
PBL			X	X		X				X		
Pharmacokinetic			X#	X#	X$	X$	X$					
HACA Response			X'			X'		X	X	X	X	X

X* = Vital signs will be obtained prior to infusion, every 30 minutes during the infusion and every 30 minutes for 2 hours after the infusion;
X' = Needs to be done prior to the infusion;
X# = Serum samples will be obtained prior to the infusion and at 1, 2, 4, 8, 12, and 24 hours after the end of the infusion;
X$ = Serum samples will be obtained to the infusion and at 2 hours after the end of the infusion.

TABLE 10

Measurement of the Index of Disease Activity (DA) Variables of Disease Activity

IDA Score	Morning Stiffness (min)	Pain (VAS, cm)*	Grip Strength (mmHg)	Ritchie Articular Index	Hemoglobin (g/dl) Male	Hemoglobin (g/dl) Female	ESR
1	<10	0–2.4	>200	0	>14.1	>11.7	0.20
2	10–30	2.5–4.4	50–200	1–7	13–14	10.8–11.6	21–45
3	31–120	4.5–6.4	30–49	8–17	10–12.9	8.4–10.7	46–80
4	>120	6.5–10	<30	>18	<9.9	<8.3	>81

*Pain was measured on a visual analog scale (VAS) 0–10 cm

Conclusions (1)

Safety of anti-TNF in RA

Anti-TNF was safe and very well tolerated:
no hemodynamic, febrile or allergic episodes;
no infections;
no clinical adverse events;
a single laboratory adverse event only, probably unrelated to anti-TNF.

Conclusions (2)

Efficacy of anti-TNF in RA

Anti-TNF therapy resulted in:
rapid and marked improvements in EMS, pain and articular index in most patients;
slower but marked improvement in swollen joint score, maximal by 3-4 weeks;
rapid and impressive falls in serum CRP, and a slower fall in ESR;
normalization of CRP and ESR in some patients;
rapid falls in serum C4d (a complement breakdown product) and IL-6 in patients where these indices were elevated at entry.

Duration of clinical improvements variable, with rebound in some patients at 6-8 weeks.

Accordingly, the present invention has been shown to have clinical efficacy in human patients for treating TNF involved pathologies using TNF MAbs of the present invention, such as for treating rheumatoid arthritis. Additionally, the human clinical use of TNF antibodies of the present invention in humans is also shown to correlate with in vitro data and in vivo animal data for the use of anti-TNF antibodies of the present invention for treating TNF-related pathologies.

EXAMPLE XXI

Treatment of Crohn's Disease in Humans Using Anti-TNFα Antibodies

Case History SB.

This 16 year old patient has a history of Crohn's disease since age 12. She was suffering from diarrhoea, rectal blood loss, abdominal pain, fever and weight loss. She showed perianal lesions, severe colitis and irregularity of the terminal ileum. She was treated with prednisolone (systemic and local) and PENTASA®. This resulted in remission of the disease, but she experienced extensive side effects of the treatment. She experienced severe exacerbations at age 12 and 12 yrs, 5 months, (IMMURAN™ added), 12 yrs, 9 months, 13 yrs, 5 months, and 14 yrs, 10 months. She experienced severe side effects (growth retardation, morbus Cushing, anemia, muscle weakness, delayed puberty, not able to visit school).

At 15 yrs, 11 months, she was diagnosed with a mass in the right lower quadrant. She had a stool frequency of 28 times per week (with as much as 10 times per day unproductive attempts). The Crohn's index was 311, the pediatric score 77.5. The sedimentation rate was elevated. Albumen and hemoglobin reduced. Before the first treatment the score was 291 and pediatric score was 60, and she would possibly have to lose her colon. She was infused on compassionate grounds with 10 mg/kg cA2, without any side effects

noticed. One week after treatment her sedimentation rate was reduced from 66 to 32 mm. The Crohn's index was 163 and pediatric score 30. She was reported to feel much better and the frequency of the stools was reduced greatly. There was apparently no more diarrhoea, but normal faeces. On October 15th, before the second infusion she had gained weight, had a sedimentation rate of 20 mm, an albumen of 46 h/l, Crohn's index 105, pediatric score 15. There seemed to be improvement on video endoscopy. A second infusion was performed at 16 yrs.

The patient was greatly improved after the second infusion. A endoscopy showed only 3 active ulcers and scar tissue.

This is in contrast with her colon on admission when the thought was that her colon should be removed. This case history shows a dramatic improvement of severe Crohn's disease upon treatment with cA2 anti-TNF antibody.

TABLE 11

Case History SB		
11y, 8m	Physical Examination	Diarrhoea, rectal blood loss, abdominal pain, fever (40%) weight loss perianal lesions
	Sigmoidoscopy	Severe colitis, probably M. Crohn
	Enterolysis	Irregularity terminal ileum
	Therapy	Prednisolone 10 mg 3 dd PENTASA ® 250 mg 3 dd Enema (40 mg prednisone, 2 g 5 ASA) ml 1 dd
	Result	Remission, however: extensive side effects of prednisone and stunting growth
	Action	Prednisone
11y, 11m	Exacerbation	Same clinical picture as 11y, 8 m
	Sigmoidoscopy	Recurrence of colitis (grade IV) in last 60 cm and anus
	Therapy	Prednisolone 40 mg 1 dd PENTASA ® 500 mg 3 dd Enema 1 dd
	Result	Better
12y, 5m	Severe Exacerbation	Despite intensive treatment
	Sigmoidoscopy	Extensive perianal and sigmoidal lesions; active disease
	Therapy	Continued + IMMURAN ™ 25 mg 1 dd
	Result	Slight improvement, however still growth retardation, cushing, anaemia, muscle weakness
	Action	Prednisone
12y, 9m	Exacerbation	
	Sigmoidoscopy	Extensive (active colitis, polyps)
	Action	Prednisone: 30 mg 1 dd, IMMURAN ™ 50 mg 1 dd PENTASA ® 500 mg 3 dd Enema 2 dd
	Result	Still needs enemas with prednisone and oral prednisone. Delayed puberty, stunting growth
14y, 10m	Severe Exacerbation	Weight loss, abdominal pain, fever
	Ileoscopy	Active colitis (grade IV), perianal lesions. Terminal ileum normal
	Result	No remission still fever, poor appetite, weight loss, diarrhea, not able to visit school
Important Findings		
14y, 11m	151.9 cm; 34 kg; t = 38° C., Abdominal mass in right lower quadrant; stool frequency 28 per week (however goes 10–15 times a day but most often without success); ESR 55 mm; Hb 6.2 mmol/1; Ht 0, 29 l/l; alb. 38.4 g/l Crohn's Dis./Act Index: 311 Pediatric score: 77.5	

TABLE 11-continued

Case History SB	
14y, 11.2m	151,8 cm; 34.6 kg (before 1st infusion) Crohn's Dis/Act Index: 291 Pediatric score: 60
14y, 11.4m	151,8 cm; 34.6 kg; ESR 332 mm; Hb 5.7 mmol/l Crohn's Dis/Act Index: 163 Pediatric score: 30
15y, 0m	152,1 cm: 34.8 kg (before 2nd infusion) Feels like she has never felt before. Parents also very enthusiastic; ESR 30 mm: Hb 6.3 mol/l; Ht 0, 32 11; Alb 46 g/l Crohn Dis/Act Index: 105 Pediatric Score: 15 Videoendoscopy: Improvement No problems or side effects observed during and following infusion.

Accordingly, anti-TNF antibodies according to the present invention, as exemplified by cA2, are shown to provide successful treatment of TNF related pathologies, as exemplified by Crohn's disease, in human patients with no or little side effects.

EXAMPLE XXII

Treatment of Arthritis in Humans Using Chimeric Immunoglobulin Chain of the Present Invention

Patient Selection

Twenty patients were recruited, each of whom fulfilled the revised American Rheumatism Association criteria for the diagnosis of RA (Arnett et al., *Arthritis Rheum.* 31:315-324 (1988). The clinical characteristics of the patients are shown in Table 12. The study group comprised 15 females and 5 males, with a median age of 51 years (range 23-72), a median disease duration of 10.5 years (range 3-20) and a history of failed therapy with standard disease—modifying anti-rheumatic drugs (DMARDs; median number of failed DMARDs: 4, range 2-7). Seventeen were seropositive at entry or had been seropositive at some stage of their disease, all had erosions on X-Rays of hands or feet, and 3 had rheumatoid nodules. All patients had active disease at trial entry, as defined by an Index of Disease Activity (IDA; Mallya et al., *Rheumatol. Rehab.* 20:14-17 (1981) of at least 1.75, together with at least 3 swollen joints, and were classed as anatomical and functional activity stage 2 or 3' (Steinbrocker et al., *JAMA* 140:659-662 (1949). The pooled data for each of the clinical and laboratory indices of disease activity at the time of screening for the trial (up to 4 weeks prior to trial entry), and on the day of trial entry itself (week 0), are shown in Tables 13 and 14.

TABLE 12

Demographic Features of 20 Patients with Refractory Rheumatoid Arthritis

Pat	Age/Sex	DD (yr)	Previous DMARDs	Concomitant therapy
1	48/F	7	SSZ, DP, GST, AUR, MTX, AZA, HCQ	Pred 5 mg

TABLE 12-continued

Demographic Features of 20 Patients with Refractory Rheumatoid Arthritis

Pat	Age/Sex	DD (yr)	Previous DMARDs	Concomitant therapy
2	63/F	7	SSZ, GST, DP	Para 1–2 g
3	59/M	3	AUR, HCQ, GST, MTX, SSZ	Pred 10 mg; Indo 225 mg
4	56/M	10	GST, DP, AZA, SSZ	Pred 12.5 mg; Ibu 2 g; Para 1–2 g
5	28/F	3	GST, SSZ, DP, AZA	Pred 8 mg; Para 1–2 g; Cod 16 mg
6	40/M	3	SSZ, HCQ, AUR	Nap 1 g
7	54/F	7	DP, GST, SSZ, AZA, MTX	Para 1–2 g; Cod 16–32 mg
8	23/F	11	HCQ, GST, SSZ, MTX, AZA	Pred 7.5 mg; Dicl 100 mg; Para 1–2 g; Dex 100–200 mg
9	51/F	15	GST, HCQ, DP, MTX	Pred 7.5 mg; Dicl 125 mg; Para 1–3 g
10	47/F	12	SSZ, CYC, MTX	Ben 4 g
11	34/F	10	DP, SSZ, MTX	Pred 10 mg; Para 1.5 g; Cod 30–90 mg
12	57/F	12	GST, MTX, DP, AUR	Asp 1.2 g
13	51/F	7	SSZ, AZA	Para 1–4 g
14	72/M	11	GST, DP, AZA, MTX	Pred 5 mg; Para 1–4 g; Cod 16–63 mg
15	51/F	17	HCQ, DP, SSZ, MTX	Asp 0.3 g
16	62/F	16	GST, DP, SSZ, MTX, AZA	Para 1–4 g; Cod 16–64 mg
17	56/F	11	SSZ, DP, GST, MTX, HCQ, AZA	Pred 7.5 mg; Eto 600 mg; Para 1–2 g; Dext 100–200 mg
18	48/F	14	GST, MTX, DP, SS, ZAUR, AZA	Pred 7.5 mg; Indo 100 mg; Para 1–3 g
19	42/F	3	SSZ, MTX	Fen 450 mg; Ben 6 g; Cod 30 mg
20	47/M	20	GST, DP, SSZ, AZA	Pred 10 mg; Para 1–3 g

Pat. = Patient;
DD(yrs) = Disease duration (years);
DMARDs = disease-modifying anti-rheumatic drugs;
SSZ = sulphasalazine;
DP = D-penicillamine;
GST = gold sodium thiomalate;
AUR = auranofin;
MTX = methotrexate;
AZA = azathioprine;
HCQ = (hydroxy)chloroquine;
CYC = cyclophosphamide.
Pred = prednisolone (dose/day);
Para = paracetamol;
Indo = Indomethacin;
Ibu = ibuprofen;
Cod = codeine phosphate;
Nap = naprosyn;
Dicl = diclofenac;
Dext = dextropropoxyphene;
Ben = benorylate;
Asp = aspirin;
eto = etodolac;
Fen = fenbufen.

TABLE 13

Changes in Clinical Assessments Following Treatment of Rheumatoid Arthritis Patients with cA2

Week of Trial	Morning Stiffness (min)	Pain Score (0–10) cm	Ritchie Index (0–69)	Swollen Joints (0–28) number	Grip Strength (L) (0–300) mm Hg	Grip Strength (R) (0–300) mm Hg	IDA (1–4)	Patient Assessment (grades improved 0–3)
Screen	135 (0–600)	7.4 (4–9.7)	23 (4–51)	16 (4–28)	84 (45–300)	96 (57–300)	3 (2.3–3.3)	NA
p value 0	180 (20–600)	7.1 (2.7–9.7)	28 (4–52)	18 (3–27)	77 (52–295)	92 (50–293)	2 (2–3.5)	NA
p value 1	20 (0–180) <0.001	2.6 (0.6–7.8) <0.001	13 (2–28) <0.001; <0.002	13.5 (1–25) >0.05	122 (66–300) >0.05	133 (57–300) >0.05	2 (1.5–3.3) <0.001	1 (1–3) NA
p value 2	15 (0–150) <0.001	3.0 (0.2–6.4) <0.001	13 (1–28) <0.001	11.5 (1–22) <0.003; <0.02	139 (75–300) <0.03; >0.05	143 (59–300) >0.05 <0.001	2 (1.5–3.2)	1.5 (1–3) NA
p value 3	5 (0–150) <0.001	2.2 (0.2–7.4) <0.001	8 (0–22) <0.001	6 (1–19) <0.001; <0.002	113 (51–300) >0.05	142 (65–300) >0.05	2 (1.2–3.2) <0.001	2 (1–2) NA
p value 4	15 (0–90) <0.001	1.90 (0.1–5.6) <0.001	10 (0–17) <0.001	6 (0–21) <0.001; <0.002	124 (79–300) <0.02; >0.05	148 (64–300) <0.03; >0.05	1.8 (1.3–2.7) <0.001	2 (1–2) NA
p value 6	5 (0–90) <0.001	1.9 (0.1–6.2) <0.001	6 (0–18) <0.001	5 (1–14) <0.001	119 (68–300) <0.04; >0.05	153 (62–300) <0.05; >0.05	1.7 (1.3–2.8) <0.001	2 (1–2) NA

TABLE 13-continued

Changes in Clinical Assessments Following Treatment of Rheumatoid Arthritis Patients with cA2

Week of Trial	Morning Stiffness (min)	Pain Score (0–10) cm	Ritchie Index (0–69)	Swollen Joints (0–28) number	Grip Strength (L) (0–300) mm Hg	Grip Strength (R) (0–300) mm Hg	IDA (1–4)	Patient Assess-ment (grades improved 0–3)
p value 8	15 (0–60) <0.001	2.1 (0–2–7.7) <0.001	8 (1–28) <0.001	7 (1–18) <0.001	117 (69–300) <0.03; >0.05	167 (52–300) <0.03; >0.05	1.8 (1.5–2.8) <0.001	2 (1–3) NA

Datas are expressed as the median (range) of values from 20 patients; data from patient 15 were not included after week 2 (dropout);
P values show significance by Mann-Whitney test compared with week 0 values; adjusted for multiple statistical comparisons.
IDA = Index of disease activity;
NA = not applicable.

TABLE 14

Changes in Laboratory Measures Following Treatment of Rheumatoid Arthritis patients with cA2

Week of Trial	Hgb g/liter	WBC × 10/liter	Platelet Count × 10/liter	ESR mm/hour	CRP mg/liter	SAA mg/ml	RF Inverse titer
Screen	117 (98–146)	7.9 (3.9–15.2)	353 (274–631)	59 (18–87)	42 (9–107)	ND	ND
P value 0	113 (97–144)	9.0 (4.9–15.7)	341 (228–710)	55 (15–94)	39.5 (5–107)	245 (18–1900)	2,560 (160–10,240)
p value 1	114 (96–145) >0.05	8.5 (3.6–13.6) >0.05	351 (223–589) >0.05	26 (13–100) >0.05	5 (0–50) <0.001	58 (0–330) <0.001; <0.003	ND
p value 2	112 (95–144) >0.05	8.2 (4.3–12.7) >0.05	296 (158–535) <0.04; >0.05	27 (10–90) <0.02; >0.05	5.5 (0–80) <0.001; <0.003	89 (11–900) <0.02; <0.04	ND
p value 3	110 (89–151) >0.05	9.0 (3.7–14.4) >0.05	289 (190–546) <0.03; >0.05	27 (12–86) <0.04; >0.05	7 (0–78) <0.01; <0.002	ND	ND
p value 4	112 (91–148) >0.05	8.2 (4.7–13.9) >0.05	314 (186–565) >0.05	23 (10–87) <0.04; >0.05	10 (0–91) <0.004; <0.02	ND	ND
p value 6	116 (91–159) >0.05	9.1 (2.9–13.9) >0.05	339 (207–589) >0.05	23 (12–78) <0.03; >0.05	8 (0–59) <0.001	ND	ND
p value 8	114 (94–153) >0.05	7.6 (4.2–13.5) >0.05	339 (210–591) >0.05	30 (7–73) >0.05	6 (0–65) <0.001	ND	480 (40–05,120) >0.05

Data are expressed as the median (range) of values from 20 patients; data from patient 15 were not included after week 2 (dropout). For rheumatoid factor (RF), only those patients with week 0 titers >1/160 in the particle agglutination assay were included (No. = 14).
P values show significance by Mann-Whitney test compared with week 0 values; adjusted for multiple statistical comparisons;
ND = not done.
Normal ranges: hemoglobin (Hgb) 120–160 g/liter (F), 135–175 g/liter (M); white blood cell count (WBC) 3–11 × 10^9/liter; platelet count 150–400 × 10^9/liter; erythrocyte sedimentation rate (ESR) < 15 mm/hour (F), <10 mm/hour (M); C-reactive protein (CRP) < 10 mg/liter; serum amyloid A(SAA) < 10 mg/ml.

All DMARDs were discontinued at least 1 month prior to trial entry. Patients were allowed to continue on a nonsteroidal anti-inflammatory drug and/or prednisolone (<12.5 mg/day) during the trial. The dosage of these agents was kept stable for 1 month prior to trial entry and during the course of the trial, and no parenteral corticosteroids were allowed during these periods. Simple analgesics were allowed ad libitum. Patients with other serious medical conditions were excluded. Specific exclusions included serum creatinine >150 mmol/liter (normal range 60-120 mmol/liter), hemoglobin (Hgb) <90 gm/liter (normal range 120-160 gm/liter [females]; 135-175 gm/liter [males]), white blood cell count (WBC) <4×10 g/liter (normal range 4-11×10^9/liter), platelet count <100×10 g/liter (normal range

150-400×10^9/liter), and abnormal liver function tests or active pathology on chest X-Ray.

All patients gave their informed consent for the trial, and approval was granted by the local ethics committee.

Treatment

The cA2 antibody was stored at 4° C. in 20 ml vials containing 5 mg of cA2 per milliliter of 0.01 M phosphate buffered saline in 0.15M sodium chloride at a pH of 7.2 and was filtered through a 0.2 μm sterile filter before use. The appropriate amount of cA2 was then diluted to a total volume of 300 ml in sterile saline and administered intravenously via a 0.2 μm in-line filter over a 2 hour period.

Patients were admitted to hospital for 8-24 hours for each treatment, and were mobile except during infusions. The trial was of an open, uncontrolled design, with a comparison of two treatment schedules. Patients 1 to 5 and 11 to 20 received a total of 2 infusions, each of 10 mg/kg cA2, at entry to the study (week 0) and 14 days later (week 2). Patients 6 to 10 received 4 infusions of 5 mg/kg activity included complete blood counts, C-reactive protein (CRP; by rate nephelometry) and the erythrocyte sedimentation rate (ESR; Westergren). Follow-up assessments were made at monthly intervals after the conclusion of the formal trial period, in order to assess the duration of response.

Analysis of improvement in individual patients was made using two separate indices. Firstly, an index of disease activity (IDA) was calculated for each time point according to the method of Mallya and Mace (Mallya et al., *Rheumatol. Rehab.* 20:14-17 (1981), with input variable of morning stiffness, pain score, Ritchie Index, grip strength, ESR and Hgb. The second index calculated was that of Paulus (Paulus et al., *Arthritis Rheum.* 33:477-484 (1990) which uses input variables of morning stiffness, ESR, joint pain/tenderness, joint swelling, patient's and physician's global assessment of disease severity. In order to calculate the presence or otherwise of a response according to this index, two approximations were made to accommodate our data. The 28 swollen joint count used by us (nongraded; validated in Fuchs et al., *Arthritis Rheum.* 32:531-537 (1989)) was used in place of the more extensive graded count used by Paulus, and the patient's and physician's global assessments of response recorded by us were approximated to the global assessments of disease activity used by Paulus infra. In addition to determining response according to these published indices, we selected 6 disease activity assessments of interest (morning stiffness, pain score, Ritchie index, swollen joint count, ESR and CRP) and calculated their mean percentage improvement. We have used FIGS. **24** and **25** to give an indication of the degree of improvement seen in responding patients.

Immunological Investigations

Rheumatoid factors were measured using the rheumatoid arthritis particle agglutination assay (PAPA, FujiBerio Inc., Tokyo, Japan), in which titers of 1/160 or greater were considered significant. Rheumatoid factor isotypes were measured by ELISA (Cambridge Life Sciences, Ely, UK). The addition of cA2 at concentrations of up to 200 μg/ml to these assay cA2, at entry, and days 4, 8 and 12. The total dose received by the 2 patient groups was therefore the same at 20 mg/kg.

Assessment Safety Monitoring

Vital signs were recorded every 15 to 30 minutes during infusions, and at intervals for up to 24 hours post infusion. Patients were questioned concerning possible adverse events before each infusion and at weeks 1, 2, 3, 4, 6, and 8 of the trial. A complete physical examination was performed at screening and week 8. In addition, patients were monitored by standard laboratory tests including complete blood count, C3 and C4 components of complement, IgG, IgM and IgA, serum electrolytes, creatinine, urea, alkaline phosphatase, aspartate transaminase and total bilirubin. Sample times for these tests were between 0800 and 0900 hours (pre-infusion) and 1200-1400 hours (24 hours post completion of the infusion). Blood tests subsequent to day 1 were performed in the morning, usually between 0700 and 1200 hours. Urine analysis and culture were also performed at each assessment point.

Response Assessment

The patients were assessed for response to cA2 at weeks 1, 2, 3, 4, 6 and 8 of the trial. The assessments were all made between 0700 and 1300 hours by the same observer. The following clinical assessments were made: duration of morning stiffness (minutes), pain score (0 to 10 cm on a visual analog scale), Ritchie Articular Index (maximum 69; Ritchie et al., *Quart. J. Med.* 147:393-406 (1968)), number of swollen joints (28 joint count; validated in Fuchs et al., *Arthritis Rheum.* 32:531-537 (1989), grip strength (0 to 300 mm Hg, mean of 3 measurements per hand by sphygmomanometer cuff) and an assessment of function (the Stanford Health Assessment Questionnaire (HAQ) modified for British patients; 34). In addition, the patients' global assessments of response were recorded on a 5-point scale (worse, no response, fair response, good response, excellent response). Routine laboratory indicators of disease systems did not alter assay results (data not shown). Antinuclear antibodies were detected by immunofluorescence on HEpo 2 cells (Biodiagnostics, Upton, Worcs. UK) and antibodies to extractable nuclear antigens were measured by counter immunoelectrophoresis with poly-antigen extract (Biodiagnostics). Sera positive by immunofluorescence were also screened for antibodies to DNA by the Farr assay (Kodak Diagnostics, Amersham, UK). Anti-cardiolipin antibodies were measured by ELISA (Shield Diagnostics, Dundee, Scotland). Serum amyloid A (SAA) was measured by sandwich ELISA (Biosource International, Camarillo, Calif., USA). Antiglobulin responses to the infused chimeric antibody were measured by an in-house ELISA, using cA2 as a capture reagent.

Cytokine Assays

Bioactive TNF was measured in sera using the WEHI 164 clone 13 cytotoxicity assay (Espevik et al., *J. Imm. Methods* 95:99-105 (1986). Total IL-6 was measured in sera using a commercial immunoassay (Medgenix Diagnostics, SA, Belgium) and by a sandwich ELISA developed "in house" using monoclonal antibodies provided by Dr. F. di Padova (Basel, Switzerland). Microtiter plates were coated with monoclonal antibody LNI 314-14 at a concentration of 3 ug/ml for 18 hours at 4° C. and blocked with 3% bovine serum albumin in 0.1M phosphate buffered saline, pH 7.2. Undiluted sera or standards (recombinant hIL 6, 0-8.1 ug/ml) were added to the wells in duplicate and incubated for 18 hours at 4° C. Bound IL-6 was detected by incubation with monoclonal antibody LNI 110-14 for 90 minutes at 37° C., followed by biotin labeled goat anti-murine IgG2b for 90 minutes at 37° C. (Southern Biotechnology, Birmingham, Ala.). The assay was developed using streptavidin—alkaline phosphatase (Southern Biotechnology) and p-nitrophenylphosphate as a substrate and the optical density read at 405 nm.

Statistics

Comparisons between week 0 and subsequent time points were made for each assessment using the Mann—Whitney

test. For comparison of rheumatoid factor (RAPA) titers, the data were expressed as dilutions before applying the test.

This was an exploratory study, in which prejudgements about the optimal times for assessment were not possible. Although it has not been common practice to adjust for multiple statistical comparisons in such studies, a conservative statistical approach would require adjustment of p values to take into account analysis at several time points. The p values have therefore been presented in two forms: unadjusted, and after making allowance for analysis at multiple time points by use of the Bonferroni adjustment. Where p values remained <0.001 after adjustment, a single value only is given. A p value of <0.05 is considered significant.

Results

Safety of cA2

The administration of cA2 was exceptionally well tolerated, with no headache, fever, hemodynamic disturbance, allergy or other acute manifestation. No serious adverse events were recorded during the 8-week trial. Two minor infective episodes were recorded, patient 15 presented at week 2 with clinical features of bronchitis and growth of normal commensals only on sputum culture. She had a history of smoking and of a similar illness 3 years previously. The illness responded promptly to treatment with amoxicillin, but her second cA2 infusion was withheld and the data for this patient are therefore not analyzed beyond week 2. Patient 18 showed significant bacteriuria on routine culture at week 6 (>10^5/ml; lactose fermenting coliform), but was asymptomatic. This condition also responded promptly to amoxicillin.

Routine analysis of blood samples showed no consistent adverse changes in hematological parameters, renal function, liver function, levels of C3 or C4 or immunoglobulins during the 8 weeks of the trial. Four minor, isolated and potentially adverse laboratory disturbances were recorded. Patient 2 experienced a transient rise in blood urea, from 5.7 mmol/liter to 9.2 mmol/liter (normal range 2.5 to 7 mmol/liter), with no change in serum creatinine. This change was associated with the temporary use of a diuretic, prescribed for a non-rheumatological disorder. The abnormality normalized within 1 week and was classified as "probably not" related to cA2. Patient 6 experienced a transient fall in the peripheral blood lymphocyte count, from 1.6 to 0.8×10^9/liter (normal range 1.0-4.8×10^9/liter). This abnormality normalized by the next sample point (2 weeks later), was not associated with any clinical manifestations and was classified as "possible related" to cA2. Patients 10 and 18 developed elevated titers of anti-DNA antibodies at weeks 6 and 8 of the trial, with elevated anti-cardiolipin antibodies being detected in patient 10 only. Both patients had a pre-existing positive antinuclear antibody and patient 10 had a history of borderline lymphocytopenia and high serum IgM. There were no clinical features of systemic lupus erythematosus and the laboratory changes were judged 'possibly related' to cA2.

Efficacy of cA2 Disease Activity

The pattern of response for each of the clinical assessments of disease activity and the derived IDA are shown in Table 13. All clinical assessments showed <improvement following treatment with cA2, with maximal responses from week 3. Morning stiffness fell from a median of 180 minutes at entry to 5 minutes at week 6 (p<0.001, adjusted), representing an improvement of 73%. Similarly, the Ritchie Index improved from 28 to 6 at week 6, (p<0.001, adjusted, 79% improvement) and the swollen joint count fell from 18 to 5, (p<0.001, adjusted, 72% improvement). The individual swollen joint counts for all time points are shown in FIG. **24**. Grip strength also improved; the median grip strength rose from 77 (left) and 92 (right) mm Hg at entry to 119 (left) and 153 (right) mmHg at week 6 (p<0.04, p<0.05, left and right respectively; p>0.05 after adjustment for multiple comparisons). The IDA showed a fall from a median of 3 at entry to 1.7 at week 6 (p<0.001, adjusted). Patients were asked to grade their responses to cA2 on a 5 point scale. No patient recorded a response of "worse" or "no change" at any point in the trial. "Fair", "good" and "excellent" responses were classed as improvements of 1, 2 and 3 grades respectively. At week 6, the study group showed a median of 2 grades of improvement (Table 13).

We also measured changes in the patients' functional capacity, using the HAQ modified for British patients (range 0-3). The median (range) HAQ score improved from 2(0.9-3) at entry to 1.1 (0-2.6) by week 6, (p<0.001; p<0.002 adjusted).

The changes in the laboratory tests which reflect disease activity are shown in Table 14. The most rapid and impressive changes were seen in serum CRP, which fell from a median of 39.5 mg/liter at entry to 8 mg/liter by week 6 of the trial (p<0.001, adjusted; normal range <10 mg/liter), representing an improvement of 80%. Of the 19 patients with elevated CRP at entry, 17 showed falls to the normal range at some point during the trial. The improvement in CRP was maintained in most patients for the assessment period (Table 14 and FIG. **25**); the exceptions with high values at 4 and 6 weeks tended to be those with the highest starting values (data not shown). The ESR also showed improvement, with a fall from 55 mm/hour at entry to 23 mm/hour at week 6 (p<0.03; p>0.05 adjusted; 58% improvement; normal range <10 mm/hour, <15 mm/hour, males and females respectively). SAA levels were elevated in all patients at trial entry, and fell from a median of 245 mg/ml to 58 mg/ml at week 1 (p<0.003, adjusted; 76% improvement; normal range <10/mg/ml) and to 80 mg/ml at week 2 (p<0.04, adjusted). No significant changes were seen in Hgb, WBC or platelet count at week 6, although the latter did improve at weeks 2 and 3 compared with trial entry (Table 14).

The response data have also been analyzed for each individual patient. The majority of patients had their best overall responses at week 6, at which time 13 assessed their responses as "good" while 6 assessed their responses as "fair". Eighteen of the 19 patients who completed the treatment schedule achieved an improvement in the index of Disease Activity (Mallya et al., *Rheumatol. Rehab.* 20:14-17 (1981) of 0.5 or greater at week 6, and 10 achieved an improvement of 1.0 or greater. All patients achieved a response at week 6 according to the index of Paulus (Paulus et al., *Arthritis Rheum.* 33:477-484 (1990). Finally, all patients showed a mean improvement at week 6 in the 6 selected measures of disease activity (as presented above) of 30% or greater, with 18 of the 19 patients showing a mean improvement of 50% or greater.

Although the study was primarily designed to assess the short-term effects of cA2 treatment, follow-up clinical and laboratory data are available for those patients followed for sufficient time (number=12). The duration of response in these patients, defined as the duration of a 30% (or greater) mean improvement in the 6 selected disease activity measures, was variable, ranging from 8 to 25 (median 14) weeks.

Comparison of the clinical and laboratory data for patients treated with 2 infusions of cA2 (each at 10 m/kg) compared

with those treated with 4 infusions (each at 5 mg/kg) showed no significant differences in the rapidity or extent of response (data not shown).

Immunological Investigations and Cytokines

Measurement of rheumatoid factor by RAPA showed 14 patients with significant titers (>1/160) at trial entry. Of these, 6 patients showed a fall of at least 2 titers on treatment with cA2, while the remaining patients showed a change of 1 titer or less. No patient showed a significant increase in RF titer during the trial. The median RF titer in the 11 patients fell from ½, 560 at entry to 1/480 by week 8 ($p>0.05$; Table 14). Specific RF isotypes were measured by ELISA, and showed falls in the 6 patients whose RAPA had declined significantly, as well as in some other patients. Median values for the three RF isotypes in the 14 patients seropositive at trial entry were 119, 102 and 62 IU/ml (IgM, IgG and IgA isotypes respectively) and at week 8 were 81, 64 and 46 IU/ml ($p>0.05$).

We tested sera from the first 9 patients for the presence of bioactive TNF, using the WEHI 164 clone 13 cytotoxicity assay (Espevik et al., *J. Imm. Methods* 95:99-105 (1986). In 8 patients, serum sets spanning the entire trial period were tested, while for patient 9, one pre-trial, one period were tested, patient, one pre, one intermediate and the last available sample only were tested. The levels of bioactive TNF were below the limit of sensitivity of the assay in the presence of human serum (1 pg/ml). Since production of CRP and SAA are thought to be regulated in large part by IL-6, we also measured serum levels of this cytokine, using 2 different assays which measure total IL-6. In the Medgenix assay, IL-6 was significantly elevated in 17 of the 20 patients at entry. In this group, levels fell from 60 (18-500) pg/ml to 40 (0-230) pg/ml at week 1 ($p>0.05$; normal range <10 pg/ml) and to 32 (0-210) pg/ml at week 2 ($p<0.005$, $p<0.01$, adjusted). These results were supported by measurement of serum IL-6 in the first 16 patients in a separate ELISA developed in-house. IL-6 was detectable in 11 of the 16, with median (range) levels falling from 210 (25-900) pg/ml at entry to 32 (01,700) pg/ml at week 1 ($p<0.02$, $p<0.04$, adjusted; normal range <10 pg/ml) and to 44 (0-240) pg/ml at week 2 ($p<0.02$, $p<0.03$, adjusted).

We tested sera from the first 10 patients for the presence of anti-globulin responses to the infused chimeric antibody, but none were detected. In many patients however, cA2 was still detectable in serum samples taken at week 8 and this can have interfered with the ELISA.

Discussion

This is the first report describing the use of anti-TNFα antibodies in human autoimmune disease. Many cytokines are produced in rheumatoid synovium, but we chose to target specifically TNFα because of mounting evidence that it was a major molecular regulator in RA. The study results presented here support that view and allow three important conclusions to be drawn.

First, treatment with cA2 was safe and the infusion procedure was well tolerated. Although fever, headache, chills and hemodynamic disturbance have all been reported following treatment with anti CD4 or anti CDw52 in RA, such features were absent in our patients. Also notable was the absence of any allergic event despite repeated treatment with the chimeric antibody, although the interval between initial and repeat infusions can have been too short to allow maximal expression of any anti-globulin response. The continuing presence of circulating cA2 at the conclusion of the trial may have precluded detection of antiglobulin responses, but also implied that any such responses were likely to be of low titre and/or affinity. Although we recorded 2 infective episodes amongst the study group, these were minor and the clinical courses were unremarkable. TNFα has been implicated in the control of listeria and other infections in mice (Havell et al., *J. Immunol.* 143:2894-2899 (1989), but our limited experience does not suggest an increased risk of infections after TNFα blockade in man.

The second conclusion concerns the clinical efficacy of cA2. The patients we treated had long-standing, erosive, and for the most part seropositive disease, and had each failed therapy with several standard DMARDs. Despite this, the major clinical assessments of disease activity and outcome (morning stiffness, pain score, Ritchie index, swollen joint count and HAQ score) showed statistically significant improvement, even after adjustment for multiple comparisons. All patients graded their response as at least "fair", with the majority grading it as "good". In addition, all achieved a response according to the criteria of Paulus and showed a mean improvement of at least 30% in 6 selected disease activity measures.

The improvements in clinical assessments following treatment with cA2 appear to be at least as good as those reported following treatment of similar patients with anti-leukocyte antibodies. The two therapeutic approaches can already be distinguished, however, by their effects on the acute phase response, since in several studies of antileukocyte antibodies, no consistent improvements in CRP or ESR were seen. In contrast, treatment with cA2 resulted in significant falls in serum CRP and SAA, with normalization of values in many patients. The changes were rapid and marked, and in the case of CRP, sustained for the duration of the study (Table 14). The falls in ESR were less marked, achieving statistical significance only when unadjusted (Table 14).

These results are consistent with current concepts that implicate TNFα in the regulation of hepatic acute phase protein synthesis, either directly, or by control of other, secondary, cytokines such as IL-6 (Fong et al., *J. Exp. Med.* 170:1627-1633 (1989); Guerne et al., *J. Clin. Invest.* 83:585-592 (1989)). In order to investigate the mechanism of control of the acute phase response in our patients, we measured serum TNFα and IL-6 before and after cA2 treatment. Bioactive TNFα was not detectable in baseline or subsequent sera. We used 2 different assays for IL-6, in view of previous reports of variability between different immunoassays in the measurement of cytokines in biological fluids (Roux-Lombard et al., *Clin. Exp. Rheum.* 10:515-520 (1992), and both demonstrated significant falls in serum IL-6 by week 2. These findings support the other objective laboratory changes induced by cA2, and provide in vivo evidence that TNFα is a regulatory cytokine for IL-6 in this disease. Amongst the other laboratory tests performed, rheumatoid factors fell significantly in 6 patients.

Neutralization of TNFα can have a number of beneficial consequences, including a reduction in the local release of cytokines such as IL-6 and other inflammatory mediators and modulation of synovial endothelial/leukocyte interactions. cA2 can also bind directly to synovial inflammatory cells expressing membrane TNFα, with subsequent in situ cell lysis.

The results obtained in this small series have important implications, both scientifically and clinically. At the scientific level, the ability of the neutralizing antibody, cA2, to reduce acute phase protein synthesis, reduce the production of other cytokines such as IL-6, and significantly improve the clinical state demonstrates that it is possible to interfere with the cytokine network in a useful manner without

untoward effects. Due to the many functions and overlapping effects of cytokines such as IL-1 and TNFα, and the fact that cytokines induce the production of other cytokines and of themselves, there had been some pessimism as to whether targeting a single cytokine in vivo would have any beneficial effect (Kingsley et al., *Immunol. Today* 12:177-179 (1991), Trentham, *Curr. Opin. Rheumatol.* 3:369-372 (1991)). This view is clearly refuted. On the clinical side, the results of short-term treatment with cA2 are significant and confirm that TNFα is useful as a new therapeutic target in RA.

EXAMPLE XXIII

Treatment with Chimeric Anti-TNF in a Patient with Severe Ulcerative Colitis

The patient is a 41 year old woman with long term ulcerative colitis, which was diagnosed by endoscopy and histology. She has a pancolitis, but the main disease activity was left-sided. There were no extra-intestinal complications in the past. Maintenance therapy consisted of ASACOL®. Only one severe flair-up occurred 4 years previously and was successfully treated with steroids.

At beginning month one, she was admitted elsewhere because of a very severe flair-up of the ulcerative colitis. Treatment consisted of high doses of steroids intravenously, antibiotics, ASACOL® and Total Parental Nutrition. Her clinical condition worsened and a colectomy was considered.

At end of month one, she was admitted at the internal ward of the AMC. Her main complaints consisted of abdominal pains, frequent water stools with blood and mucopus and malaise.

Medication: ASACOL® 2 dd 500 mg, orally
Di-Adresone-T 1 dd 100-mg, intravenously
Flagyl 3 dd 500 mg, intravenously
Fortum 3 dd 1 gram, intravenously
Total parental nutrition via central venous catheter

On physical examination the patient was moderately ill with a weight of 55 kg and a temperature of 36° C. Jugular venous pressure was not elevated. Blood pressure was 110/70 mm Hg with a pulse rate of 80 per minute. No lymphadenopathy was found. Oropharynx was normal. Central venous catheter was inserted in situ with no signs of inflammation at the place of insertion. Normal auscultation of the lungs and heart. The abdomen was slightly distended and tender. Bowel sounds where reduced. Liver and spleen where not enlarged. No signs of peritonitis. Rectal examination was normal.

All cultures of the stools where negative.

Plain x-ray of the abdomen; slightly dilated colon. No thumb-printing, no free air, no toxic megacolon.

Sigmoidoscopy; (video-taped) Very severe inflammation with deep ulcers. Dilated rectum and sigmoid. Because of danger of perforation the color, the endoscopy was limited to the racto-sigmoid. No biopsies where taken.

Conclusion at time of admission: Severe steroid resistant flair-up of ulcerative colitis.

Antibiotics were stopped, because no improvement was noticed and there was no temperature.

After informed consent of the patient, treatment was started with 10 mg/kg bodyweight (a 550 mg) of cA2 chimeric monoclonal anti-TNF (Centocor) given intravenously over 2 hours (according the protocol of cA2 used in severe Crohn's disease).

During the infusion there were no complaints. Vital signs were monitored and were all normal. Before and after infusion blood samples were drawn. Two days after infusion she had less abdominal pain, the stool frequency decreased and no blood was seen in the stools any more. However she developed high temperature (40° C.). Blood cultures were positive for *Staphylococcus epidermidis*. Infection of the central venous catheter was suspected. The catheter was removed and the same Staphylococcus was cultured from the tip of the central venous catheter. During this period she was treated with antibiotics for three days. After this her temperature dropped and she recovered substantially. Steroids were tapered off to 40 mg of prednisone daily.

After 14 days sigmoidoscopy was repeated and showed a remarkable improvement of the mucosa with signs of reepithelization. There were no signs of bleeding, less mucopus and even some normal vascular structures were seen.

At four months she was discharged.

At the outpatient clinic further monitoring was done weekly. Patient is still improving. Stool frequency is two times per day without blood or mucopus. Her laboratory improved, but there is still anaemia, probably due to iron deficiency. A colonoscopy is planned in the nearby future.

Our conclusion is that this patient had a very severe flair-up of her ulcerative colitis. She was refractory to treatment and a total colectomy was seriously considered. After infusion of cA2 the clinical course improved dramatically in spite of the fact that there was a complication of a sepsis which was caused by the central venous catheter.

EXAMPLE XXIV

Treatment of Rheumatoid Arthritis in Humans with cA2 Antibody Patients

Patients were recruited from the clinics of four cooperating trial centers or after referral from outside physicians. Patients aged 18-75 were included if they met the criteria of the American College of Rheumatology for the diagnosis of rheumatoid arthritis, had had disease for at least six months, had a history of failed treatment with at least one disease modifying anti-rheumatic drug (DMARD) and had evidence of erosive disease on radiography of hands and feet. In addition, patients had to have active disease, as defined by the presence of six or more swollen joints plus at least three of four secondary criteria (duration of morning stiffness ≧45 minutes; ≧6 tender or painful joints; erythrocyte sedimentation rate (ESR) ≧28 mm/h; C-reactive protein (CRF) ≧20 mg/L). Patients with severe physical incapacity (Steinbrocker class IV) or with clinically evident joint ankylosis were excluded. Other exclusion criteria included: severe anaemia (haemoglobin <8.5 g/dL); leucopenia (white cells $<3.5\times10^9$/L, neutrophils $<1.5\times10^9$/L) or thrombocytopenia (100×10^9/L); elevation of liver function tests to over three times the upper limit of normal or of serum creatine to over 150 μmol/L; or active pathology on chest film. Patients were also excluded if they had a history of previous administration of murine monoclonal antibodies, a history of cancer or HIV infection, or current other serious medical conditions. Female patients of child-bearing age had to be using an effective method of birth control and to have a negative pregnancy test before entry.

No patient had received other experimental drugs targeted to TNF (e.g., oxpentifylline) in the previous three months. Patients taking disease-modifying anti-rheumatic drugs at screening were withdrawn from their therapy at least four weeks before entry. Patients taking low-dose oral corticosteroids (prednisolone ≦12.5 mg per day) or non-steroidal anti-inflammatory drugs at screening were allowed to continue on stable doses. Additional steroids by injection or other routes were not allowed. Simple analgesics were freely allowed.

All patients gave their informed consent for the trial, which was approved by each of the local regional ethics committees.

Study Infusions

The cA2 antibody was supplied as a sterile solution containing 5 mg cA2 per ml of 0.01 mol/L phosphate-buffered saline in 0.15 mol/L sodium chloride with 0.01% polysorbate 80, pH 7.2. The placebo vials contained 0.1% human serum albumin in the same buffer. Before use, the appropriate amount of cA2 or placebo was diluted to 300 mL in sterile saline by the pharmacist, and administered intravenously via a 0.2 μm in-line filter over 2 hours. The characteristics of the placebo and cA2 infusion bags were identical, and the investigators and patients did not know which infusion was being administered.

Assessments

Patients were seen at an initial screening visit and if eligible, were entered within four weeks. On the day of entry, patients were admitted to the hospital and randomized (in blocks of 6, stratified for center) to one of three groups (24 per group). The first group received a single infusion of placebo. The other two groups received one infusion of cA2, 1 mg/kg ("low dose") and 10 mg/kg ("high dose"). The doses of cA2 were chosen on the basis of experience in the open-label trial and by extrapolation from the anti-TNF-treated collagen-arthritis mice.

Patients were monitored for adverse events during infusions and regularly thereafter, by interviews, physical examination, and laboratory testing.

Before the start of the trial, all clinical observers agreed on a standard technique to assess joints, and to establish protocols for the collection of other clinical data. In each center, patients were assessed by the same clinical observer at each evaluation visit, usually between 0800 and 1100 hour. Clinical observers were additionally blinded to the results of laboratory testing for acute-phase measures (ESR and CRP).

The six primary disease-activity assessments were chosen to allow analysis of the response in individual patients according to the Paulus index. The assessments contributing to this index were the tender and swollen joint scores (60 and 58 joints, respectively, hips not assessed for swelling; graded 0-3), the duration of morning stiffness (minutes), the patient's and observer's assessment of disease severity (on a 5 point scale, ranging from 1 (symptom-free) to 5 (very severe) and ESR. Patients were considered to have responded if at least four of the six variables improved, defined as at least 20% improvement in the continuous variables, and at least two grades of improvement or improvement from grade 1 to 1 in the two disease-severity assessments (Paulus 20% response). Improvements of at least 50% in the continuous variables were also used (Paulus 50%).

Other disease-activity assessments included the pain score (0-10 cm on a visual analogue scale (VAS)), an assessment of fatigue (0-10 cm VAS), and grip strength (0-300 mm Hg, mean of three measurements per hand by sphygmomanometer cuff).

The ESR was measured at each study site with a standard method (Westergen). CRP (Abbott fluorescent polarizing immunoassay) and rheumatoid factor (rheumatoid-arthritis particle-agglutination assay (RAPA, FujiBerio, Tokyo); titres $\geqq$160 were taken to be important) were measured in stored frozen serum samples shipped to a central laboratory.

Statistics

The analysis was on the basis of intention to treat. The sample size was chosen as having an 80% probability of achieving a statistically significant ($p<0.05$) result if the true response rates were 10% and 40% in the placebo and 10 mg/kg cA2 groups, respectively. Fisher's exact test was used to compare the groups for baseline sex ratio and rheumatoid factor status and for Paulus response rates. Comparisons between groups for other demographic features and for individual disease activity assessments were by analysis of variance, or Cochran-Mantel-Haenszel statistics where appropriate (baseline comparison of disease-modifying anti-rheumatic drugs usage, patient's and observer's assessments of disease severity/activity). The Paulus 20% response rate at week 4 was defined as the primary efficacy endpoint, with other time points and variables considered supportive. Levels of significance were therefore not adjusted for multiple comparisons.

Results

Seventy-two patients were initially randomized. One patient presented two weeks after treatment with 1 mg/kg cA2 with probable pneumonia that required admission to the hospital. The patient was withdrawn and, according to protocol, another patient was recruited. Thus, the intention-to-treat analysis brought the number analyzed in the 1 mg/kg group to 25 patients and the total number to 73.

The three groups were well-matched at entry, with no significant differences in age, sex ratio, disease duration, number of failed disease-modifying anti-rheumatic drugs, or percentage of patients with significant titre of rheumatoid factor (Table 15). Demographic data were similar between the four sites. The patients had active disease at entry, as judged by the presence of multiple tender and swollen joints, high pain scores, substantial morning stiffness, raised acute-phase measures (Table 16). Comparison between groups revealed no significant differences for any of the clinical and laboratory indices of disease activity at entry.

The response rates at Paulus 20% and 50% are shown in Table 17. Only 2 of 24 placebo recipients achieved a 20% response at week 4. By contrast, 19 of 24 patients treated with 10 mg/kg cA2 achieved a response by week 4 (p0.0001 compared with placebo). The response rates in the 1 mg/kg group were intermediate, with 11 of 25 patients responding at week 4 ($p=0.0083$). Analysis of the Paulus 50% response showed similar differences between the groups, with 14 of 24 high-dose cA2 patients responding ($p=0.0005$), compared with 2 of 24 patients in the placebo group. Analysis of the response data for corticosteroid use showed that patients who were taking steroids behaved no differently in their responses from non-steroid-treated patients.

Although secondary to the differences in overall response rates, analysis of changes in individual disease-activity assessments was also of interest (Table 16). Significant improvements were seen in both cA2 groups for each of the clinical assessments. For many assessments, maximum mean improvements in cA2-treated groups exceeded 60%. Among the laboratory measures, significant falls were seen in both cA2 groups for ESR, CRP, and platelet counts, with the best improvements seen in the high-dose group. The changes in CRP were particularly rapid in onset and impressive in extent, with many individual patients achieving normal concentrations (10 mg/L, data not shown). In addition, significant improvements relative to placebo were seen for haemoglobin, especially in the high-dose cA2 group. Trends towards a fall in white cell count (from increased counts at entry) in both cA2 groups supported the changes in other laboratory measures, but did not reach statistical significance (Table 16).

Detailed time response profiles for six disease-activity assessments common to the American College of Rheumatology and the European League Against Rheumatism core-sets showed rapid and highly significant falls in the cA2-treated groups compared with placebo, with significant

inter-group differences evident as early as 24 and 72 h (CRP and all other assessments, respectively).

Seeking possible dose-response relations, we compared response rates between the cA2 groups. We found no difference in 20% or 50% Paulus responses at week 2, but significantly higher response rates for the high-dose group at week 4 (likelihood ratio 1.8, 95% CI 1.1, 2.9, p=0.0186; 2.1, 1.1, 4.1, p=0.0450, for Paulus 20% and 50%, respectively). A similar analysis for each of the individual disease-activity assessments showed no greater benefit with the higher dose at week 2 of the study, except for haemoglobin (least squares mean difference 0.5, 95% CI 0.1, 0.9, p=0.021). By week 4, however, some diminution of the response in the 1 mg/kg group was evident for several assessments; responses in the 120 mg/kg group were maintained (Table 16). As a result, significantly better responses were seen at this time in the high-dose group, including pain score (least-squares mean difference −1.8, 95% CI −3.4-0.2, p=0.036), right (28.4, 5.4, 51.3, p=0.018) and left grip strength (20.6, 3.3, 37.9, p=0.022), observer's assessment of disease severity (−0.8, −1.3, −0.4, p<0.035), ESR (−15.0, −23.6 to −1.4 p=0.035), CRP (−20.7, −32.1, −9.2, p<0.001), and haemoglobin (0.5, 0.0, 1.0, p=0.042).

The infusions of cA2 and placebo were well tolerated, with no episodes of fever or hemodynamic disturbance. The adverse events recorded during the 4 weeks after treatment are shown in Table 18. In all, two-thirds of the adverse events occurred in the cA2 groups. Infections formed the largest group, with 5 infections recorded in the 1 mg/kg group and 1 each in those receiving 10 mg/kg cA2 and placebo.

Of the 72 initially randomized, 2 patients had severe adverse events. One was the patient with probable pneumonia. The patient recovered fully with treatment, but was withdrawn and replaced. This event was judged "possibly" related to cA2. A second patient presented 1 week after treatment with 10 mg/kg cA2 with a pathological fracture of the clavicle, but continued in the study. In retrospect, a minor bony abnormality was evident on an X-ray film taken pretreatment, and the event was judged "probably not" related to cA2.

TABLE 15

Demographic Features	Group		
	Placebo (n = 24)	1 mg/kg cA2 (n = 25)	10 mg/kg cA2 (n = 24)
Age (yr)	48-1 (11-9)	56-2 (12-2)	50-6 (13-1)
M/F	7/17	5/25	4/20
Disease Duration	9-0 (7-3)	7-5 (4-8)	7-3 (5-2)
Previous Drugs*	3-7 (1-9)	2-8 (1-5)	3-1 (1-7)
Rheumatoid Factor (seropositive)	7%	96%	75%

Mean (SC).

*Number of disease-modifying anti-rheumatic drugs previously used.

TABLE 16

	Disease Activity Assessments						
	Data Summary			Statistical analysis vs. Placebo			
		1 mg/kg	10 mg/kg	1 mg/kg cA2		10 mg/kg cA2	
Assess Wk	Placebo	cA2	cA2	Least-sq 95% CI	p	Least-sq 95% CI	p
Tender joint count							
0	27.8 (13.5)	29.1 (14.1)	28.1 (12.7)				
2	25.7 (16.6)	12.1 (10.2)	11.1 (6.9)	−14.8 −21.2, −8.4	<0.001	−14.8 −20.2, −9.5	<0.001
4	26.2 (15.5)	16.9 (12.1)	11.3 (9.8)	−10.9 −16.4, −5.3	<0.001	−15.2 −21.2, −9.2	<0.001
Swollen Joint count (0–58)							
0	23.4 (10.5)	21.4 (10.6)	21.8 (11.5)				
2	24.2 (12.1)	11.1 (8.1)	8.2 (5.5)	−10.9 −15.6, −6.3	<0.001	−14.4 −19.6, −9.2	<0.001
4	23.0 (11.2)	12.9 (8.8)	8.6 (6.4)	−8.2 −12.8, −3.6	0.001	−12.7 −17.8, −7.5	<0.001
Pain Score (0–10 cm)							
0	6.8 (1.8)	6.6 (2.6)	6.7 (2.5)				
2	6.9 (2.6)	2.5 (2.6)	2.6 (2.1)	−1.3 −5.7, −2.9	<0.001	−4.3 −5.8, −2.8	<0.001
4	6.9 (2.5)	4.2 (2.9)	2.5 (1.8)	−2.6 −4.2, −0.9	0.003	−4.3 −5.8, −2.9	<0.001
Morning Stiffness (min)							
0	132.3 (286.7)	142.0 (122.0)	143.1 (106.5)				
2	150.6 (284.0)	27.4 (48.7)	10.3 (14.9)	−88.9 −147.5, −30.3	0.004	−101.2 −156.4, −16.1	<0.001
4	172.3 (300.1)	99.6 (286.3)	8.3 (13.6)	−33.4 −156.4, 89–6	0.592	−124.8 −188.9, −60.8	<0.001
Fatigue Score (0–10 cm)							
0	6.3 (2.3)	6.5 (2.6)	5.6 (2.4)				
2	5.8 (2.9)	3.2 (2.7)	2.8 (2.3)	−2.6 −4.3, −1.0	−0.003	−2.3 −3.9, −0.7	0.006
4	5.6 (3.0)	3.8 (2.8)	2.3 (1.7)	−1.9 −3.6, −0.2	0.028	−2.6 4.3, −1.0	0.003

TABLE 16-continued

	Disease Activity Assessments						
	Data Summary			Statistical analysis vs. Placebo			
		1 mg/kg	10 mg/kg	1 mg/kg cA2		10 mg/kg cA2	
Assess Wk	Placebo	cA2	cA2	Least-sq 95% CI	p	Least-sq 95% CI	p
Grip Strength, right (0–300 mm Hg)							
0	120.7 (50.2)	102.4 (48.8)	117.0 (64.1)				
2	122.7 (51.5)	161.8 (78.3)	175.3 (79.1)	55.8 32.6, 79.0	<0.001	56.4 35.0, 77.3	<0.001
4	119.1 (50.2)	131.8 (65.0)	175.2 (78.6)	31.3 15.6, 46.9	<0.001	59.9 35.9, 83.9	<0.001
Grip Strength, left (0–300 mm Hg)							
0	120.0 (58.4)	100.8 (46.8)	108.4 (50.5)				
2	123.3 (64.9	152.4 (72.0)	157.2 (65.1)	46.7 23.7, 69.6	<0.001	45.4 28.9, 52.0	<0.001
4	120.9 (58.4)	126.6 (65.8)	155.1 (60.9)	25.0 6.5, 43.5	0.010	45.8 28.9, 62.7	<0.001
Disease Severity, patient (1–5)							
0	3.8 (0.5)	3.7 (0.5)	3.6 (0.6)				
2	3.8 (0.8)	3.5 (0.7)	2.6 (1.0)	−1.2 −1.6, −0.8	<0.001	−1.1 −1.6 −0.6	<0.001
4	3.3 (0.8)	3.0 (0.8)	2.6 (0.8)	−0.7 −1.2, −0.3	0.002	−1.2 −1.7 −0.8	<0.001
Disease Severity, Observer							
0	3.7 (0.7)	3.7 (0.5)	3.6 (0.7)				
2	3.5 (0.8)	2.5 (0.8)	2.3 (0.6)	−1.0 −1.5, −0.6	<0.001	−1.2 −1.6, −0.8	<0.001
4	3.6 (2.0)	3.0 (1.0)	2.2 (0.6)	−0.6 −1.1, −0.1	0.036	−1.4 −1.9, −1.0	<0.001
ESR (mm/h)							
0	63.1 (24.8)	58.1 (25.5)	63.1 (27.6)				
2	67.0 (27.4)	41.8 (24.6)	42.4 (25.2)	−23.1 −35.9, −10.4	<0.001	−24.9 −39.0, −10.9	<0.001
4	65.1 (29.8)	52.4 (32.3)	42.7 (24.6)	−10.7 −26.4, 5.1	0.185	−22.5 −38.7, −6.3	0.009
CRP (mg/L)							
0	64 (42)	67 (41)	64 (36)				
2	53 (30)	39 (39)	28 (29)	−19.1 −34.1, −4.1	0.016	−24.3 −38.8, −9.8	0.002
4	60 (42)	58 (39)	35 (29)	−7.7 −20.5, 5.1	0.239	−28.8 −33.7, −12.9	<0.001
Haemoglobin g/dL							
0	11.6 (1.6)	11.8 (1.3)	11.0 (1.1)				
2	10.9 (1.5)	11.5 (1.2)	11.2 (1.1)	0.4 0.0, 0.7	0.052	0.8 0.5, 1.2	<0.001
4	10.9 (1.7)	11.7 (1.2)	11.4 (1.2)	0.6 0.1, 1.0	0.022	1.1 0.6, 1.5	<0.001
WBC ($\times 10^9$/L)							
0	10.7 (3.5)	10.1 (3.5)	9.0 (2.1)				
2	10.5 (2.9)	9.2 (3.2)	8.4 (2.5)	−0.8 −1.9, 0.4	0.202	−0.4 −1.4, 0.6	0.414
4	10.3 (3.2)	9.3 (4.3)	7.7 (2.0)	−0.4 −1.6, 0.8	0.500	−0.9 −1.9, 0.2	0.096
Platelets ($\times 10^9$/L)							
0	447 (126)	421 (132)	400 (127)				
2	471 (135	375 (111)	368 (117)	−69 −108, −30	0.001	−56 −89, −22	0.002
4	462 (115)	406 (131)	345 (120)	−29 −073, 16	0.208	−69 −103, −36	<0.001

Mean(SD)

*0.2 1 = baseline and after 2 and 4 weeks of study.

Least sq. = least-squares mean difference.

WBC = white blood cells. Normal values: ESR, female < 15, male < 10; CRP < 10; haemoglobin, female 12–16, male 13.5–17.5; WBC 4–11, platelets 150–400.

TABLE 17

Responses According to Paulus 20% and 50% Criteria at Each Evaluation Point

	Data Summary			Statistical Analysis vs. Placebo					
		1 mg/kg	10 mg/kg	1 mg/kg cA2			10 mg/kg cA2		
	Placebo	cA2	cA2						
	n = 24	n = 25	n = 24	LR	95% CI	p	LR	95% CI	p
Paulus 20%									
Day 3	2 (8%)	8 (32%)	7 (29%)	3.8	1.1, 14.0	0.0738	3.5	0.9, 13.4	0.1365
Wk 1	2 (8%)	13(52%)	16(67%)	6.2	2.1, 18.6	0.0015	8.0	3.0, 21.5	0.0001
Wk 2	3(13%)	15(60%)	18(75%)	5.0	2.1, 12.2	0.0008	6.0	2.7, 13.5	<0.0001
Wk 3	4(17%)	12(48%)	21(88%)	2.9	1.2, 7.1	0.0322	5.3	2.7, 10.3	<0.0001
Wk 4	2 (8%)	11(44%)	19(79%)	5.3	1.7, 16.9	0.0083	9.5	3.9, 23.4	<0.0001
Paulus 50%									
Day 3	1(4%)	6 (24%)	2 (9%)	5.8	1.0, 33.1	0.0983	2.0	0.2, 20.0	1.000
Wk 1	1(4)	11(44%)	12(50%)	10.6	2.5, 44.6	0.0019	12.0	3.0, 47.5	0.007
Wk 2	0	11(44%)	12(50%)	NA	NA	0.0002	NA	NA	<0.0001
Wk 3	2(8%)	7 (28%)	13(54%)	3.4	0.9, 13.0	0.1383	6.5	2.2, 19.2	0.0013
Wk 4	2(8%)	7 (28%)	14(58%)	3.4	0.9, 13.0	0.1383	7.0	2.5, 20.0	0.0005

LR = likelihood ratio,
NA = not applicable (ratio could not be calculated because no placebo recipients responded at that time).

TABLE 18

All Adverse Events Recorded During 4 Weeks After Entry

System	Event	Pla-cebo	1 mg/kg cA2	10 mg/kg cA2
Respiratory	URTI	1(0)	2(0)	1(1)
	Probable pneumonia	—	1(1)	—
	Pleuritis	—	1(0)	—
Gastro intestinal	Nausea	2(0)	—	—
	Diarrhoea	1(1)	—	—
	Abdominal Pain	—	2(0)	—
	Peptic Ulcer	—	1(0)	—
	Blood loss per rectum	—	1(0)	—
Cardiovascular	Hypertension	1(0)	1(1)	1(1)
	Peripheral oedema	—	1(0)	1(0)
Skin	Rash	3(1)	1(0)	—
	Infection	—	2(2)	—
	Injection site reaction	—	1(1)	—
Neurological	Dizziness	3(1)	—	—
	Headache	—	—	1(0)
Musculoskeletal	Rheumatoid nodules	—	1(0)	—
	Popliteal cyst	—	1(0)	—
	Fracture	—	—	1(0)
Other	Malaise	—	1(0)	—
	Rigors	—	1(1)	—
	Facial oedema	—	1(0)	—
	Scleritis/conjunctivitis	—	1(0)	—
	Vasculitis	1(0)	—	—

URTI = upper respiratory tract infection. Those events judged by blinded observers to be reasonably related to infusion shown in brackets.

EXAMPLE XXV

Treatment of Crohn's Disease with cA2 Antibody

This patient is a 25 year old female known with Crohn's disease with an eight year history, who has had several exacerbations of Crohn's disease. Following the birth of a child the patient developed again an exacerbation. Prednisone treatment was increased to 30 mg about 15 months earlier. It was not possible to sufficiently taper off prednisone, and azathioprine was added to the therapy six months prior to antibody treatment. Remission could not be achieved, and in the end the patient was enrolled in this trial.

At enrollment her Crohn's medication had been stable for four months and consisted of mesalazine 3×250 mg, prednisone 20 mg, and azathioprine 100 mg. Her main symptoms were frequent diarrhoea, abdominal cramps and poor general well being. Her CDAI was 216, CRP 14 and ESR 32. Endoscopy prior to treatment showed severe inflammation with pseudopolyps and deep ulcerations of the ascending and transverse colon.

The patient was infused with 840 mg of cA2 (10 mg/kg). From day 5 and onwards through week 8 there was considerable improvement of her symptoms, as is also reflected by a marked decrease in the CDAI. For the first time in many years the patient had formed stools again. CRP and ESR also decreased a little, although not as markedly as the CDAI, but these were not that much elevated prior to the treatment. This improvement is also objectivated by the endoscopic findings, which show a greatly improved image at week 4 and a complete remission at week 8.

Prior to the infusion of cA2, the patient already had on her buttocks skin eruptions which were identified as pyoderma gangrenosum. These skin lesions also improved substantially during treatment, although they have not vanished completely. Before the treatment with cA2, the patient already had problems with decreased vision but she did not report this. During the follow up after three weeks she reported this and she was immediately sent to the ophthalmologist who concluded that the reduced vision was a result of cataract due to prednisone. This was the rationale for tapering off prednisone to 15 mg at week 6.

Colon biopsies taken during the 8 week endoscopy showed a mild to moderate focal epithelial dysplasia in one of the biopsy specimens. This is a common finding in patients with chronic inflammatory bowel disease. However,

differentiation between epithelial dysplasia associated with inflammatory bowel disease or a fragment of tubular adenoma could not be made.

Pre-Treatment Endoscopy

Smooth introduction of the scope till the bottom of the cecum. Because of slight edema it is not possible to enter the terminal ileum. Also the patient indicates that these attempts are quite painful. While retracting the scope the colon is inspected. Especially the promixal part till approximately the flexural lienalis is severely inflamed and has pseudopolyps and deep ulcerations. Also the mucosa is edematous. More distally the mucosa looks more normal. At about 15 cm, there is a small lesion, which could be the exit of a fistula. Biopsies are taken and the entire endoscopy is taken on video. Conclusion: Unchanged image compared to previous endoscopy.

Four Week Endoscopy

Smooth introduction of the scope till in the terminal ileum. While withdrawing, the terminal ileum and cecum are inspected. The inflammation is considerably less than compared to the endoscopy of 4 weeks ago. There are still a few pseudopolyps and left and right a small ulceration, but compared to the endoscopy of 4 weeks ago, there is a dramatic improvement of the endoscopic image. Distally the mucosa looks normal. A diligent search has been made for the fistula opening which was seen last time at 15 cm of the anal ring. At this location we do see a scar with a slightly indurated edge, but at this moment it does not appear to be a real fistula opening. Conclusion: Greatly improved endoscopic image, compared to the endoscopy of 4 weeks ago.

Eight Week Endoscopy

There are only pseudopolyps left. Conclusion: Complete remission.

EXAMPLE XXVI

p55 Fusion Protein Structure

The extracellular domains of the p55 and p75 receptors were expressed as Ig fusion proteins from DNA constructs designed to closely mimic the structure of naturally occurring, rearranged Ig genes. Thus, the fused genes included the promoter and leader peptide coding sequence of a highly expressed chimeric mouse-human antibody (cM-T412, Looney et al., *Hum. Antibody Hybridomas* 3:191-200 (1992)) on the 5' side of the TNF receptor insert, and codons for eight amino acids of human J sequence and a genomic fragment encoding all three constant domains of human IgG1 on the 3' side of the receptor insert position (FIGS. **27** and **29**).

Minor changes were introduced at the N-terminal ends of the heavy chain fusion proteins so that the first two amino acids would be identical or similar to the first two amino acids (Gln-Ile) encoded by the cM-T412 antibody gene (from which the leader peptide originated). This was done to increase the likelihood that any interactions between the N-terminal end of the mature protein and the leader peptide would still result in efficient transport into the lumen of the endoplasmic reticulum. Boyd et al., *Cell* 62: 1031-1033 (1990). Therefore, the Asp^1 and Ser^2 residues of naturally occurring p55 were replaced with a Gln residue, and the Leu^1 residue of p75 was preceded by a Gln residue in all p75 constructs. No amino acid changes were introduced at the N-terminal end of the p55 light chain fusion.

Expression Vectors

PCR methodology was used to engineer cloned genes. Oligonucleotides were purchased from National Biosciences (Plymouth, Minn.). PCR amplification kits were from Perkin Elmer (CA) and DNA sequencing kits from U.S. Biochemical Corporation (Cleveland, Ohio). Alkaline phosphatase-conjugated goat anti-human IgG was purchased from Jackson ImmunoResearch (West Grove, Pa.). ^{125}I-labeled human TNF was obtained from Du Pont Company, NEN (Boston, Mass.) and unlabeled recombinant human TNF from R&D Systems (Minneapolis, Minn.). Protein A-Sepharose beads was purchased from PHARMACIA (Piscataway, N.J.).

PCR methodology was used to engineer two cloned genes encoding the heavy chain or light chain of an efficiently expressed murine antibody, cM-T412 (see Looney et al.), for the purpose of directing the expression of foreign genes in a mammalian cell system. The approaches were to effectively delete the coding region of the antibody variable region and to place a unique restriction site in its place (StuI for the heavy chain vector and EcoRV for the light chain vector).

The resulting vector contained 2.5 kb of 5' flanking genomic DNA, the promoter, the leader peptide coding sequence (including the leader intron), a StuI cloning site to introduce inserts, coding sequence for eight amino acids of human J sequence Gly Thr Leu Val Thr Val Ser Ser (SEQ ID NO:6) followed by genomic sequences for the human IgG1 constant region. An analogous vector was made from the cM-T412 light chain gene except that an EcoRV cloning site was introduced at the carboxyl terminal end of the light chain leader peptide and a different human J sequence was encoded by the vector Gly Thr Lys Leu Glu Ile Lys (SEQ ID NO:7). Both vectors are based on plasmid pSV2-gpt and subsequent vector derivatives that contain genomic sequences for either the heavy chain or light chain constant regions. See Mulligan et al., *Science* 209:1422-1427 (1980). The *E. coli* gpt gene allows selection of transfected cells with mycophenolic acid.

Heavy Chain Vector

A previously cloned EcoRI fragment containing the cM-T412 heavy chain gene (Goeddel et al., *Cold Spring Harbor Symp. Quant. Biol.* 51: 597-609 (1986)) was subcloned into pUC19. This recombinant plasmid was used as a template for two PCR reactions. In one reaction, an oligo corresponding to the "reverse" primer of the pUC plasmids and the 3' oligo 5'-CCTGGATACCTGTGAAAAGA-3' (SEQ ID NO:8) (with half of a StuI site; oligo was phosphorylated prior to the PCR reaction) were used to amplify a fragment containing 3 kb of 5' flanking DNA, the promoter, transcription start site and leader peptide coding sequence (including the leader intron). In the second reaction, the 5' oligo 5'CCTGGTACCTTAGTCACCGTCT CCTCA-3' (SEQ ID NO:9) (with half of a StuI site; oligo phosphorylated prior to the PCR reaction) and an oligo corresponding to the "forward" primer of pUC plasmids amplified a fragment encoding eight amino acids of human J sequence Gly Thr Leu Val Thr Val Ser Ser (SEQ ID NO:6) and a splice donor to allow splicing to the human constant region coding sequence provided in another vector. The two PCR fragments were digested with EcoRI and then simultaneously ligated into EcoRI-digested pUC19 to make pHC684 (FIG. **27**).

Because the StuI site formed at the junction of the two PCR fragments was followed by a "GG" dinucleotide sequence, a dcm methylation site was formed preventing StuI from digesting that site when the DNA was grown in HB 101 strain of *E coli*. Therefore, the plasmid DNA was

introduced into dcm-JM110 *E. Coli* cells and reisolated. StuI was then able to cut at the junction but a second StuI site in the 5' flanking DNA was a apparent (DNA sequencing showed that StuI site to also be followed by a GG dinucleotide and therefore also methylated). To make the StuI cloning site at the junction be unique, a 790 bp XbaI fragment that included only one of the two StuI sites was subcloned into pUC19 to make the vector pHC707 (FIG. **27**) which was then grown in JM110 cells. The StuI cloning site formed at the junction of the two PCR fragments second and third nucleotides (i.e., "CA") of the last codon (Ala) of the signal sequence in order to maintain the appropriate translation reading frame (FIG. **27**).

A PCR fragment encoding a protein of interest can then be ligated into the unique StuI site of pHC707. The insert can include a translation stop codon that would result in expression of a "non-fusion" protein. Alternatively, a fusion protein could be expressed by the absence of a translation stop codon, thus allowing translation to proceed through additional coding sequences positioned downstream of the StuI cloning site. pH 730 contains coding sequences for all three constant domains of human IgG1 and was designed to accomodate the Xba**1** fragments of pHC707 at a unique Xba**1** site upstream of the IgG1 coding sequences (FIG. **28**). Coding sequences in the Stu**1** site of pHC707 would not be fused directly to the IgG1 coding sequences in pHC730 but would be separated by an intron sequence that partially originates from pHC707 and partially from pHC730. These intron sequences would be deleted in the cell following transcription resulting in an RNA molecule that is translated into a chimeric protein with the protein of interest fused directly to the IgG1 constant domains.

The plasmid pHC730 was a modified form of an IgG1 expression, pSV2gpt-hCy1 vector described previously (Goeddel et al., *Cold Spring Harbor Symp. Quant. Biol.* 51:597-609 (1986)) (FIG. **28**). The modifications were (1) removal of the unique Sal**1** and Xba**1** sites upstream of the constant region coding sequence, (2) insertion of a Sal**1** linker into the unique BamHI site to allow use of Sal**1** to linearize the plasmid prior to transfections, and (3) ligation into the unique EcoRI site the cloned cM-T412 EcoRI fragment but with the Xba**1** fragment flanking the V gene deleted (FIG. **28**). The resulting expression vector had a unique Xba**1** site for inserting the Xba**1** fragments from pHC707.

Light Chain Vector

A previously cloned HindIII fragment containing the cM-T412 light chain gene (Goeddel et al., *Cold Spring Harbor Symp. Quant. Biol.* 51:597-609 (1986)) was subcloned into pUC19 and the resulting plasmid used as template for PCR reactions. In one PCR reaction the "reverse" pUC primer and the 3' oligo 5'-AATAGATATCTCCTTCAACACCTGCAA-3' (SEQ ID NO:10) (with an EcoRV site) were used to amplify a 2.8 kb fragment containing 5' flanking DNA, the promoter, transcription start site and leader peptide coding sequence (including the leader intron) of the cloned light chain gene. This fragment was then digested with HindIII and EcoRV. In a second PCR reaction, the 5' oligo 5'-ATCGGG ACAAAGTTGG AAATA-3' (SEQ ID NO:11) (with half of an EcoRV site) and the "forward" pUC primer were used to amplify a fragment encoding seven amino acids of human J sequence (Gly Thr Lys Leu Glu Ile Lys) and an intron splice donor sequence. This fragment was digested with HindIII and ligated along with the other PCR fragment into pUC cut with HindIII. The resulting plasmid, pLC671 (FIG. **29**), has a unique EcoRV cloning site at the junction of the two PCR fragments with the EcoRV site positioned such that the first three nucleotides of the EcoRV site encoded the first amino acid of the mature protein (Asp).

The pLC671 HindIII insert was designed to be positioned upstream of coding sequences for the human kappa light chain constant region present in a previously described expression vector, pSV2gpt-hCk (FIG. **30**). However, pSV2gpt-hCk contained an EcoRV site in its gpt gene. Because it was desired that the EcoRV site in the pLC671 HindIII fragment be a unique cloning site after transferring the fragment into pSV2gpt-hCk, the EcoRV site in pSV2gpt-hCk was first destroyed by PCR mutagenesis. Advantage was taken of the uniqueness of this EcoRV site in pSV2gpt-hCk and a Kpn1 site 260 bp upstream of the EcoRV site. Therefore, the 260 bp Kpn1-EcoRV fragment was removed from pSV2gpt-hCk and replaced with a PCR fragment that has identical DNA sequence to the 260 bp fragment except for a single nucleotide change that destroys the EcoRV site. The nucleotide change that was chosen was a T to a C in the third position of the EcoRV recognition sequence (i.e., GATATC changed to GACATC). Because the translation reading frame is such that GAT is a codon and because both GAT and GAG codons encode an Asp residue, the nucleotide change does not change the amino acid ended at that position. Specifically, pSV2gpt-hCk was used as template in a PCR reaction using the 5' oligo 5'GGCGGTCT GGTACCGG-3' (SEQ ID NO:12) (with a Kpn1 site) and the 3' oligo 5'-GTCAACAACATAGTCATCA-3' (SEQ ID NO:13) (with the complement of the Asp codon). The 260 bp PCR fragment was treated with the Klenow fragment of DNA polymerase to fill-in the DNA ends completely and then digested with Kpn1. The fragment was ligated into pSV2gpt-hCk that had its KpnI-EcoRV fragment removed to make pLC327 (FIG. **30**).

The HindIII fragment of pLC671 was cloned into the unique HindIII site of pLC327 to make the light chain expression vector, pLC690 (FIG. **30**). This plasmid can be introduced into cells without further modifications to encode a truncated human kappa light chain, JCk, that contains the first two amino acids of the cM-T412 light chain gene, seven amino acids of human J sequences, and the light chain constant region. Alternatively, coding sequence of interest can be introduced into the unique EcoRV site of pLC690 to make a light chain fusion protein.

TNF Receptor DNA Constructs

For the p55 heavy chain fusion, amino acids 3-159 of the p55 extracellular domain were encoded in a PCR fragment generated using the 5' oligo 5' CACAGGTGTGTCCCCAAGGAAAA-3' (SEQ ID NO:14) (with the Val^3 codon) and the 3' oligo 5'-AATCTGGGGTAGGCACAA-3' (SEQ ID NO:15) (with the complement of the Ile^{159} codon). For the p55 light chain fusion, amino acids 2-159 were encoded in a PCR fragment made using the 5' oligo 5,-AGTGTGTGTCCCCAAGG3' (SEQ ID NO:16) (with the Ser^2 codon) and the same 3' oligo shown above. The light chain vector contained the codon for Asp^1 of p55. The DNA template for these PCR reactions was a previously reported human p55 cDNA clone. (Gray et al., *Proc. Natl. Acad. Sci. USA* 87:7380-7384 (1990)).

A truncated light chain that lacked a variable region was expressed by transfecting cells with the light chain vector with no insert in the EcoRV cloning site. The resulting protein, termed JC_K, consisted of the first two amino acids of the cM-T412 light chain gene, seven amino acids of

human J sequence (Gly Thr Lys Leu GluIle Lys) (SEQ ID NO:7), and the human light chain constant region.

A non-fusion form of p55 (p55-nf) was expressed in CHO—K1 cells using the CMV-major immediate early promoter after introducing a translation stop codon immediately after Ile^{159}. Secreted p55 was purified by affinity chromatography on a TNFα column.

Transfections and ELISA Assays

All plasmids were linearized with a restriction enzyme prior to introducing them into cells. Cells of the myeloma cell line X63-Ag8.653 were transfected with 12 μg of DNA by electroporation. Cell supernatants were assayed for IgG domains. Briefly, supernatants were incubated in plates coated with anti-human IgG Fe and then bound protein detected using alkaline phosphatase-conjugated anti-human and light chains.

Purification of Fusion Proteins

Cell supernatants were clarified by centrifugation followed by passage through a 0.45 micron filter. Supernatants were adjusted to 20 mM Tris-HCl, pH 8.3, 150 mM NaCl, and 1 mM EDTA (1×protein A buffer) and passed over a column of protein A-Sepharose beads. The column was washed in IX protein A buffer followed by 100 mM Na Citrate, pH 5.0 to elute bound bovine IgG originating from the cell media. Bound fusion protein was then eluted in 100 mM Na Citrate, pH 3.5, neutralized with 0.2 volumes 1 M Tris, and dialyzed against PBS.

TNF Cytotoxicity Assays

TNF-sensitive WEHI-164 cells (Espevik et al., *J. Immunol. Methods* 95:99-105 (1986)) were plated in 1 μg/ml actinomycin D at 50,000 cells per well in 96-well microtiter plates for 3-4 hours. Cells were exposed to 40 pM TNFα or TNFβ and varying concentrations of fusion protein. The mixture was incubated overnight at 37° C. Cell viability was determined by adding 3-[4,5-dimethyl-thiazol-2-yl]-2, 5diphenyltetrazolium bromide dye (MTT) to a final concentration of 0.5 mg/ml, incubating for 4 hours at 37° C., lysing the cells in 0.1 N HCl, 0.1% SDS and measuring the optical density at 550 nm wavelength.

Saturation Binding Analyses

Fusion proteins were captured while at a concentration of 10 ng/ml in 96-well microtiter plates coated with goat anti-human Fe antibodies. Varying concentrations of ^{125}I-TNF (34.8 μCi/μg) were added in PBS/1% BSA and allowed to bind for two hours at room temperature. Plates were washed and bound cpm determined. Non-specific binding was determined using an irrelevant antibody.

Several different versions of the p55 fusion proteins were expressed. Unlike what was reported for CD4 (Capon et al., *Nature* 337:525-531 (1989)) and IL-2 (Landolfi, *J. Biol. Chem.* 146:915-919 (1991)) fusion proteins that also included the CHI domain of the heavy chain, inclusion of a light chain proved to be necessary to get secretion of the Ig heavy chain fusion proteins from the murine myeloma cells. The light chain variable region was deleted to enable the TNF R domain on the heavy chain to bind TNF without steric hindrance from the light chain.

The "double fusion" (df) protein, p55-df2, has p55 fused to both the heavy chain and light chain and is therefore tetravalent with regard to p55. p55-sf3 has the p55 receptor (and the same eight amino acids of human J sequence present in p55-sf2 and p55-df2) linked to the hinge region, i.e., the C_H1 domain of the constant region is deleted.

After one or two rounds of subcloning, spent cell supernatant from the various cell lines were yielding 20 μg/ml (for p55-sf2) of fusion protein. The proteins were purified from the spent supernatant by protein A column chromatography and analyzed by SDS-PAGE with or without a reducing agent. Each fusion protein was clearly dimeric in that their M_r estimates from their migration through a non-reducing gel was approximately double the estimated M_r from a reducing gel. However, two bands were seen for p55-sf2 (lane 1) and p55-df2. Two lines of evidence indicated that, in each case, the lower bands did not include a light chain while the upper bands did include a light chain. First, when p55-sf2 containing both bands were passed over an anti-kappa column, the upper band bound to the column (lane 3) while the lower band passed through the column. Second, Western blots have shown that only the upper bands were reactive with anti-kappa antibodies.

It is believed that the versions of these fusion proteins that do not have a light chain (k) were not secreted to a significant degree but rather were primarily released from dead cells because 1) supernatants from cells transfected with the p55 heavy chain fusion gene and no light chain gene did not have detectable fusion protein until after there was significant cell death, and 2) the ratio of the k– to k+ versions of p55-sf2 increased as cell cultures went from 95% viability to 10% viability.

EXAMPLE XXVII

p75

To make a p75 heavy chain fusion (p75-sf2), amino acids 1-235 (Smith et al., *Science* 248: 1019-1023 (1990) and Kohno et al., *Proc. Natl. Acad. Sci.* 87:8331-8335 (1990)) were encoded in a fragment prepared using the 5' oligo 5'CACAGCTGCCCGCCCAGGTGGCAT-3' (SEQ ID NO:17) (with the Leu^1 codon) and the 3' oligo 5'-GTCGC-CAGTGCTCCC TT-3' (SEQ ID NO: 18) (with the complement of the Asp^{235} codon). Two other p75 heavy chain fusions (p75P-sf2 and p75P-sf3) were made using the same 5' oligo with the 3' oligo 5'ATCGGACGTGGACGTG-CAGA-3' (SEQ ID NO:19). The resulting PCR fragment encoded amino acids 1-182. The PCR fragments were blunt-end ligated into the StuI or EcoRV site of the appropriate vector and checked for the absence of errors by sequencing the inserts completely.

Several different versions of the p75 fusion proteins were also expressed. p75-sf2 has the complete extracellular domain of p75 fused to the heavy chain while p75P-sf2 lacks the C-terminal 53 amino acids of the p75 extracellular domain. p75P-sf3 is the same as p75P-sf2 except that it lacks the C_H1 domain. The region deleted in p75P-sf2 and -sf3 contains sites of O-linked glycosylation and a proline-rich region, neither of which is present in the extracellular domain of p55. Seckinger et al., *Proc. Nat. Acad. Sci. USA* 87:5188-5192 (1990).

Similar to pS5-sf2, two bands were seen for p75-sf2 (lane 7) and p75P-sf2 (lane 8).

WEHI Cytotoxicity Assays

The ability of the various fusion proteins to bind and neutralize human TNFα or TNFβ was tested in a TNF-mediated cell killing assay. Overnight incubation of the murine fibrosarcoma cell line, WEHI 164 (Espevik et al., *J. Immunol. Methods* 95:99-105 (1986)), with 20 pM (1 ng/ml) TNFα results in essentially complete death of the culture. When the fusion proteins were pre-incubated with TNFα (FIGS. **31**A, B and C and Table 1 above) or TNFβ (FIG. **32**) and the mixture added to cells, each fusion protein demon-

strated dose-dependent protection of the cells from TNF cytotoxicity. Comparison of the viability of control cells not exposed to TNF to cells incubated in both TNF and fusion protein showed that the protection was essentially complete at higher concentrations of fusion protein.

Tetravalent p55-df2 showed the greatest affinity for TNFα requiring a concentration of only 55 pM to confer 50% inhibition of 39 pM (2 ng/ml) TNFα (FIG. **31**A and Table 1). Bivalent p55-sf2 and p75P-sf2 were nearly as efficient, requiring concentrations of 70 pM to half-inhibit TNFα. Approximately two times as much p75-sf2 was required to confer 50% inhibition compared to p55-sf2 at the TNF concentration that was used. The monomeric, non-fusion form of p55 was much less efficient at inhibiting TNFα requiring a 900-fold molar excess over TNFα to inhibit cytotoxicity by 50%. This much-reduced inhibition was also observed with a monomeric, Fab-like p55 fusion protein that was required at a 2000-fold molar excess over TNFα to get 50% inhibition. The order of decreasing inhibitory activity was therefore p55-df2>p55-sf2=p75P-sf2>p75-sf2>>>monomeric p55.

Surprisingly, the order of decreasing inhibitory activity was different for TNFβ, as presented in FIG. **32**. p75P-sf2 was most efficient at inhibition requiring a concentration of 31 pM to half-inhibit human TNFβ at 2 pM. Compared to p75P-sf2, three times as much p75-sf2 and three times as much p55-sf^2 were necessary to obtain the same degree of inhibition. The order of decreasing inhibitory activity was therefore p75P-sf2>p75-sf2=p55-sf2.

Affinity Measurements

A comparison was made of the binding affinity of various fusion proteins and TNFα by saturation binding (FIGS. **33**A and **33**B) and Scatchard analysis (FIGS. **33**C-**33**H). A microtiter plate was coated with excess goat anti-Fc polyclonal antibody and incubated with 10 ng/ml of fusion protein in TBST buffer (10 mM Tris-HCl, pH 7.8, 150 NaCl, 0.05% TWEEN® 20) for 1 hour. Varying amounts of ^{125}I labeled TNFα (specific activity—34.8 μCi/μg) was then incubated with the captured fusion protein in PBS (10 mM Na Phosphate, pH 7.0, 150 mM NaCl) with 1% bovine serum albumin for 2 hours. Unbound TNFα was washed away with four washes in PBS and the cpm bound was quantitated using a y-counter. All samples were analyzed in triplicate. The slope of the lines in (FIGS. **33**C-H) represent the affinity constant, K_a. The dissociation constant (K_d) values (see Table 1) were derived using the equation K_d=1/K.

EXAMPLE XXVIII

In Vivo Results

C3H mice were challenged with 5 μg of human TNFα after treatment with an immunoreceptor molecule of the invention. The effect of the treatment was compared with two control treatments. The first control, cA2, is a chimeric mouse/human IgG_1 monoclonal antibody that binds human TNF, and thus is a positive control. The second control, c17-1A, is a chimeric mouse/human IgG_1 irrelevant monoclonal antibody and is thus a negative control. The results of the treatments were as presented in the following Table 19.

TABLE 19

Treatment	Dead Fraction	% Dead
1 μg cA2	5/14	36%
10 μg cA2	1/15	7%
50 μg c17-1A	13/15	87%
1 μg p55-sf2	8/15	53%
10 μg p55-sf2	0/15	0%
50 μg p55-sf2	0/15	0%

Mice were injected with 25 μg of p55 fusion protein or a control antibody and 1 hour later were challenged with 1 μg lipopolysaccharide (type J5). Mice were checked 24 hourts later. The results are presented in the following Table 20.

TABLE 20

Treatment	Dead Fraction	% Dead
Control Antibody	11/11	100%
p55-sf2	0/13	0%

All references cited herein, including journal articles or abstracts, published or corresponding U.S. or foreign patent applications, issued U.S. or foreign patents, or any other references, are entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited references. Additionally, the entire contents of the references cited within the references cited herein are also entirely incorporated by reference.

Reference to known method steps, conventional methods steps, known methods or conventional methods is not in any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the relevant art.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance presented herein, in combination with the knowledge of one of ordinary skill in the art.

Equivalents

Those skilled in the art will know, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. These and all other equivalents are intended to be encompassed by the following claims.

SEQUENCE LISTING

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<212> TYPE: PRT
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            20                  25                  30

Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu
        35                  40                  45

Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe
    50                  55                  60

Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile
65                  70                  75                  80

Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala
                85                  90                  95

Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys
            100                 105                 110

Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu Lys
        115                 120                 125

Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp Phe
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Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu
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Glu Arg Val Ser Phe Ser Cys Arg Ala Ser Gln Phe Val Gly Ser Ser
             20                  25                  30

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Lys Tyr Ala Ser Glu Ser Met Ser Gly Ile Pro Ser Arg Phe Ser Gly
     50                  55                  60

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Lys Tyr Ala Ser Glu Ser Met Ser Gly Ile Pro Ser Arg Phe Ser Gly
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Ser Met Lys Leu Ser Cys Val Ala Ser Gly Phe Ile Phe Ser Asn His
             20                  25                  30

tgg atg aac tgg gtc cgc cag tct cca gag aag ggg ctt gag tgg gtt      144
Trp Met Asn Trp Val Arg Gln Ser Pro Glu Lys Gly Leu Glu Trp Val
         35                  40                  45

gct gaa att aga tca aaa tct att aat tct gca aca cat tat gcg gag      192
Ala Glu Ile Arg Ser Lys Ser Ile Asn Ser Ala Thr His Tyr Ala Glu
     50                  55                  60

tct gtg aaa ggg agg ttc acc atc tca aga gat gat tcc aaa agt gct      240
Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asp Ser Lys Ser Ala
 65                  70                  75                  80

gtc tac ctg caa atg acc gac tta aga act gaa gac act ggc gtt tat      288
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What is claimed is:

1. A method of inhibiting TNFα in a human patient, wherein said human patient has fistulas in Crohn's disease, comprising administering to the human at least four doses provided as single or divided 0.1-50 mg/kg doses of a TNFα-inhibiting amount of an anti-TNFα antibody or antigen-binding fragment thereof, said antibody comprising a human constant region, wherein said anti-TNFα antibody or antigen-binding fragment (i) competitively inhibits binding of A2 (ATCC Accession No. PTA-7045) to human TNFα, and (ii) binds to a neutralizing epitope of human TNFα with an affinity of at least 1×10^8 liter/mole, measured as an association constant (Ka), as determined by Scatchard analysis.

2. The method of claim **1**, wherein the single or divided doses are selected from 0.5, 0.9, 1, 1.1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14or 15 mg/kg per day on at least one of day 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 or at least one of week 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20.

3. The method of claim **1**, wherein the doses are administered for a period of time until the fistulas are improved substantially or in complete remission.

4. The method of claim **1**, wherein the doses are increased upon loss of response to the anti-TNFα antibody in the human.

5. The method of claim **1**, wherein said anti-TNFα antibody comprises a non-human variable region comprising an amino acid sequence selected from the group consisting of SEQ ID NO:3 and SEQ ID NO:5.

6. The method of claim **5**, wherein the non-human variable region is murine.

7. The method of claim **5**, wherein the non-human variable region comprises a polypeptide encoded by a nucleic acid sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4.

8. The method of claim **1**, wherein said anti-TNFα antibody comprises an IgG1 constant region.

9. The method of claim **1**, wherein said anti-TNFα antibody comprises a non-human variable region comprising an amino acid sequence selected from the group consisting of SEQ ID NO:3 and SEQ ID NO:5 and an IgG1 human constant region.

10. The method of claim **9**, wherein the non-human variable region is murine.

11. The method of claim **9**, wherein the non-human variable region comprises a polypeptide encoded by a nucleic acid sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4.

12. The method of claim **1**, wherein the anti-TNFα antibody is administered to the human patient by means of parenteral administration.

13. The method of claim **1**, wherein the anti-TNFα antibody is administered to the human patient by means of intravenous administration, subcutaneous administration, or intramuscular administration.

14. The method of claim **1**, wherein the anti-TNFα antibody is of immunoglobulin class IgG1, IgG2, IgG3, IgG4 or IgM.

15. The method of claim **1**, wherein the anti-TNFα antibody is a fragment selected from the group consisting of Fab, Fab', $F(ab')_2$ and Fv.

16. The method of claim **1**, wherein said antibody or antigen-binding fragment comprises a human constant region and a human variable region.

17. The method of claim 1, wherein said antibody or antigen-binding fragment comprises at least one human light chain and at least one human heavy chain.

18. The method of claim 17, wherein the light chain comprises all antigen-binding regions of the light chain of A2 (ATCC Accession No. PTA-7045).

19. The method of claim 17, wherein the heavy chain comprises all antigen-binding regions of the heavy chain of A2 (ATCC Accession No. PTA-7045).

20. The method of claim 17, wherein the light chain comprises all antigen-binding regions of the light chain of A2 (ATCC Accession No. PTA-7045) and the heavy chain comprises all antigen-binding regions of the heavy chain of A2 (ATCC Accession No. PTA-7045).

21. A method of inhibiting TNFα in a human patient, wherein said human patient has fistulas in Crohn's disease, comprising administering to the human an effective TNFα-inhibiting amount of an anti-TNFα antibody or antigen-binding fragment thereof, wherein said anti-TNFα antibody comprises a human IgG1 constant region and wherein said anti-TNFα antibody or antigen-binding fragment (i) competitively inhibits binding of human A2 (ATCC Accession No. PTA-7045) te human TNFα, and (ii) binds to a neutralizing epitope of human TNFα with an affinity of at least 1×10^{8} liter/mole, measured as an association constant (Ka), as determined by Scatchard analysis.

22. The method of claim 21, wherein the antibody or antigen-binding fragment comprises a human constant region and a human variable region.

23. The method of claim 21, wherein said antibody or antigen-binding fragment comprises at least one human light chain and at least one human heavy chain.

24. The method of claim 23, wherein the light chain comprises all antigen-binding regions of the light chain of A2 (ATCC Accession No. PTA-7045).

25. The method of claim 23, wherein the heavy chain comprises all antigen-binding regions of the heavy chain of A2 (ATCC Accession No. PTA-7045).

26. The method of claim 23, wherein the light chain comprises all antigen-binding regions of the light chain of A2 (ATCC Accession No. PTA-7045) and the heavy chain comprises all antigen-binding regions of the heavy chain of A2 (ATCC Accession No. PTA-7045).

27. A method of inhibiting TNFα in a human patient, wherein said human patient has fistulas in Crolin's disease, comprising administering to the human an anti-TNFα antibody or antigen-binding fragment thereof, said antibody comprising a human constant region, wherein said antibody or antigen-binding fragment (i) comprises the antigen-binding regions of A2 (ATCC Accession No. PTA-7045), and (ii) binds to a neutralizing epitope of human TNFα with an affinity of at least 1×10^{8} liter/mole, measured as an association constant (Ka), as determined by Scatchard analysis.

28. The method of claim 1, further comprising administering a composition comprising the antibody or antigen-binding fragment of claim 1 and a pharmaceutically acceptable carrier.

29. The method of claim 1, wherein said antibody or antigen-binding fragment has specificity for a neutralizing epiwpe of human TNFα.

30. The antibody or antigen-binding fragment of claim 1, wherein said Scatchard analysis comprises labeling the arni-TNFα antibody or antigen-binding fragment thereof and measuring direct binding of ^{125}I labeled anti-TNFα antibody or antigen-binding fragment thereof to immobilized rh TNFα, and wherein said antibodies are labelled to a specific activity of about 9.7 μCi/μg by the iodogen method.

31. A method of inhibiting TNFα in a human patient, wherein said human patient has fistulas in Crohn's disease, comprising administering to the human at least one single or divided 0.5-50 mg/kg dose of an anti-TNFα antibody or antigen-binding fragment thereof, said antibody comprising a human constant region, wherein said anti-TNFα antibody or antigen-binding fragment (i) competitively inhibits binding of A2 (ATCC Accession No. PTA-7045) to human TNF-α, and (ii) hinds to a neutralizing epitope of human TNFα with an affinity of at least 1×10^{8} liter/mole, measured as an association constant (Ka), as determined by Scatchard analysis.

32. The method of claim 31, wherein said single or divided dose is 1-10 mg/kg.

* * * * *

EXHIBIT C

US005807715A

United States Patent [19]

Morrison et al.

[11] **Patent Number: 5,807,715**

[45] **Date of Patent: Sep. 15, 1998**

[54] **METHODS AND TRANSFORMED MAMMALIAN LYMPHOCYTE CELLS FOR PRODUCING FUNCTIONAL ANTIGEN-BINDING PROTEIN INCLUDING CHIMERIC IMMUNOGLOBULIN**

[75] Inventors: **Sherie L. Morrison**, Scarsdale, N.Y.; **Leonard A. Herzenberg**, Stanford; **Vernon T. Oi**, Menlo Park, both of Calif.

[73] Assignee: **The Board of Trustees of The Leland Stanford Junior University**, Stanford, Calif.

[21] Appl. No.: **266,154**

[22] Filed: **Jun. 27, 1994**

Related U.S. Application Data

[63] Continuation of Ser. No. 893,610, Jun. 3, 1992, abandoned, which is a continuation of Ser. No. 675,106, Mar. 25, 1991, abandoned, which is a continuation of Ser. No. 441,189, Nov. 22, 1989, abandoned, which is a continuation of Ser. No. 90,669, Aug. 28, 1987, abandoned, which is a continuation-in-part of Ser. No. 644,473, Aug. 27, 1984, abandoned.

[51] **Int. Cl.**[6] **C12N 15/00**; C12N 15/13; C07K 16/00

[52] **U.S. Cl.** **435/69.6**; 435/172.3; 435/326; 530/387.1; 530/387.3; 536/23.53

[58] **Field of Search** 435/69.6, 246.27, 435/320.1, 172.3, 326; 530/387.3, 387.1; 935/15; 536/23.53

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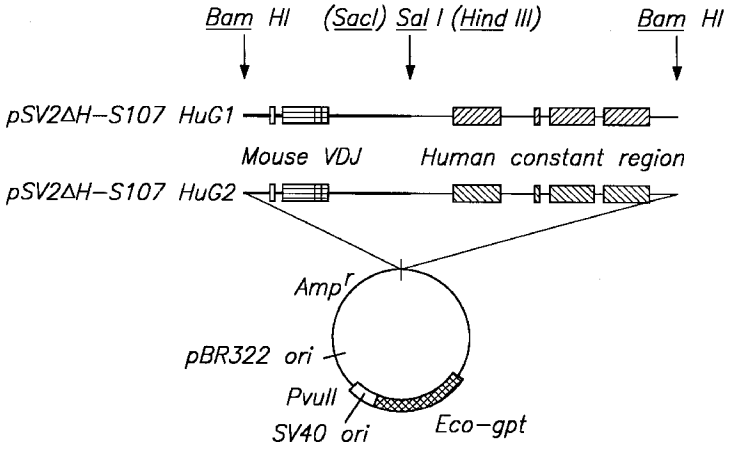
Primary Examiner—Lila Feisee
Assistant Examiner—Julie E. Reeves
Attorney, Agent, or Firm—Fish & Neave; Vicki S. Veenker; Edward F. Mullowney

[57] **ABSTRACT**

Methods for producing functional immunoglobulin are provided. The methods involve transfecting and expressing exogenous DNA coding for the heavy and light chains of immunoglobulin. In some embodiments, chimeric immunoglobulins are provided having variable regions from one species and constant regions from another species by linking DNA sequences encoding for the variable regions of the light and heavy chains from one species to the constant regions of the light and heavy chains respectively from a different species. Introduction of the resulting genes into mammalian host cells under conditions for expression provides for production of chimeric immunoglobulins having the specificity of the variable region derived from a first species and the physiological functions of the constant region from a different species.

62 Claims, 2 Drawing Sheets



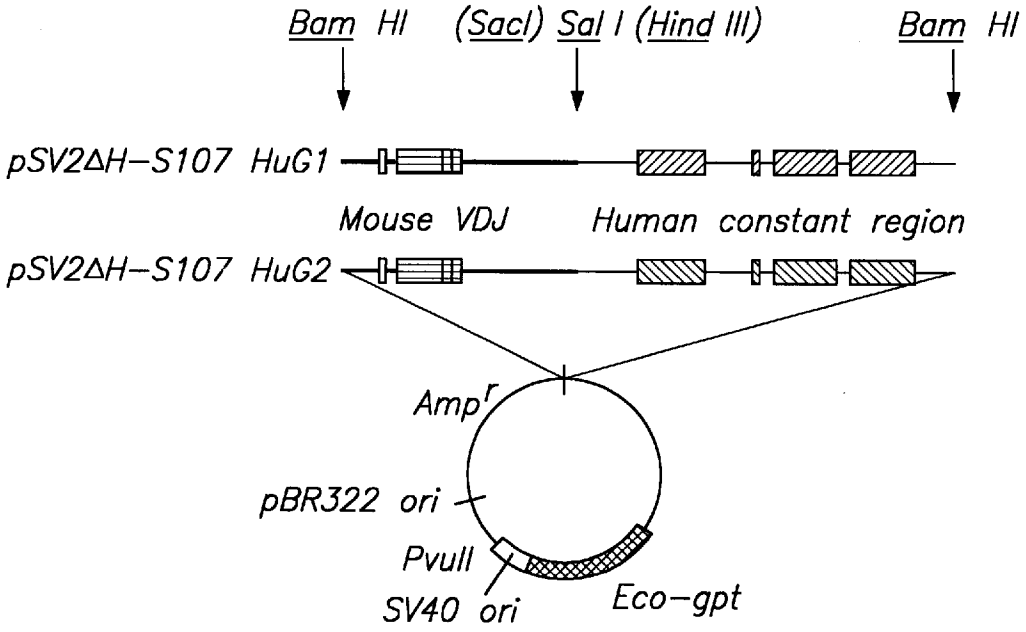

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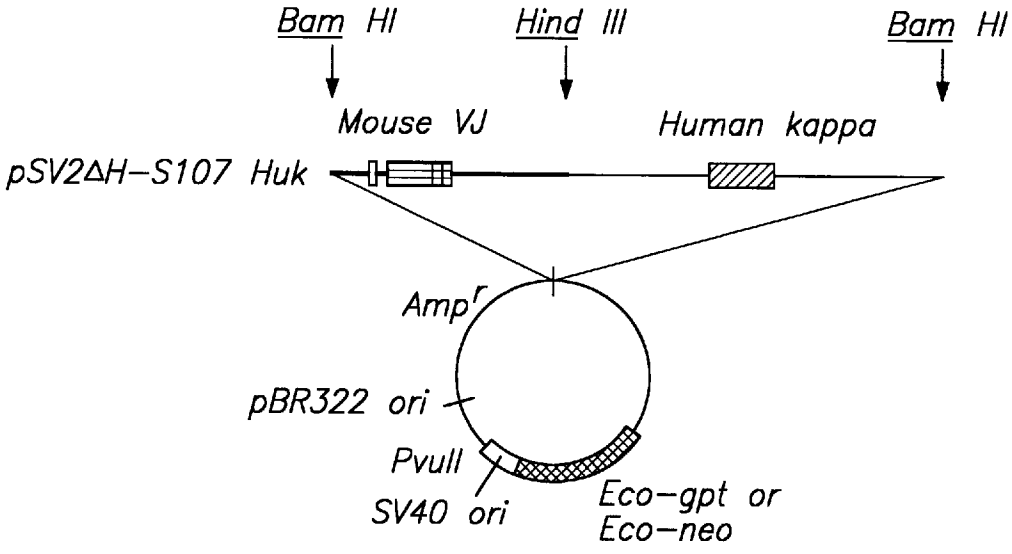
FIG. 1A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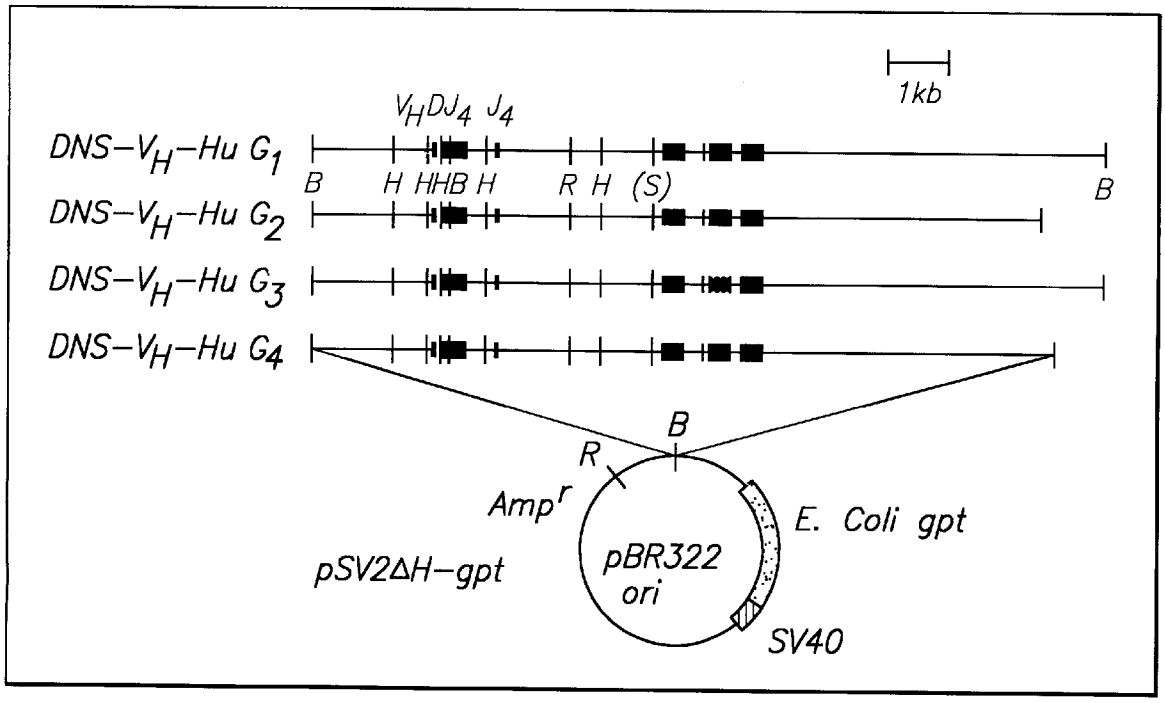
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FIG. 2

METHODS AND TRANSFORMED MAMMALIAN LYMPHOCYTE CELLS FOR PRODUCING FUNCTIONAL ANTIGEN-BINDING PROTEIN INCLUDING CHIMERIC IMMUNOGLOBULIN

This is a continuation of application Ser. No. 07/893,610, filed Jun. 3, 1992, now abandoned, which is a continuation of application Ser. No. 07/675,106, filed Mar. 25, 1991, now abandoned, which is a continuation of application Ser. No. 07/441,189, filed Nov. 22, 1989, now abandoned, which is a continuation of application Ser. No. 07/090,669, filed Aug. 28, 1987, now abandoned, which is a continuation-in-part of application Ser. No. 06/644,473, filed Aug. 27, 1984 now abandoned.

BACKGROUND OF THE INVENTION

1. Field of the Invention

Naturally occurring receptors, such as immunoglobulins, enzymes, and membrane proteins have seen an extraordinary expansion in commercial applications over the last decade. With the advent of monoclonal antibodies, the usefulness of immunoglobulins has been greatly expanded and in many situations has greatly extended prior uses employing polyclonal antibodies. However, in many applications, the use of monoclonal antibodies is severely restricted where the monoclonal antibodies are to be used in a physiological (in vivo) environment. Since, for the most part, monoclonal antibodies are produced in rodents, e.g., mice, the monoclonal antibodies are immunogenic to other species.

While the constant regions of immunoglobulins are not involved in ligand binding, the constant regions do have a number of specific functions, such as complement binding, immunogenicity, cell receptor binding, and the like. There will, therefore, be situations where it will be desirable to have constant regions which bind to cells or proteins from a particular species having binding regions for a particular ligand.

2. Relevant Literature

Kwan et al., *J. Exp. Mod.* (1981) 153:1366–1370 and Clarke et al., *Nucl. Acids Res.* (1982) 10:7731–7749 describe V_H and V_κ exons from the mouse phosphocholine-binding antibody-producing S107 myeloma cell line. Oi et al., *Proc. Natl. Acad. Sci. USA* (1983) 80:825–829, report that the mouse light chain gene is not expressed efficiently in a rat myeloma cell.

SUMMARY OF THE INVENTION

Chimeric multi-subunit receptors are provided, where each of the subunits is an expression product of a fused gene. Each fused gene comprises a DNA sequence from one host species encoding the region involved with ligand binding joined to a DNA sequence from a different source, either the same or a different host species, encoding a "constant" region providing a structural framework and biological properties. Introduction of the fused genes into an appropriate eukaryotic host cell under conditions for expression and processing provides for a functional assembled multi-subunit receptor product.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A is a schematic diagram of the chimeric mouse-:human heavy chain gene vector; and FIG. 1B is the chimeric light chain vector.

FIG. 2 is a schematic diagram of chimeric human IgG anti-DNS expression vectors.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Novel methods and compositions are provided, for production of polypeptide products having specific binding affinities for a predetermined ligand and predetermined biological, particularly physiological, properties, each of which are not normally associated with the binding region peptide sequences. Particularly, multi-subunit chimeric receptors are provided which result from fused genes having the portion of the polypeptide involved with binding of a predetermined ligand having an amino acid sequence substantially the same (>90% conserved) as an amino acid sequence having the same function from one host, while the portion involved with providing structural stability, as well as other biological functions, being analogously derived from a different host. The resulting composition can be either an inter- or intraspecies chimera. At least two fused genes are involved, which genes are introduced into an appropriate eukaryotic host under conditions for expression and processing, whereby the fused genes are expressed and the resulting subunits bound together, resulting in an assembled chimeric receptor.

The receptors prepared in accordance with the subject invention will be multi-subunit, where the units are held together either by non-covalent binding or a combination of non-covalent and covalent binding, particularly disulfide linkages through cysteine, and having at least one binding site, usually at least two binding sites, and not more than about ten binding sites. Receptors of interest include both B-cell and T-cell receptors, more particularly, immunoglobulins, such as IgM, IgG, IgA, IgD and IgE, as well as the various subtypes of the individual groups. The light chain may be κ or λ. The heavy chains are referred to as μ, γ, α, δ, and ϵ.

In discussing the two regions of each subunit, the two regions will be referred to as "variable" and "constant" by analogy to immunoglobulins. The variable region is the region involved with ligand binding and, therefore, will vary in conformation and amino acid sequence depending upon the ligand. The region will usually be composed of a plurality of smaller regions (hypervariable or complementary determining regions), involving a region having as its primary function binding to the ligand (V) and a region associated with joining the V region to the constant region, the joining region (J). There may also be a hypervariable region joining the V and J regions, the diversity region (D). These regions are related to gene segments observed in the genes encoding immunoglobulin variable regions.

The constant region will not be associated with ligand binding and will be relatively limited in the variations in its conformation and amino acid sequence within any one species and within any one class, each class generally having from 1 to 4 subclasses. Each constant region is specific for a species. Within the classes there will be allotypes, individual polymorphisms within a class within a species.

The varible region of the immunoglobulins will be derived from a convenient mammalian source, which may be a rodent, e.g., mouse or rat, rabbit, or other vertebrate, mammalian or otherwise, capable of producing immunoglobulins. The constant region of the immunoglobulin, as well as the J chain for IgM and IgA (not the same as the J region of the heavy or light immunoglobulin chain), will be derived from a vertebrate source different from the source of the

variable region, particularly a mammalian source, more particularly primate or domestic animal, e.g., bovine, porcine, equine, canine, feline, or the like, and particularly, humans. The different source of the constant region can be either from a different species or from the same species as the mammalian source utilized to provide the variable region. Thus, the constant region of the receptor will normally be chosen in accordance with the purpose of the receptor. For example, where the receptor is to be introduced into the host, the constant portion will be selected so as to minimize the immune response of the host to the receptor and to optimize biological efficiency, such as complement fixation or physiological half-life (catabolism). Where the receptor is to bind to particular cell membrane surface receptors, the constant region will be chosen in accordance with the host of the receptor recognition site.

The fused gene derived from the two host sources will be prepared by joining the 5'-end of a sequence encoding the constant region in reading frame to the 3'-end of a sequence encoding the variable region. (In referring to 5' or 3' for a double strand, the direction of transcription is with 5' being upstream from 3'.) With immunoglobulins, two fused genes will be prepared, one for the light chain and one for the heavy chain. With T-cell receptors, the two fused genes will be for each of the two chains involved in the formation of the T-cell receptor. The DNA sequences employed for preparation of the fused gene may be derived from a variety of sources. These sources include genomic DNA, cDNA, synthetic DNA, and combinations thereof. The genomic DNA may or may not include naturally occurring introns.

The DNA obtained from natural sources, namely the genomic DNA or CDNA, may be obtained in a variety of ways. Host cells coding for the desired sequence may be isolated, the genomic DNA may be fragmented, conveniently by one or more restriction endonucleases, and the resulting fragments may be cloned and screened with a probe for the presence of the DNA sequence coding for the polypeptide sequence of interest. For the variable region, the rearranged germline heavy chain DNA will include V, D, and J regions, including the leader sequence, which may be subsequently removed as well as any introns. The rearranged germline light chain coding DNA will include the V and J regions including the leader sequence, as well as any introns which may be subsequently removed. The particular source of the exons defining the domains and the manner of splicing, where introns are present, is not germane to this invention. Once the cloned fragment has been identified which contains the desired DNA sequence, this fragment may be further manipulated to remove superfluous DNA, modify one or both termini, remove all or a portion of intervening sequences (introns), or the like.

In providing a fragment encoding the variable region, it will usually be desirable to include all or a portion of the intron downstream from the J region. Where the intron is retained, it will be necessary that there be functional splice acceptor and donor sequences at the intron termini. The gene sequence between the J (joining region) and the constant region of the fused gene may be primarily the intron sequence associated with (1) the constant region, (2) the J region, or (3) portions of each. The last may be a matter of convenience where there is a convenient restriction site in the introns from the two sources. In some instances, all or a portion of the intron may be modified by deletion, nucleotide substitution(s) or insertion, to enhance ease of manipulation, expression, or the like. When the variable region is chosen to be syngeneic with the host cells employed for expression, all or at least about 80% of the intron sequence can be

selected from the naturally occurring intron sequence associated with the J region. In some instances it will be necessary to provide adapters to join the intron or truncated intron to the constant region. By cleaving within the intron, the variable region will be separated from its natural constant region.

Alternatively, it may be desirable to have the fused gene free of the intron between the variable and constant regions. Thus, the 3' terminus will be at or in the joining region. Normally all or a portion of the J region will be associated with the host providing the variable region. By restriction enzyme analysis or sequencing of the J region, one can select for a particular site for the 3' terminus of the variable region.

Alternatively, one can use an exonuclease and by employing varying periods of digestion, one can provide for varying 3'-termini, which can then be used for linking to the constant region and selection made for a functional product in a variety of ways. For example, where joining of the variable region to the constant region results in a unique restriction site, the fused DNA fragments may be screened for the presence of the restriction site.

Alternatively, it may be found desirable to include an adapter or linker to join the variable region to the constant region, where the adapter or linker may have the same or substantially the same sequence, usually at least substantially the same sequence, of the DNA sequence of the two fragments adjacent the juncture. The adapter or linker will be selected so as to provide for the two sequences to be in common reading frame. Furthermore, by employing adapters, one could add an additional degree of variability in the binding affinity of the chimeric receptor, by providing for the expression of different amino acids in the J region.

The joining of the various fragments is performed in accordance with conventional techniques, employing blunt-ended or staggered-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and ligation with appropriate ligases.

For cDNA, the cDNA may be cloned and the resulting clone screened with an appropriate probe for cDNA coding for the desired variable or constant region. Once the desired clone has been isolated, the CDNA may be manipulated in substantially the same manner as the genomic DNA. However, with cDNA there will be no introns or intervening sequences. The cDNA is cleaved at or near the juncture of the variable region with the constant region so that the variable region is separated from the constant region and the desired region retained. Where a convenient restriction site exists, the CDNA may be digested to provide for a fragment having the appropriate terminus. The restriction site may provide a satisfactory site or be extended with an adapter. Alternatively, primer repair may be employed, where for the variable region a complementary sequence to the site of cleavage and successive nucleotides in the 3' direction of the complementary sequence is hybridized to the sense strand of the CDNA and the nonsense strand replicated beginning with the primer and removal of the single-stranded DNA of the sense strand 3' from the primer. The reverse is true for the constant region. Other techniques may also suggest themselves. Once the fragment has been obtained having the predetermined 3' or 5' terminus, as appropriate, it may then be employed for Joining to the other region.

Finally, one or both of the regions may be synthesized and cloned for use in preparing the fused gene. For the most part,

the same or substantially the same constant region can be repetitively used, so that a library of constant regions may be established which can be selected for joining to variable regions. Thus, the constant regions would have an appropriate 5' terminus for joining directly or through an adapter to a variable region.

In order for expression of the fused gene, it will be necessary to have transcriptional and translational signals recognized by an appropriate eukaryotic host. For the most part, desirable eukaryotic hosts will be mammalian cells capable of culture in vitro, particularly leukocytes, more particularly myeloma cells, or other transformed or oncogenic lymphocyte, e.g., EBV transformed cells. Alternatively, non-mammalian cells may be employed, such as fungi, e.g., yeast, filamentous fungi, or the like.

The DNA sequence coding for the variable region may be obtained in association with the promoter region from genomic DNA. To the extent that the host cells recognize the transcriptional regulatory and translational initiation signals associated with the variable region, then the region 5' of the variable region coding sequence may be retained with the variable region coding sequence and employed for transcriptional and translational initiation regulation.

The contiguous non-coding region 5' to the variable region will normally include those sequences involved with initiation of transcription and translation, such as the TATA box, capping sequence, CAAT sequence, and the like. Usually the 5'-non-coding sequence will be at least 150 bp, more usually at least 200 bp, usually not exceeding about 2 kbp, more usually not exceeding about 1 kbp.

The non-coding region 3' to the constant region may be retained for its transcriptional termination regulatory sequences, such as termination and polyadenylation. Thus, by retaining the 3'-region naturally contiguous to the DNA sequence coding for the constant region, the transcriptional termination signals may be provided for the fused gene. Where the transcriptional termination signals are not satisfactorily functional in the expression host cell, then a 3' region functional in the host cell may be substituted. Conveniently, the non-coding 3' region may be obtained from a non-coding contiguous 3' region of a constant region from the expression host. The 3'-non-coding region may be joined to the constant region by any of the means described previously for manipulation and ligation of DNA fragments. This region could then be used as a building block in preparing the fused gene.

The fused gene for the most part may be depicted by the following formula:

$$TIR\text{-}(LS)_e\text{-}V_f\text{-}(Vi)_a\text{-}(D)_b\text{-}(Di)_c\text{-}J\text{-}(I)_d\text{-}C\text{-}TTR$$

wherein:

TIR intends the transcriptional regulatory and translational initiation region and is generally of at least about 150 bp and not more than about 2 kbp, which may be in whole or in part the sequence naturally joined to the V coding region;

LS refers to a DNA sequence encoding a leader sequence and processing signal functional in the expression host for secretion and processing for removal of the sequence; this leader sequence can contain an intron, as is known in the art to occur;

e is 0 or 1;

V is a segment coding for the variable domain in reading frame with LS, when LS is present;

f is 0 or 1;

D is a segment coding for the diversity domain and is present for the heavy chain (b=1) and is absent for the light chain (b=0);

J is a segment coding for the joining region;

Vi and Di are introns associated with the letter-indicated coding segments having functional donor and acceptor splicing sites;

a, b and c are the same or different and are 0 or 1, wherein when b is 0, c is 0; a, b, and c are all preferably 0;

I is an intron which may be naturally contiguous to the J segment or naturally contiguous to the C domain or a combination of fragments from both, or a fragment thereof, desirably including an enhancer sequence functional in said expression host, or I may be foreign in whole or in part to the J and C segments;

d is 0 or 1 (preferably 0);

C is the constant domain and may code for a μ, γ, δ, α or ϵ chain, preferably μ, γ, or α, usually including at least 80% of the constant region sequence, and may be the same as or a modified naturally occurring allotype or an altered constant region encoding an improved protein sequence; and

TTR is the transcriptional termination region providing for transcriptional termination and polyadenylation which may be naturally associated with C or may be joined to C, being functional in the expression host; usually being at least about 100 bp and may be 1 kbp or more.

Fused genes lacking, or containing modifications in, the hinge region or other immunoglobulin constant region domains can also be prepared, in like manner to the modifications described above, in which case the formula will be as shown above but with the hinge region of the constant chain being absent or modified.

The constructs for each of the different subunits may be joined together to form a single DNA segment or may be maintained as separate segments, by themselves or in conjunction with vectors.

The subunit constructs may be introduced into a cell by transformation in conjunction with a gene allowing for selection where the construct will become integrated into the host genome.

A large number of vectors are available or can be readily prepared which provide for expression in a host, either by maintenance as an extrachromosomal element or by integration into the host genome. For a mammalian host, a wide variety of vectors are known based on viral replication systems, such as Simian virus, bovine papilloma virus, adenovirus and the like. These vectors can be used as expression vectors where transcriptional and translational initiation and termination signals are present and one or more restriction sites are available for insertion of a structural gene. In addition, the vectors normally have one or more markers which allow for selection of host cells which contain the expression vector. The marker may provide for prototrophy to an auxotrophic host; biocide resistance, e.g., resistance to antibiotics, such as G418, or heavy metals, such as copper; or the like. If desired, expression vectors can be prepared by joining the various components, such as the replication system, markers, and transcriptional and translational regulatory initiation and termination signals in conjunction with the fused gene. Frequently, a vector will include a prokaryotic replication system, which allows for cloning, manipulation, purification, and expansion of the desired DNA sequence.

A wide variety of transcriptional and translational regulatory sequences may be employed, depending upon the nature of the host. The transcriptional and translational regulatory signals may be derived from viral sources, such as adenovirus, bovine papilloma virus, Simian virus, or the like, where the regulatory signals are associated with a particular gene which has a high level of expression.

Alternatively, promoters from mammalian expression products, such as actin, collagen, myosin, etc., may be employed. Transcriptional initiation regulatory signals may be selected which allow for repression or activation, so that expression of the fused genes can be modulated. Of interest are regulatory signals which are temperature-sensitive so that by varying the temperature, expression can be repressed or initiated, or are subject to chemical regulation, e.g., metabolite.

Once the vector DNA sequence containing the fused gene has been prepared for expression, the DNA construct may be introduced into an appropriate host. Various techniques may be employed, such as protoplast fusion, calcium phosphate-precipitation, or other conventional technique. After the fusion, the cells are grown in a selective medium or are phenotypically selected leaving only cells transformed with the DNA construct. Expression of the fused gene results in assembly to form the receptor. To date, expression has been accomplished in lymphocytes.

The host cells will for the most part be immortalized cells, particularly myeloma or lymphoma cells. These cells may be grown in an appropriate nutrient medium in culture flasks or injected into a syngeneic host, e.g., mouse or rat, or immunodeficient host or host site, e.g., nude mouse or hamster pouch. Particularly, the cells may be introduced into the abdominal cavity for production of ascites fluid and harvesting of the chimeric receptor. Alternatively, the cells may be injected subcutaneously and the antibodies harvested from the blood of the host. The cells may be used in the same manner as hybridoma cells. See Diamond et al., *N. Eng. J. Med.* (1981) 3034:1344 and Kennatt, McKearn and Bechtol (eds.), *Monoclonal Antibodies: Hybridomas—A New Dimension in Biologic Analysis,* Plenum, 1980, which are incorporated herein by reference.

Where a leader is present with a processing signal for secretion and selective cleavage of the leader (signal) sequence, the resulting assembled receptor will be secreted into the nutrient medium of the transformed cells and may be harvested. Where secretion does not occur, after sufficient time for the receptor to be expressed in reasonable amounts, the cells may be killed, lysed, and the receptors isolated and purified. Where transcriptional initiation can be modulated, the cells may be grown to high density under non-permissive conditions, followed by growth under permissive conditions where the receptor is expressed.

The receptors may be naturally glycosylated, unnaturally glycosylated or be free of glycosyl groups, depending on the host, conditions of cellular growth and subsequent treatment. Where a mammalian host cell is employed for expression, usually natural glycosylation will occur. Glycosylation can be prevented by an appropriate inhibitor, e.g., tunicamycin. Alternatively, glycosyl groups may be removed by hydrolysis, e.g., enzymatic hydrolysis using hydrolases. In expression hosts other than mammalian cells, unglycosylated or unnatural glycosylated receptors may be obtained.

The receptor may be isolated and purified in accordance with conventional conditions, such as extraction, precipitation, chromatography, affinity chromatography, electrophoresis, or the like. By employing antibodies specific for the constant region(s), affinity chromatography will allow for concentration and purification of the chimeric receptor.

The chimeric receptors can be used in the same manner as other receptors for binding to specific ligands in diagnostic assays, affinity chromatography or the like. In addition, because a chimeric receptor of substantially reduced immunogenicity can be produced, the chimeric receptors can find use in therapy, for passive immunization for in vivo imaging, for specific treatment of diseased cells, or the like. For in vivo imaging, the chimeric antibody will normally be conjugated to a radionuclide, e.g., technetium, rhenium, or the like. For biocidal activity, the antibody may be joined to the A-portion of toxins, liposomes containing biocidal reagents, radionuclides, or other biocidal agent. Alternatively, the antibodies can be used in combination with the host immune system, e.g., complement, due to the presence of the native constant region. In vitro, the subject chimeric antibodies can be used in conjunction with complement to remove particular cells from a mixture of cells, where the target cells have a ligand complementary to the binding site of the chimeric antibody.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLE 1

Materials and Methods

Chimeric Genes

The cloned S107 variable region (heavy) (VH) and S107 Vκ variable region (light, kappa) genes were obtained from Dr. Matthew Scharff (Dept. of Cell Biology, Albert Einstein College of Medicine, Bronx, N.Y. 10641). The S107 VH gene was spliced to human IgG1 and IgG2 constant region genes using SalI linkers as shown in FIG. **1**A. Both constructs were inserted into the vector pSV2ΔH-gpt (Oi et al., *Proc. Natl. Acad. Sci. USA* (1983) 80:825–829; Mulligan and Berg, *Science* (1980) 209:1422–1427). The S107 Vκ gene was spliced to the human κ gene at a unique HindIII site located in the large intron between the Jκ and Cκ exons as shown in FIG. **1**B. This chimeric light chain gene construct was inserted into both pSV2ΔH-gpt and pSV2-neo plasmid vectors (Mulligan and Berg, *Proc. Natl. Acad. Sci. USA* (1981) 78:2072–2076) and pSV184ΔH-neo plasmid vectors (Oi and Morrison, *Biotechniques* (1986) 4:214–221).

Transfection

Protoplast fusion and calcium-phosphate ($CaPO_4$) precipitation techniques (Oi et al., (1983) supra; Sandri-Goldin et al., *Mol. Cell. Biol.* (1981) 1:743–752; Chu and Sharp, *Gene* (1980) 13:197–202) were used to transfect these chimeric immunoglobulin genes into the J558L myeloma cell line (a lambda (λ) light chain producing mouse myeloma cell line) and the non-immunoglobulin-producing An derivative of the P3 myeloma cell line. Mycophenolic acid (Gibco Laboratories, Santa Clara, Calif. 95050) was used for selection of cells transfected with pSV2ΔH-gpt vectors as described previously (Oi et al., (1983) supra; Ochi et al., *Proc. Natl. Acad. Sci. USA* (1983) 80:6351–6355). G418 (Gibco Laboratories) at 1.0 mg/ml was used for selection of cells transfected with pSV2-neo vectors (Mulligan and Berg, (1980) supra).

When both light and heavy chimeric genes were transfected into the J558L cell line using protoplast fusion techniques, light and heavy chimeric immunoglobulin genes were transfected sequentially using G418 selection for the chimeric light chain gene vector and mycophenolic acid for the chimeric heavy chain gene vector. The protoplast fusion transfection procedure used was as described previously (Oi et al., (1983) supra).

Transfection using the calcium phosphate precipitation procedure was done by mixing 40 μg of both chimeric light and chimeric heavy chain pSV2ΔH-gpt vectors and transfecting a total of 80 μg of plasmid DNA into 5×10^6 cells. Mycophenolic acid was used to select for transformed cell lines as described previously (Oi et al., (1983) supra).

Antigen-binding

Phosphocholine (PC) binding of antibody secreted into the culture supernates of transfected cell lines was analyzed using a solid-phase radioimmunoassay described previously (Oi and Herzenberg, *Mol. Immunol.* (1979) 16:1005–1017). PC-binding antibodies in biosynthetically-labeled culture supernates and cell lysates of transfected cell lines also were analyzed by binding the biosynthetically-labeled antibody to PC-coupled to Sepharose 4B (Pharmacia Five Chamicals, Piscataway, N.J.) and then eluting the bound antibody with PC-hapten. The bound and eluted antibody was examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Biosynthetic-labeling procedures were done as described previously (Oi et al., *J. Exp. Med.* (1980) 151:1260–1274).

Idiotype Analysis

Three hybridoma anti-idiotope antibodies, obtained from Dr. Matthew Scharff (Dept. of Cell Biology, Albert Einstein College of Medicine, Bronx, N.Y.), were used to analyze the variable heavy-variable light (VH-VL) domain structure of the chimeric human anti-PC antibodies. These antibodies, recognizing three independent idiotopes, were used to immunoprecipitate biosynthetically-labeled material eluted with PC from the PC-Sepharose 4B matrix. Immunoprecipitates were analyzed by SDS-PAGE.

Immunoglobulin Chain Composition

Monoclonal anti-human IgG and anti-human κ antibodies (Becton-Dickinson Monoclonal Center, Mt. View, Calif.) were used to immunoprecipitate biosynthetically-labeled chimeric human anti-PC antibodies for analyses using two-dimensional non-equilibrium pH gradient polyacrylamide gel electrophoresis (NEPHGE) (Oi and Herzenberg, (1979) supra). PC-coupled to Sepharose 4B also was used for immunoprecipitations.

Immunoglobulin Heavy Chain Glycosylation

Tunicamycin (Calbiochem-Behring, San Diego, Calif.) was used to inhibit asparagine-linked glycosylation of biosynthetically-labeled antibody from mouse cell lines producing mouse:human chimeric immunoglobulins (Oi et al., (1980) supra). PC-binding antibody from tunicamycin-treated cells was analyzed by SDS-PAGE. Procedures used for tunicamycin treatment were as described previously (Oi et al., (1980) supra).

Chimeric Mouse:Human Antibody Production in Mice

Transformed J558L cells producing chimeric mouse:human antibody were injected subcutaneously into BALB/c mice (10^6 cells/mouse). Sera from tumor-bearing mice were analyzed for human anti-PC antibody by a solid-phase radioimmunoassay described previously (Oi and Herzenberg, (1979) supra) and by immunoelectrophoresis using a polyclonal anti-human antiserum.

Results

Expression of chimeric mouse V:human C region genes in transfected mouse myeloma cells J558L and the non-immunoglobulin-producing P3 myeloma cell lines was obtained. When both light chain and heavy chain chimeric genes were transfected into the same cell, tetrameric (H2L2) antigen-binding antibodies were obtained. Autoradiograms of two-dimensional NEPHGE analyses of the chain composition of biosynthesized and secreted antibody molecules bound and eluted from phosphocholine-Sepharose showed the formation of mixed molecules, including the endogenously produced J558L λ light chain. Each polypeptide chain had the expected charge and relative molecular weight. Identical two-dimensional gel analyses results were obtained with immunoprecipitates with monoclonal anti-human K and IgG antiodies. Similar results were obtained from immunoprecipitates of human $IgG_2(\kappa)$ antibodies produced by transfected P3 cell lines. Since the non-immunoglobulin-producing parental P3 cell line does not produce endogenous immunoglobulin polypeptide chains, as expected only the chimeric mouse:human heavy and light chains are seen on the autoradiograms.

Phosphocholine-binding by the chimeric antibody produced in the J558L cell line was the result from specific association of the chimeric immunoglobulin light and heavy polypeptide chains, i.e., the VH and Vκ domains of the S107 myeloma protein connected to human constant region polypeptides. This was determined by measuring PC-binding by immunoglobulins produced by J558L cells transfected with the mouse: human chimeric heavy chain gene. PC-binding was never observed from antibody secreted by transfected J558L cells expressing only the chimeric heavy chain and the endogenous J558L λ light chain (data not shown). Chimeric antibodies produced in the transfected non-immunoglobulin-producing P3 cell line also were shown to bind PC-Sepharose. In view of the low binding affinity of the parental mouse S107 antibody, analyses of appropriate polypeptide folding of mouse VH and Vκ domains in the novel environment of human constant regions polypeptide chains were done by determining the presence of idiotypes known to occur on the parental S107 PC-binding antibody molecule. Three monoclonal anti-idiotope antibodies, each recognizing a distinct epitope on the light and heavy variable region domains and an epitope defined by the presence of both light and heavy variable region domains, were found to react with the mouse:human chimeric anti-PC antibodies. This strongly supports the fact that the mouse S107 antigen-binding domains have folded into their intended structures.

Glycosylation of the mouse:human chimeric antibodies in mouse myeloma cells was analyzed by measuring the relative molecular weight (Mr) of antibodies biosynthesized in the presence and absence of tunicamycin, a known antibiotic inhibitor of asparagine-linked glycosylation. Autoradiograms of SDS-PAGE analysis of the chimeric heavy and light chains produced in mouse myeloma cells in the presence or absence of tunicamycin showed the lower relative Mr of the heavy chain synthesized in the presence of tunicamycin as expected if a single N-linked carbohydrate was absent from the polypeptide chain. From these data it is concluded that the mouse myeloma cell appropriately glycosylates the human heavy chain.

When transfected J558L cells producing the human IgG2 (κ) chimeric anti-PC antibody were grown as a subcutaneous tumor in BALB/c mice, analysis of the sera of these mice showed significant human IgG2(κ) anti-PC binding antibody production by radioimmunoassay. Polyclonal anti-human antiserum demonstrated the presence of significant quantities of human immunoglobulin in the sera. Based on comparison with prior experience with mouse hybri-doma-antibody production in mice, the amount of immunoglobulin visualized by immunoelectrophoresis analysis of mice bearing tumors of the transfected J558L cell line was similar to the lower levels of production seen with other mouse hybridoma tumor cell lines.

Analysis showed that fewer than about 10% of the transfected cell lines produced both chimeric heavy and light chain polypeptides. Among transformants generated by protoplast fusion, both gpt and neo biochemical markers were expressed at expected frequencies. However, chimeric light chain expression was infrequent. In co-transfection experiments using the $CaPO_4$ precipitation protocol, the same phenomenon was observed.

Based on prior experience with co-expression of gene products in transformed cell lines, it appears that the appropriate transcriptional or translational controlling elements are absent in either the chimeric light chain gene construct or in the mouse myeloma cell lines used. The mouse Vκ gene promoter is coupled to the presumed human intronic DNA sequences that are homologous to the known mouse intronic controlling element (ICE) or immunoglobulin "enhancer" element (Morrison and Oi, *Ann. Rev. Immunol.* (1984) 7:239–256). The chimeric mouse: human heavy chain gene is not constructed in this manner and, in contrast, is expressed efficiently. The mouse heavy chain intronic controlling element (ICE) sequences are included and human sequences excluded in this construct. The basis for the low level of expression of the light chain is subject to speculation.

It is evident from the above results that chimeric receptors, as illustrated by immunoglobulins, can be produced where the variable regions may be obtained from one host source and the constant regions obtained from another host source. Where the immunoglobulins are to be use in vivo, this can provide for numerous advantages, such as reduced immunogenicity, a lower catabolism, and the ability to fulfill biological effector functions associated with the constant regions. Furthermore, now that it is shown that chimeric receptors can be produced, there is the opportunity to prepare chimeric receptors with modification of V, J, and D regions so as to modify binding specificity.

EXAMPLE 2

A family of recombinant anti-DNS antibodies was prepared by cloning the mouse V_H gene expressed in the DNS1 (27–44) hybridoma cell line (Dangl et al., *Cytometry* (1982) 2:395) and joining this gene to the already cloned human IgG_1, IgG_2, IgG_3, IgG_4, rabbit IgG and mouse IgG_3 heavy chain constant region genes. These recombinant genes were inserted into the eukaryotic expression vector, pSV2(delta) H-gpt (Oi et al., *BioTechniques* (1987) 4:214). Each recombinant vector was transfected into a heavy chain loss mutant of the DNS1 hybridoma cell line, 27–44.A5C13, or co-transfected with a DNS-V_K expression vector into the Ig^- cell line SP2.0 to generate stable anti-DNS transfectoma cell lines (Morrison and Oi, *Ann Rev. Immunol.* (1984) 2:239). The expressed V_H and V_K genes from the DNS1 hybridoma (Oi et al., *Nature* (1984) 307:136; Reidler et al., *J. Mol. Biol.* (1982) 158:739) were cloned from phage lambda libraries (Dangl, thesis, Stanford University, Stanford, Calif.) and used to construct the vectors shown in FIG. **2**. Transfection by protoplast fusion and selection and screening of transfectomas has been described (Oi et al., *BioTechniques,* supra; Dangl, thesis, Stanford University, Stanford, Calif.). Anti-DNS antibodies were purified from culture supernate by affinity chromatography using a dansyl isomer 2-dimethylaminonaphthyl-5-sulfonamide-3-lysine as absorbant (coupled to AH-Sepharose-4B) and eluant. The affinity of the DNS1 combining site for DNS is 17 nM; the binding affinity of this isomer is lower by a factor of 10^3, making it suitable for use in affinity purification. Removal of bound hapten by dialysis was monitored by fluorescence emission spectroscopy. Antibodies were pure, >95% and free of aggregates as determined by size exclusion chromatography (Dangl, thesis, Stanford University, Stanford, Calif.). The chimeric mouse-human immunoglobulin heavy chain vectors are depicted in FIG. **2**. The rabbit IgG and mouse IgG_3 heavy chain vectors were similarly constructed.

The nature of the DNS combining site of the genetically engineered antibodies was monitored by measuring the fluorescence emission spectra of bound DNS-lysine. This hapten is a sensitive indicator of the dynamic polarity of its microenvironment. Independently derived mouse IgG_1 anti-DNS antibodies with different combining sites were previously shown to generate different emission spectra (Reidler et al., *J. Mol. Biol.* (1982) 158:739). The absorption and emission spectra of DNS-lysine bound to each genetically engineered antibody was identical to the DNS1 combining site of the parental hybridoma (corrected fluorescence emission spectra of each affinity purified chimeric antibody were measured using a SIM model 8000 fluorescence spectrophotometer with 340 nm excitation, essentially as described in Reidler, et al., *J. Mol. Biol.* (1982) 158:739). This indicates that each chimeric antibody has a properly folded V_H domain, despite the heterologous junction between the mouse V_H and human or rabbit C_{H1} domains. Stable transfectoma cell lines produced immunoglobulins with heavy chains of appropriate size and charge as determined by two-dimensional sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Immunoglobulins produced by each transfectoma cell line were analyzed by biosynthetic labelling, immunoprecipitation and one- or two-dimensional SDS-PAGE essentially as described by P. P. Jones in *Selected Methods in Cellular Immunology,* B. B. Mishell, S. M. Shiigi, Eds. (W. H. Freeman, San Francisco, 1980), pp. 238. Rabbit anti-human IgG anti-sera was purchased from Cappel. The recombinant heavy chains are glycosylated, a post-translation modification known to affect biosynthetic labeling in the presence or absence of tunicamycin, an antibiotic known to inhibit asparagine-linked glycosylation (Hickman and Kornfeld *J. Immunol* (1978) 121:990; Nose and Wigzell *Proc, Natl. Acad. Sci.* (1983) 80:6632; Leatherbarrow et al. *Molec. Immunol.* (1985) 22:407).

This Example demonstrates the ability to prepare inter-species chimeric antibodies from additional species using the previously described techniques as well as the ability to produce intra-species chimerics.

EXAMPLE 3

A series of intra-species hybrid mouse IgG_1-IgG_{2a} immunoglobulins all with identical light chains and variable regions were produced. Hybrid heavy chain constant region gene segments were generated by genetic recombination in *E. coli* between plasmids carrying mouse γ1 and γ2a gene segments. Crossovers occurred throughout these segments although the frequency was highest in regions of high nucleotide sequence homology. Eleven hybrid proteins were produced by transfection of a variant hybridoma cell line. Immunoglobulins produced by the transfected cell lines were assembled into H_2L_2 tetramers and properly glycosylated in addition to having identical antigen combining sites specific for the fluorescent hapten ϵ-dansyl-L-lysine. Protein A binding, used as a probe of Fc structure in these variant antibodies was consistent with the identity of protein A contact residues within the Fc. Novel receptors with new and improved functions were created, demonstrating that improvements over naturally occurring receptors can be made.

Materials and Methods

Generation of hybrid genes

Hybrid C_H gene segments coding for polypeptides with an IgG_1 amino-terminus and an IgG_2a carboxy-terminus or an IgG_{2a} amino-terminus and IgG_1 carboxy-terminus were generated by adaptation of the system described by Schneider et al., *Proc. Natl. Acad. Sci.* (1981)

78:2169–2173. Plasmid pHGX1 was constructed by digesting pBN2 (Nichols and Yanofsky, *Proc. Natl. Acad. Sci.* (1979) 76:5244–5248) with HindIII and PvuII, filling in the overhanging HindIII ends with T_4 DNA polymerase, ligating XbaI linkers to the flush ends, and recircularizing the plasmid. Plasmids pHGX1$C_{\gamma 2a}$A and pHGX1$C_{\gamma 2a}$B were generated by digesting pγ2a.9 with EcoRI and HindIII, filling in the overhanging ends, ligating XbaI linkers to the blunt ends, and inserting the $C_{\gamma 2a}$-containing fragment into XbaI-digested pHGX1. Plasmids pHGX1$C_{\gamma 2a}$A and pHGX1$C_{\gamma 2a}$B differ only in the orientation of the inserted fragment. Plasmid pHGX2 was constructed by digesting pWS1 (Schneider et al., *Proc. Natl. Acad. Sci.* (1981) 78:2169–2173) with HpaI and SalI, filling in the overhanging SalI end, ligating XbaI linkers to the flush ends, and recircularizing the plasmid. Plasmids pHGX2$C_{\gamma 1}$A and pHGX2$C_{\gamma 1}$B were generated by digesting $P_{\gamma 1}$ with KpnI, and ligating the $C_{\gamma 2a}$-containing fragment to XbaI-digested pHGX2. Plasmids pHGX2$C_{\gamma 1}$A and pHGX2$C_{\gamma 1}$B differ only in the orientation of the inserted fragment.

E. coli strain W3110 trpR ΔtrpEA2 tnaA2 rna-19 was transformed with both pHGX1$C_{\gamma 2a}$A and pHGX1$C_{\gamma 1}$A or pHGX1$C_{\gamma 2a}$B and pHGX1$C_{\gamma 1}$B to chloramohenicol resistance (Cm^r) and ampicillin resistance (Amp^r). Single colonies were transferred to L broth containing chloramphenicol (20 mg/l) and ampicillin (100 mg/l) and grown overnight. Cells were collected by centrifugation, washed with Vogel-Bonner minimal medium (Vogel and Bonner, *J. Biol. Chem.* (1956) 218:97–106), and plated on minimal plates supplemented with glucose (0.4%), acid-hydrolyzed casein (0.5%), indole (10 mg/l) and chloramphenicol. Individual colonies were transferred to identical liquid medium and grown overnight. Plasmid DNA was extracted from these cultures, and the monomeric double crossover plasmids isolated by size fractionation using agarose gel electrophoresis. DNA from monomeric plasmid fractions was used to transform *E. coli* W3110 trpR ΔtrpEA2 tnaA2 rna-19 to Trp^+ and Cm^r. Transformants were screened for ampicillin sensitivity (Amp^s) by replica plating.

The crossover site generating each hybrid was located by restriction enzyme analysis. Precise crossover junctions were identified by DNA sequencing by the method of Maxam and Gilbert *Methods Enymol.* (1980) 65:499–560 or Sanger and Coulson, *Proc. Natl. Acad. Sci.* (1977) 74:5463–5467 using M13mp9 (Messing and Vieira, *Gene* (1982) 19:269–276) to generate single-stranded DNA template.

An additional variant heavy chain gene, one lacking the hinge region exon, also was constructed. The XbaI ends of fragments containing C_H gene segments were filled in and converted to SalI ends with linkers. These fragments were ligated into the SalI site of pMLSVgpt DNS-V_H.Aγ_{2a} heavy chain constant region with a hinge exon deletion was constructed by removing a StuI-SmaI fragment from an expression vector carrying the γ_{2a} C_H coding region. This plasmid is designated pMLSVgpt DNS-$V_HC_{\gamma 2a}\Delta$ hinge.

Transfection of Hybrid Heavy Chain Genes

Plasmids designed to express anti-DNS hybrid heavy chain genes were constructed as described above. The pMLSVgpt DNA-V_H contains the *E. coli* xanthine-guanine phosphoribosyltransferase gene (gpt) which is used to biochemically-select transfected mammalian cells (Mulligan and Berg, *Proc. Natl. Acad. Sci.* (1981) 78:2072–2076). The heavy chain variable region (V_H) gene segment from the anti-DNS hybridoma 27–44 (Dangi et al., *Cytometry* (1982) 2:395–401) is located upstream of the C_H gene insertion site. Hence ligation of each recombinant heavy chain constant region gene segment into pMLSVgpt DNA-V_H in the proper orientation generates a gene encoding the corresponding variant anti-DNS heavy chain polypeptide. Hybrid anti-DNS antibody-producing cell lines were generated by transfecting these recombinant vectors into a heavy-chain-loss variant of the 27–44 hybridoma cell line, designated 27–44 A5C13 (which was provided by Dr. D. Parks of Stanford University). Other hybridoma cell lines lacking production of the heavy chain could also be used.

Expression vectors were transfected into 27–44 A5C13 using the following protoplast fusion procedure. *E. coli* strain HB101 carrying the appropriate plasmid was grown in L broth to an OD_{600} of 0.6–0.7. Plasmid copy number was amplified by addition of chloramphenicol (170 mg/l) and incubating cultures overnight. Cells from 25 ml of culture were harvested by centrifugation and gently resuspended in 1.25 ml of ice cold 20% sucrose, 50 mM Tris-HCl pH 8.0. Freshly prepared lysozyme solution (0.25 ml at 5 mg/ml in 0.25M Tris-HCl pH 8.0) was added and the suspension incubated for 6 minutes on ice. A 0.5 ml aliquot of 0.25 mM EDTA pH 8.0 was added and the mixture was incubated for 5 minutes on ice. Following addition of 0.5 ml of 50 mM Tris-HCl pH 8.0, the cells were incubated for 10 minutes at 37° C. The bateria were then diluted with 10 ml of RPMI 1640 medium supplemented to 10% sucrose and 10 mM $MgCl_2$ and warmed to 37° C. After incubation for 10 minutes at room temperature, the protoplasts were used for fusion.

The 27–44 A5C13 cell line was grown to a density of 0.3 to 1×10^6 cells/ml in RPMI 1640 medium containing 1.0-mM sodium pyruvate, 2.0 mM L-glutamine, 50 mM β-mercaptoethanol, 10% fetal calf serum (FCS), and 1% NuSerum™ (Collaborative Research, Lexington, Mass.). Cells (2×10^7) were harvested by centrifugation and resuspended in 1 ml of RPMI 1640 medium with supplements. After addition of 3 ml of protoplast suspension, the cells were pelleted by centrifugation. The pellet was resuspended by slowly adding 1 ml of 45% polyethylene glycol (PEG) in RPMI 1640 medium without supplements (warmed to 37° C.) while mixing gently for 1 to 2 minutes. Nine milliliters of 37° C. RPMI medium without supplements were added slowly to dilute the PEG solution. The suspension was centrifuged and the pellet resuspended in 12 ml of RPMI 1640 medium. Cells were then dispensed into a 24-well plate in 0.5 ml aliquots. The following day, 1 ml of RPMI 1640 medium was added to each well. Two days after fusion, transfected cell lines were selected in medium containing 1 mg/l mycophenolic acid, 15 mg/l hypoxanthine, and 200 mg/l xanthine. Cultures were fed with 1 ml of selection medium as needed.

Protein Characterization

Antibodies from transfected cell lines were biosynthetically-labelled with [^{35}S]-methionine in the absence and presence of tunicamycin. Antibodies were immunoprecipitated with rabbit anti-mouse immunoglobulin antisera and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), two-dimensional non-equilibrium pH gradient gel electrophoresis, and two-dimensional non-reducing-reducing polyacrylamide gel electrophoresis (Goding, *Handbook of Experimental Immunology, Vol.* 1. Blackwell Scientific Publications, Oxford (1986) 20:1–20.33). Fluorescence emission spectra of hybrid anti-DNS antibodies bound to ε-dansyl-L-lysine were measured using 340 nm excitation on a SLM model 8000 fluorescence spectrophotometer.

Production and Purification of Genetically Engineered Antibodies

Hybrid anti-DNS antibodies were purified from sera of tumor-bearing mice or from culture supernates of transfected cell lines grown in serum-free medium (Hana Biologicals, Berkeley, Calif.). Hybridoma cell lines 27–44 and 27–13 were used to produce parental IgG_1 and IgG_{2a} anti-DNS antibodies (Dangl et al., *Cytometry* (1982) 2:395–401).

Anti-DNS antibodies were purified by affinity chromatography using a DNS analog (2-dimethylaminonaphthyl-5-sulfonyl chloride; Molecular Probes, Eugene, Oreg.) coupled to AH-Sepharose 4B (Pharmacia, Piscataway, N.J.). N-(5-Carboxypentyl)-2-dimethylaminonaphthyl-5-sulfonamide was used to elute bound antibody.

Protein A Binding

Binding of each hybrid anti-DNS antibody to *Staphylococcus aureus* protein A was measured at pH 7.0. Purified protein A (Pharmacia) and a two-fold molar excess of IgG were incubated in 150 mM sodium phosphate pH 7.0 for 20 minutes at room temperature. IgG-protein A complexes were analyzed using Superose 6 column chromatography (Pharmacia).

Results

Generation of Hybrid C_H Genes

Genetic recombination between pHGX1$C_{\gamma 2a}$A and pHGX1$C_{\gamma 1}$A or pHGX1$C_{\gamma 2a}$B and pHGX1$C_{\gamma 2a}$B yielded TRP^+ colonies at frequencies similar to that reported previously (Schneider et al., supra). The Trp^+ phenotype results from a single crossover between trpB segments in the two parental plasmids to generate an intact, functional gene. The single crossover produces a dimeric plasmid, containing the entire genetic material of both starting plasmids. The dimeric $trpB^+$ Cm^r Amp^r plasmid is expected to sustain a second recombinational event in any region of homology. A second crossover, between $C_\gamma 1$ and $C_\gamma 2a$ gene segments, yields a monomeric $trpB^+$ Cm^r Amp^s plasmid with a hybrid C_H segment. Transformation of *E. coli* W3110 trpR ΔtrpEA2 tnaA2 rna-19 with size-fractionated monomeric plasmid DNA from $trpB^+$ cells, followed by selection for Trp^+ and Cm^r, yielded only monomeric $trpB^+$ Cm^r Amp^s plasmids that always carried hybrid C_H gene segments.

Seventy independently derived hybrid C_H gene segments were recovered. Ten of the most diverse recombinant immunoglobulin heavy chain constant region gene segments were selected for further study. Six have an IgG_1 amino-terminus and an IgG_{2a} carboxy-terminus (designated $\gamma_1\gamma_{2a}^{-2\ through\ -7}$). Five of these six recombinant molecules were generated by crossovers in the C_H2 exon; the sixth was generated by a crossover in the intron between the C_H1 and hinge exons. Two recombinants (designated $\gamma_{2a}\gamma_1^{-1\ and\ -2}$) are IgG_{2a}-IgG_1 hybrids; one generated by a crossover in the C_H2 exon and the other by a crossover in the intron between the C_H1 and hinge exons. Two other recombinants ($\gamma_1\gamma_{2a}^{-1\ and\ -8}$) were included for study as controls. They encode parental γ_1 and γ_{2a} heavy chain polypeptides and arose from crossovers in DNA sequences flanking immunoglobulin coding regions.

Precise crossover points were determined by DNA sequencing. The amino acid sequences of the parental IgG_1 and IgG_{2a} polypeptides as well as the deduced sequence junctions of the ten recombinant heavy chains were determined. Nine of the ten hybrid genetic segments appear to have been generated by single crossover events, while $\gamma_1\gamma_{2a}^{-1}$ appears to have arisen by a triple exchange within the intron between C_H1 and hinge exons. The length of nucleotide sequence identity between $C_{\gamma 1}$ and $C_{\gamma 2a}$ gene segments within which exchanges occurred varied from 5 to 21 base pairs.

Expression of Recombinant Immunoglobulins Genes

All eleven heavy chain genes are expressed when transfected into the 27–44 A5C13 cell line. SDS-PAGE analyses show that each cell line synthesizes and secretes, in addition to light chain (L), an immunoglobulin heavy chain (H) polypeptide of the appropriate relative molecular weight (Mr). Two-dimensional nonequilibrium pH gradient gel electrophoresis analysis confirmed the identity of the endogenous light chain produced by each transfected cell line, as well as the expected charge (deduced from the amino acid sequence) for each recombinant heavy chain polypeptide. Each recombinant antibody, when synthesized in the presence of tunicamycin showed an altered mobility upon reducing SDS-PAGE relative to each antibody synthesized in the absence of tumicamycin indicating that each heavy chain is glycosylated.

The DNS fluorescence emission spectrum of all eleven recombinant antibodies are identical to the hybridoma IgG_1 and IgG_{2a} anti-DNS antibody fluorescence emission spectra. This is an accurate and sensitive means of identifying the antigen-combining sites of anti-DNS antibodies (Reidler et al., *J. Mol. Biol.* (1982) 158:739–746, Oi et al., *Nature* (1984) 307:136–140) and demonstrates that the original DNS combining sites have been re-created in the hybrid antibodies.

Interchain Disulfide Bridges

Each genetically engineered anti-DNS antibody was assembled into H_2L_2 tetramers as determined using Superose 6 column chromatography. The presence of interchain disulfide bridges was ascertained using non-reducing SDS-PAGE. Eight of ten hybrid immunoglobulins have mobilities characteristic of H_2L_2 molecules, having both H-L and H-H disulfide bridges. Analysis of the hingeless IgG_{2a} antibody shows H-L molecules and the absence of H_2L_2 tetramers. This was expected since the cysteines forming the H-H disulfide bridges are located in the hinge region. The two remaining hybrid immunoglobulins have unusual disulfide bridges.

Protein A Binding

The protein A binding of each of the eleven variant antibodies was assessed and used as a probe of Fc structural integrity. Table 1 summarizes the results of this analysis.

TABLE 1

Amino acid residues at positions of protein A contact and protein A binding of hybrid and hinge deleted antibodies.

	Contact Residues												Protein A
Antibody	252	253	254	308	309	310	311	312	433	434	435	436	Binding
$\gamma_1\gamma_{2a}^{-1}$(IgG2a)	Met	Ile	Ser	Ile	Gln	His	Gln	Asp	His	Asn	His	His	+
$\gamma_1\gamma_{2a}^{-2}$	–	–	–	–	–	–	–	–	–	–	–	–	+

TABLE 1-continued

Amino acid residues at positions of protein A contact and protein A binding of hybrid and hinge deleted antibodies.

	Contact Residues												Protein A
Antibody	252	253	254	308	309	310	311	312	433	434	435	436	Binding
$\gamma_1\gamma_{2a}{}^{-3}$	–	–	–	–	–	–	–	–	–	–	–	–	+
IgG_{2a}Ahinge	–	–	–	–	–	–	–	–	–	–	–	–	+
$\gamma_1\gamma_{2a}{}^{-8}$(IgG1)	Thr	–	Thr	–	Met	–	–	–	–	–	–	–	–
$\gamma_1\gamma_{2a}{}^{-6}$	Thr	–	Thr	–	Met	–	–	–	–	–	–	–	–
$\gamma_1\gamma_{2a}{}^{-7}$	Thr	–	Thr	–	Met	–	–	–	–	–	–	–	–
$\gamma_1\gamma_1{}^{-1}$	Thr	–	Thr	–	Met	–	–	–	–	–	–	–	–
$\gamma_1\gamma_1{}^{-2}$	Thr	–	Thr	–	Met	–	–	–	–	–	–	–	–
$\gamma_1\gamma_{2a}{}^{-4}$	Thr	–	Thr	–	–	–	–	–	–	–	–	–	–
$\gamma_1\gamma_{2a}{}^{-5}$	Thr	–	Thr	–	–	–	–	–	–	–	–	–	–

Contact residues in $\gamma_1\gamma_{2a}$ are given. EU numbering is used.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

What is claimed is:

1. A method for producing a functional immunoglobulin comprising a heavy chain and a light chain, which comprises the steps of:

(a) transfecting a transformed mammalian lymphocytic cell with a first DNA molecule coding for a first chain of the immunoglobulin;

(b) transfecting the cell with a second DNA molecule, said second DNA molecule coding for a second chain of the immunoglobulin, said second chain being a chain other than the first chain and said first and second chains being either the heavy chain or the light chain; and

(c) maintaining the cell in a nutrient medium, so that the cell expresses the first and second DNA molecules and the resultant chains are intracellularly assembled together to form the immunoglobulin which is then secreted in a form capable of specifically binding to antigen

wherein prior to step (a) the cell does not express a functional immunoglobulin capable of specifically binding antigen.

2. A method as recited in claim **1** wherein the cell is transfected via protoplast fusion.

3. A method as recited in claim **1** wherein the cell is transfected via calcium phosphate precipitation.

4. A method as recited in claim **1** wherein the cell is a myeloma cell.

5. A method as recited in claim **4** wherein the cell is a murine myeloma cell.

6. A method as recited in claim **1** wherein the cell does not endogenously produce any immunoglobulin chains.

7. A method as recited in claim **6** wherein the cell is a murine P_3 cell.

8. A method as recited in claim **1** wherein prior to step (a) the cell endogenously produces an immunoglobulin light chain or an immunoglobulin heavy chain, but not both.

9. A method as recited in claim **8** wherein the cell is a murine J558L cell.

10. A method as recited in claim **1** wherein the immunoglobulin comprises the variable region found in a first mammalian species and comprises the constant region found in a second mammalian species, said second mammalian species being other than the first mammalian species.

11. A method for producing a functional immunoglobulin comprising a heavy chain and a light chain, which comprises the steps of:

(a) transfecting a transformed mammalian lymphocytic cell with a plasmid comprising a first DNA molecule coding for a first chain of the immunoglobulin and a second DNA molecule coding for a second chain of the immunoglobulin, said second chain being a chain other than the first chain and said first and second chains being either the heavy chain or the light chain; and

(b) maintaining the cell in a nutrient medium so that the cell expresses said first DNA molecule and said second DNA molecule and the resultant chains are intracellularly assembled together to form the immunoglobulin which is then secreted in a form capable of specifically binding to antigen

wherein prior to step (a) the cell does not express a functional immunoglobulin capable of specifically binding antigen.

12. A method as recited in claim **11** wherein the cell is transfected via protoplast fusion.

13. A method as recited in claim **11** wherein the cell is transfected via calcium phosphate precipitation.

14. A method as recited in claim **11** wherein the cell is a myeloma cell.

15. A method as recited in claim **14** wherein the cell is a murine myeloma cell.

16. A method as recited in claim **11** wherein the cell does not endogenously produce any immunoglobulin chains.

17. A method as recited in claim **16** wherein the cell is a murine P_3 cell.

18. A method as recited in claim **11** wherein prior to step (a) the cell endogenously produces an immunoglobulin light chain or an immunoglobulin heavy chain, which endogenously-produced heavy chain is not secreted in a form capable of specifically binding to antigen, but not both.

19. A method as recited in claim **18** wherein the cell is a murine J558L cell.

20. A method as recited in claim **11** wherein the immunoglobulin comprises the variable region found in a first mammalian species and comprises the constant region found

in a second mammalian species, said second mammalian species being other than the first mammalian species.

21. A method for producing a functional immunoglobulin comprising a heavy chain and a light chain which comprises the steps of:

(a) maintaining in a nutrient medium a transformed mammalian lymphocytic cell, said cell having been transfected with a first DNA molecule coding for a first chain of the immunoglobulin and a second DNA molecule coding for a second chain of the immunoglobulin, said second chain being a chain other than the first chain and said first and second chains being either the heavy chain or the light chain;

(b) expressing from said cell the heavy chain and the light chain functionally assembled together to form said immunoglobulin which is then secreted in a form capable of binding antigen; and

(c) recovering said immunoglobulin wherein prior to being transfected, the cell does not express a functional immunoglobulin capable of specifically binding antigen.

22. A method as recited in claim **21** wherein the cell is transfected via protoplast fusion.

23. A method as recited in claim **21** wherein the cell is transfected via calcium phosphate precipitation.

24. A method as recited in claim **21** wherein the cell is a myeloma cell.

25. A method as recited in claim **24** wherein the cell is a murine myeloma cell.

26. A method as recited in claim **21** wherein the cell does not endogenously produce any immunoglobulin chains.

27. A method as recited in claim **26** wherein the cell is a murine P_3 cell.

28. A method as recited in claim **21** wherein prior to being transfected the cell endogenously produces an immunoglobulin light chain or an immunoglobulin heavy chain, but not both.

29. A method as recited in claim **28** wherein the cell is a murine J558L cell.

30. A method as recited in claim **21** wherein the immunoglobulin comprises the variable region found in a first mammalian source and comprises the constant region found in a second mammalian species, said second mammalian species being other than the first mammalian species.

31. A method for producing a functional antigen-binding protein comprising

i) a first chain comprising an immunoglobulin heavy chain variable domain and an immunoglobulin heavy chain constant domain and

ii) a second chain comprising an immunoglobulin light chain variable domain and an immunoglobulin light chain constant domain,

wherein the method comprises the steps of:

(a) transfecting a transformed mammalian lymphocytic cell with a first DNA molecule coding for the first chain of the protein;

(b) transfecting the cell with a second DNA molecule, said second DNA molecule coding for the second chain of the protein; and

(c) maintaining the cell in a nutrient medium, so that the cell expresses the first and second DNA molecules and the resultant chains are intracellularly assembled together to form the protein which is then secreted in a form capable of specifically binding to antigen

wherein prior to step (a) the cell does not express a functional antigen-binding protein.

32. A method as recited in claim **31** wherein prior to step (a) the cell does not endogenously produce any immunoglobulin chains.

33. A method as recited in claim **31** wherein prior to step (a) the cell endogenously produces an immunoglobulin light chain or an immunoglobulin heavy chain, but not both.

34. A method as recited in claim **31** wherein the first chain comprises a constant region.

35. A method as recited in claim **31** wherein the heavy and light chain variable domains are from a first mammalian species and the heavy and light chain constant domains are from a second mammalian species, said second mammalian species being other than the first mammalian species.

36. A method as recited in claim **34** wherein the heavy and light chain variable domains are from a first mammalian species and the heavy and light chain constant regions are from a second mammalian species, said second mammalian species being other than the first mammalian species.

37. A method for producing a functional antigen-binding protein comprising

i) a first chain comprising an immunoglobulin heavy chain variable domain and an immunoglobulin heavy chain constant domain and

ii) a second chain comprising an immunoglobulin light chain variable domain and an immunoglobulin light chain constant domain,

wherein the method comprises the steps of:

(a) transfecting a transformed mammalian lymphocytic cell with a plasmid comprising a first DNA molecule coding for the first chain of the protein and a second DNA molecule coding for the second chain of the protein; and

(b) maintaining the cell in a nutrient medium so that the cell expresses said first DNA molecule and said second DNA molecule and the resultant chains are intracellularly assembled together to form the protein which is then secreted in a form capable of specifically binding to antigen

wherein prior to step (a) the cell does not express a functional immunoglobulin capable of specifically binding antigen.

38. A method as recited in claim **37** wherein prior to step (a) the cell does not endogenously produce any immunoglobulin chains.

39. A method as recited in claim **37** wherein prior to step (a) the cell endogenously produces an immunoglobulin light chain or an immunoglobulin heavy chain, but not both.

40. A method as recited in claim **37** wherein the first chain comprises a constant region.

41. A method as recited in claim **39** wherein the antigen-binding protein comprises the heavy and light chain variable domains are from a first mammalian species and comprises the heavy and light chain constant domains are from a second mammalian species, said second mammalian species being other than the first mammalian species.

42. A method as recited in claim **40** wherein the antigen-binding protein comprises the heavy and light chain variable domains are from a first mammalian species and comprises the heavy and light chain constant regions are from a second mammalian species, said second mammalian species being other than the first mammalian species.

43. A method for producing a functional antigen-binding protein comprising

i) a first chain comprising an immunoglobulin heavy chain variable domain and an immunoglobulin heavy chain constant domain and

ii) a second chain comprising an immunoglobulin light chain variable domain and an immunoglobulin light chain constant domain,

wherein the method comprises the steps of:

(a) maintaining in a nutrient medium a transformed mammalian lymphocytic cell, said cell having been transfected with a first DNA molecule coding for the first chain of the protein and a second DNA molecule coding for the second chain of the protein;

(b) expressing from said cell the first and second chains functionally assembled together to form said protein which is then secreted in a form capable of binding antigen; and

(c) recovering said antigen-binding protein,

wherein prior to being transfected, the cell does not express a functional immunoglobulin capable of specifically binding antigen.

44. A method as recited in claim **43** wherein prior to step (a) the cell does not endogenously produce any immunoglobulin chains.

45. A method as recited in claim **43** wherein prior to step (a) the cell endogenously produces an immunoglobulin light chain or an immunoglobulin heavy chain, but not both.

46. A method as recited in claim **43** wherein the first chain comprises a constant region.

47. A method as recited in claim **43** wherein the heavy and light chain variable domains are from a first mammalian species and the heavy and light chain constant domains are from a second mammalian species, said second mammalian species being other than the first mammalian species.

48. A method as recited in claim **46** wherein the heavy and light chain variable domains arc from a first mammalian species and the heavy and light chain constant regions are from a second mammalian species, said second mammalian species being other than the first mammalian species.

49. A transformed mammalian lymphocytic cell producing a functional antigen-binding protein comprising

i) a first chain comprising an immunoglobulin heavy chain variable domain and an immunoglobulin heavy chain constant domain and

ii) a second chain comprising an immunoglobulin light chain variable domain and an immunoglobulin light chain constant domain,

wherein the transformed mammalian lymphocytic cell comprises:

(a) a first exogenous DNA molecule coding for the first chain of the protein; and

(b) a second exogenous DNA molecule, said second DNA molecule coding for the second chain of the protein;

wherein without the exogenous DNA molecules the cell does not express a functional antigen-binding protein.

50. A transformed mammalian lymphocytic cell as recited in claim **49** wherein without the exogenous DNA molecules the cell does not endogenously produce any immunoglobulin chains.

51. A transformed mammalian lymphocytic cell as recited in claim **49** wherein without the exogenous DNA molecules the cell endogenously produces an immunoglobulin light chain or an immunoglobulin heavy chain, but not both.

52. A transformed mammalian lymphocytic cell as recited in claim **49** wherein the first chain comprises a constant region.

53. A transformed mammalian lymphocytic cell as recited in claim **49** wherein the heavy and light chain variable domains are from a first mammalian species and the heavy and light chain constant domains are from a second mammalian species, said second mammalian species being other than the first mammalian species.

54. A transformed mammalian lymphocytic cell as recited in claim **52** wherein the heavy and light chain variable domains are from a first mammalian species and the heavy and light chain constant regions are from a second mammalian species, said second mammalian species being other than the first mammalian species.

55. A transformed mammalian lymphocytic cell producing a functional antigen-binding protein comprising

i) a first chain comprising an immunoglobulin heavy chain variable domain and an immunoglobulin heavy chain constant domain and

ii) a second chain comprising an immunoglobulin light chain variable domain and an immunoglobulin light chain constant domain,

wherein the transformed mammalian lymphocytic cell comprises:

a plasmid comprising a first exogenous DNA molecule coding for the first chain of the protein and a second exogenous DNA molecule coding for the second chain of the protein; and

wherein without the exogenous DNA molecules the cell does not express a functional antigen-binding protein.

56. A transformed mammalian lymphocytic cell as recited in claim **55** wherein without the exogenous DNA molecules the cell does not endogenously produce any immunoglobulin chains.

57. A transformed mammalian lymphocytic cell as recited in claim **55** wherein without the exogenous DNA molecules the cell endogenously produces an immunoglobulin light chain or an immunoglobulin heavy chain, but not both.

58. A transformed mammalian lymphocytic cell as recited in claim **55** wherein the first chain comprises a constant region.

59. A transformed mammalian lymphocytic cell as recited in claim **55** wherein the heavy and light chain variable domains are from a first mammalian species and the heavy and light chain constant domains arc from a second mammalian species, said second mammalian species being other than the first mammalian species.

60. A transformed mammalian lymphocytic cell as recited in claim **58** wherein the heavy and light chain variable domains are from a first mammalian species and the heavy and light chain constant regions are from a second mammalian species, said second mammalian species being other than the first mammalian species.

61. A transformed mammalian lymphocytic cell produced by steps (a) and (b) of claim **31**.

62. A transformed mammalian lymphocytic cell produced by step (a) of claim **37**.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,807,715
DATED : September 15, 1998
INVENTOR(S) : S.L. Morrison, Herzenberg, L.A. and Oi, V.T.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page, Item [54], and Column 1, lines 2 and 5,
Delete "**LYMPHOCYTE**" and replace with -- **LYMPHOCYTIC** --; after "**IMMUNOGLOBULIN**", add -- **AND FRAGMENTS** --.

Item [75], Inventors, delete "Scarsdale, N.Y." and replace with -- Los Angeles --; delete "Menlo Park" and replace with -- Mountain View --; delete "both" and replace with -- all --.

Item [56], in **References Cited**, after "Seno et al 1983 Nucleic", delete "Acid" and replace with -- Acids --; after "Research 11 (3)", delete ";" and replace with -- : --; and after "Dolby et al 1980 PNAS 77 (10)" add -- : --.

Column 1,
Line 16, add the paragraph -- The work described herein was supported in part by grants from the National Institutes of Health (NIH), including AI-00408, AI-08917, CA-04681, and CA-16858. The United States Government has certain rights in the invention. --
Line 19, delete "1.".
Line 41, delete "2.".
Line 61, after "functional", add -- , --; after "assembled", add -- , --.
Line 66, delete "mouse-" and replace with -- mouse: --.
Line 67, delete ":".

Column 3,
Line 26, delete "prepar" and replace with -- prepara --.
Line 32, delete "CDNA" and replace with -- cDNA --.

Column 4,
Line 45, delete "CDNA" and replace with -- cDNA --.
Line 52, delete "CDNA" and replace with -- cDNA --.
Line 59, delete "CDNA" and replace with -- cDNA --.
Line 65, delete "Joining" and replace with -- joining --.

Column 8,
Line 2, after "immunization", add -- , --.
Line 21, delete "VH" and replace with -- V_H --.
Line 22, delete "Vκ" and replace with -- V_κ --.
Line 24, delete "VH" and replace with -- V_H --.
Line 29, delete "Vκ" and replace with -- V_κ --.
Line 31, delete "Jκ" and replace with -- J_κ --; delete "Cκ" and replace with -- C_κ --.

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,807,715
DATED : September 15, 1998
INVENTOR(S) : S.L. Morrison, Herzenberg, L.A. and Oi, V.T.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 9,
Line 9, delete "Five" and replace with -- Fine --; delete "Chamicals" and replace with -- Chemicals --.
Line 19, delete "VH-VL" and replace with -- V_H-V_L --.
Line 57, delete "H2L2" and replace with -- H_2L_2 --.
Line 67, delete "K" and replace with -- $_K$ --; delete "antiodies" and replace with -- antibodies --.

Column 10,
Line 5, after "expected" add -- , --.
Line 10, delete "VH" and replace with -- V_H --; delete "Vκ" and replace with -- V_κ --.
Line 14, delete "mouse: human" and replace with -- mouse:human --.
Line 22, delete "VH" and replace with -- V_H --; delete "Vκ" and replace with -- V_κ --.
Line 36, delete "Mr" and replace with -- M_r --.
Line 42, delete "Mr" and replace with -- M_r --.
Line 54, delete "hybri-doma-" and replace with -- hybridoma --.

Column 11,
Line 5, delete "Vκ" and replace with -- V_κ --.
Line 10, delete "mouse: human" and replace with -- mouse:human --.
Line 21, delete "use" and replace with -- used --.
Line 35, delete "IgG_1, IgG_2, IgG_3, IgG_4, rabbit IgG and mouse IgG_3" and replace with -- IgG1, IgG2, IgG3, IgG4, rabbit IgG and mouse IgG3 --.
Line 37, delete "(delta)" and replace with -- Δ --.
Line 41, delete "V_κ" and replace with -- V_κ --
Line 43, after "*Ann*" add -- . --.
Line 44, delete "V_κ" and replace with -- V_κ -- .
Line 60, delete "pure, >95%" and replace with -- pure (>95%) --.
Line 63, delete "mouse-human" and replace with -- mouse:human --.
Line 64, delete "IgG_3" and replace with -- IgG3 --.

Column 12,
Line 3, delete "IgG_1" and replace with -- IgG1 --.
Line 10, delete "affinity purified" and replace with -- affinity-purified --.
Line 22, delete "labelling" and replace with -- labeling --.
Line 26, delete "pp." and replace with -- p. --.
Line 31, after "Kornfeld" add -- , --.
Line 32, delete "Wigzell *Proc*," and replace with -- Wigzell, *Proc.* --.
Line 33, after "et al." add -- , --.
Line 42, delete "IgG_1-IgG_{2a}" and replace with -- IgG1-IgG2a --.

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,807,715
DATED : September 15, 1998
INVENTOR(S) : S.L. Morrison, Herzenberg, L.A. and Oi, V.T.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 12 cont'd,
Line 64, delete "IgG_1" and replace with -- IgG1 --; delete "IgG_{2a}" and replace with -- IgG2a --.
Line 65, delete "IgG_{2a}" and replace with -- IgG2a --; delete "IgG_1" and replace with -- IgG1--.

Column 13,
Line 4, delete "T_4" and replace with -- T4 --.
Line 43, after "Gilbert" add -- , --.
Line 65, delete "Dangi" and replace with -- Dangl --

Column 14,
Line 24, delete "bateria" and replace with -- bacteria --.
Line 41, delete "C." and replace with -- C --.
Line 53, delete "labelled" and replace with -- labeled --.

Column 15,
Line 5, delete "IgG_1" and replace with -- IgG1 --; delete "IgG_{2a}" and replace with -- IgG2a --.
Line 25, delete "pHGX1$C_{\gamma 2}$aB" and replace with -- pHGX1$C_{\gamma 1}$B --.
Line 27, delete "Trp^+" and replace with -- TRP^+ --.
Line 39, delete "Trp^+" and replace with -- TRP^+ --.
Line 44, delete "IgG_1" and replace with -- IgG1 --.
Line 45, delete "IgG_{2a}" and replace with -- IgG2a --
Line 46, delete "-7" and replace with -- $^{-7}$ --.
Line 49, delete "IgG_{2a}" and replace with -- IgG2a --
Line 50, delete "IgG_1" and replace with -- IgG1 --.

Column 16,
Line 2, delete "IgG_1" and replace with -- IgG1 --.
Line 3, delete "IgG_{2a}" and replace with -- IgG2a --.
Line 18, delete "Mr" and replace with -- M_r --.
Line 29, delete "are" and replace with -- is --; delete "IgG_1" and replace with -- IgG1 --.
Line 30, delete "IgG_{2a}" and replace with -- IgG2a --.
Line 44, delete "IgG_{2a}" and replace with -- IgG2a --.

Column 17,
Line 63, delete "P_3" and replace with -- P3 --.

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,807,715
DATED : September 15, 1998
INVENTOR(S) : S.L. Morrison, Herzenberg, L.A., and Oi, V.T.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 18,
Line 57, delete "P_3" and replace with -- P3 --.

Column 19,
Line 33, delete "P_3" and replace with -- P3 --.

Column 20,
Line 50, delete "39" and replace with -- 37 --.
Line 52, delete "are".
Line 53, delete "are".
Line 58, delete "are".
Line 59, delete "are".

Column 21,
Line 13, shift line beginning with "express" to left margin.
Line 14, shift line beginning with "specifically" to left margin.
Line 29, delete "arc" and replace with -- are --.

Column 22,
Line 43, delete "arc" and replace with -- are --.

Signed and Sealed this

Ninth Day of July, 2002

Attest:

Attesting Officer

JAMES E. ROGAN
Director of the United States Patent and Trademark Office

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,807,715
DATED : September 15, 1998
INVENTOR(S) : S.L. Morrison, Herzenberg, L.A. and Oi, V.T.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page, Item [54] and Column 1, Lines 1 and 5,
Item [54], delete "**LYMPHOCYTE**" and replace with **-- LYMPHOCYTIC** --; after "**IMMUNOGLOBULIN**", add -- **AND FRAGMENTS** --.
Item [75], Inventors, delete "Scarsdale, N.Y." and replace with -- Los Angeles --; delete "Menlo Park" and replace with -- Mountain View --; delete "both" and replace with -- all --.
Item [73], Assignee, delete "Assignee" and replace with -- Assignees --; after "Calif." add -- , **and The Trustees of Columbia University**, New York, N.Y. --.
Item [56], **References Cited**, after "Seno, et al 1983 Nucleic", delete "Acid" and replace with **--** Acids --; after "Research 11 (3)", delete ";" and replace with -- . --; and after "Dolby et al 1980 PNAS 77 (10)" add -- : --.

Column 1,
Line 16, add the paragraph -- The work described herein was supported in part by grants from the National Institutes of Health (NIH), including AI-00408, AI-08917, CA-04681, and CA-16858. The United States Government has certain rights in the invention. --
Line 19, delete "1.".
Line 41, delete "2.".
Line 61, after "functional", add -- , --; after "assembled", add -- , --.
Line 66, delete "mouse-" and replace with -- mouse: --
Line 67, delete ":".

Column 3,
Line 26, delete "prepar" and replace with -- prepara --.
Line 32, delete "CDNA" and replace with -- cDNA --.

Column 4,
Line 45, delete "CDNA" and replace with -- cDNA --.
Line 52, delete "CDNA" and replace with -- cDNA --.
Line 59, delete "CDNA" and replace with -- cDNA --.
Line 65, delete "Joining" and replace with -- joining --..

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,807,715

DATED : September 15, 1998
INVENTOR(S) : S.L. Morrison, Herzenberg, L.A. and Oi, V.T.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 8,
Line 2, after "immunization", add -- , --.
Line 21, delete "VH" and replace with -- V_H --.
Line 22, delete "Vκ" and replace with -- V_κ -- (Greek letter kappa subscripted).
Line 24, delete "VH" and replace with -- V_H --.
Line 29, delete "Vκ" and replace with -- V_κ -- (Greek letter kappa subscripted).
Line 31, delete "Jκ" and replace with -- J_κ -- (Greek letter kappa subscripted); delete "Cκ" and replace with -- C_κ --. (Greek letter kappa subscripted).

Column 9,
Line 9, delete "Five" and replace with -- Fine --; delete "Chamicals" and replace with -- Chemicals --.
Line 19, delete "VH-VL" and replace with -- V_H-V_L --.
Line 57, delete "H2L2" and replace with -- H_2L_2 --.
Line 67, delete "K" and replace with -- κ --; delete "antiodies" and replace with -- antibodies --.

Column 10,
Line 5, after "expected" add -- , --.
Line 10, delete "VH" and replace with -- V_H --; delete "V_K" and replace with -- V_κ -- (Greek letter kappa subscripted).
Line 14, delete "mouse: human" and replace with -- mouse:human --.
Line 22, delete "VH" and replace with -- V_H --; delete "Vκ" and replace with -- V_κ -- (Greek letter kappa subscripted).
Line 36, delete "Mr" and replace with -- M_r --.
Line 42, delete "Mr" and replace with -- M_r --.
Line 54, delete "hybri-doma-" and replace with -- hybridoma --.

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,807,715
DATED : September 15, 1998
INVENTOR(S) : S.L. Morrison, Herzenberg, L.A. and Oi, V.T.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 11,
Line 5, delete "Vκ" and replace with -- V_κ -- (Greek letter kappa subscripted).
Line 10, delete "mouse: human" and replace with -- mouse:human --.
Line 21, delete "use" and replace with -- used --.
Line 35, delete "IgG_1, IgG_2, IgG_3, IgG_4, rabbit IgG and mouse IgG_3" and replace with -- IgG1, IgG2, IgG3; IgG4, rabbit IgG and mouse IgG3 --.
Line 37, delete "(delta)" and replace with -- Δ -- (Greek letter capital delta).
Line 41, delete "VK" and replace with -- V_κ -- (Greek letter kappa subscripted).
Line 43, after "Ann" add -- . --.
Line 44, delete "Vκ" and replace with -- V_κ -- (Greek letter kappa subscripted).
Line 60, delete "pure, >95%" and replace with -- pure (>95%) --.
Line 63, delete "mouse-human" and replace with -- mouse:human --.
Line 64, delete "IgG_3" and replace with -- IgG3 --.

Column 12,
Line 3, delete "IgG_1" and replace with -- IgG1 --.
Line 10, delete "affinity purified" and replace with -- affinity-purified --.
Line 22, delete "labelling" and replace with -- labeling --.
Line 26, delete "pp." and replace with -- p. --.
Line 31, after "Kornfeld" add -- , --.
Line 32, delete "Wigzell *Proc*," and replace with -- Wigzell, *Proc.* --.
Line 33, after "et al." add -- , --.
Line 42, delete "IgG_1-IgG_{2a}" and replace with -- IgGl-IgG2a --.
Line 64, delete "IgG_1" and replace with -- IgG1 --; delete "IgG_2a" and replace with -- IgG2a --.
Line 65, delete "IgG_{2a}" and replace with -- IgG2a --; delete "IgG_1" and replace with -- IgG1 --.

Column 13,
Line 4, delete "T_4" and replace with -- T4 --.
Line 43, after "Gilbert" add -- , --.
Line 65, delete "Dangi" and replace with -- Dangl --.

Column 14,
Line 24, delete "bateria" and replace with -- bacteria --.
Line 41, delete "C." and replace with -- C --.
Line 53, delete "labelled" and replace with -- labeled --.

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,807,715
DATED : September 15, 1998
INVENTOR(S) : S.L. Morrison, Herzenberg, L.A. and Oi, V.T.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 15,
Line 5, delete "IgG_1" and replace with -- IgG1 --; delete "IgG_{2a}" and replace with -- IgG2a --.
Line 25, delete "pHGXIC$_{\gamma 2}$aB" and replace with -- pHGXlC$_{\gamma 1}$B -- .
Line 27, delete "Trp·" and replace with -- TRP^+ --.
Line 39, delete "Trp·" and replace with -- TRP^+ --.
Line 44, delete "IgG_1" and replace with -- IgG1 --.
Line 45, delete "IgG_{2a}" and replace with -- IgG2a --.
Line 49, delete "IgG_{2a}" and replace with -- IgG2a --.
Line 50, delete "IgG_1" and replace with -- IgG1 --.
Line 46, delete "-7" and replace with -- $^{-7}$ --.

Column 16,
Line 2, delete "IgG_1" and replace with -- IgG1 --.
Line 3, delete "IgG_{2a}" and replace with -- IgG2a --.
Line 18, delete "Mr" and replace with -- M_r --.
Line 29, delete "are" and replace with -- is --; delete "IgG_1" and replace with -- IgG1 --.
Line 30, delete "IgG_{2a}" and replace with -- IgG2a --.
Line 44, delete "IgG_{2a}" and replace with -- IgG2a --.

Column 17,
Line 63, delete "P_3" and replace with -- P3 --.

Column 18,
Line 57, delete "P_3" and replace with -- P3 --.

Column 19,
Line 33, delete "P_3" and replace with -- P3 --.

Column 20,
Line 50, delete "39" and replace with -- 37 --.
Line 52, delete "are".
Line 53, delete "are".
Line 58, delete "are".
Line 59, delete "are"

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,807,715
DATED : September 15, 1998
INVENTOR(S) : S.L. Morrison, Herzenberg, L.A. and Oi, V.T.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 21,
Line 13, shift line beginning with "express" to left margin.
Line 14, shift line beginning with "specifically" to left margin.
Line 29, delete "arc" and replace with -- are --.

Column 22,
Line 43, delete "arc" and replace with -- are --.

This certificate supersedes the Certificate of Correction issued July 9, 2002.

Signed and Sealed this

Sixth Day of August, 2002

Attest:

Attesting Officer

JAMES E. ROGAN
Director of the United States Patent and Trademark Office

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

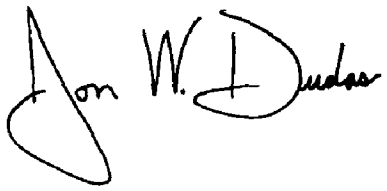
PATENT NO. : 5,807,715
DATED : September 15, 1998
INVENTOR(S) : Sherie L. Morrison et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page,
Item [73], Assignee, should read as follows:
-- **The Board of Trustees of The Leland Stanford Junior University**, Stanford, CA; and **The Trustees of Columbia University in the City of New York**, New York, NY --.

Signed and Sealed this

Eighteenth Day of May, 2004



JON W. DUDAS
Acting Director of the United States Patent and Trademark Office

EXHIBIT D

US007598083B2

(12) **United States Patent**
Epstein et al.

(10) **Patent No.: US 7,598,083 B2**
(45) **Date of Patent: Oct. 6, 2009**

(54) **CHEMICALLY DEFINED MEDIA COMPOSITIONS**

(75) Inventors: **David Epstein**, Philadelphia, PA (US); **Roger Monsell**, Willistown, PA (US); **Joseph Horwitz**, Swarthmore, PA (US); **Susan Lenk**, Devon, PA (US); **Sadettin Ozturk**, Paoli, PA (US); **Christopher Marsh**, Audubon, PA (US)

(73) Assignee: **Centocor, Inc.**, Malvern, PA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 468 days.

(21) Appl. No.: **11/260,788**

(22) Filed: **Oct. 27, 2005**

(65) **Prior Publication Data**

US 2006/0094113 A1 May 4, 2006

Related U.S. Application Data

(60) Provisional application No. 60/623,718, filed on Oct. 29, 2004.

(51) **Int. Cl.**
C12N 5/00 (2006.01)
C12N 5/02 (2006.01)

(52) **U.S. Cl.** **435/404**; 435/325

(58) **Field of Classification Search** ..... None
See application file for complete search history.

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Primary Examiner—Lora E Barnhart
(74) *Attorney, Agent, or Firm*—Kirk Baumeister

(57) **ABSTRACT**

Chemically defined media compositions for the culture of eukaryotic cells are disclosed. The compositions are useful for eukaryotic cell culture in perfusion bioreactors and other vessels.

11 Claims, 3 Drawing Sheets

Fig. 1

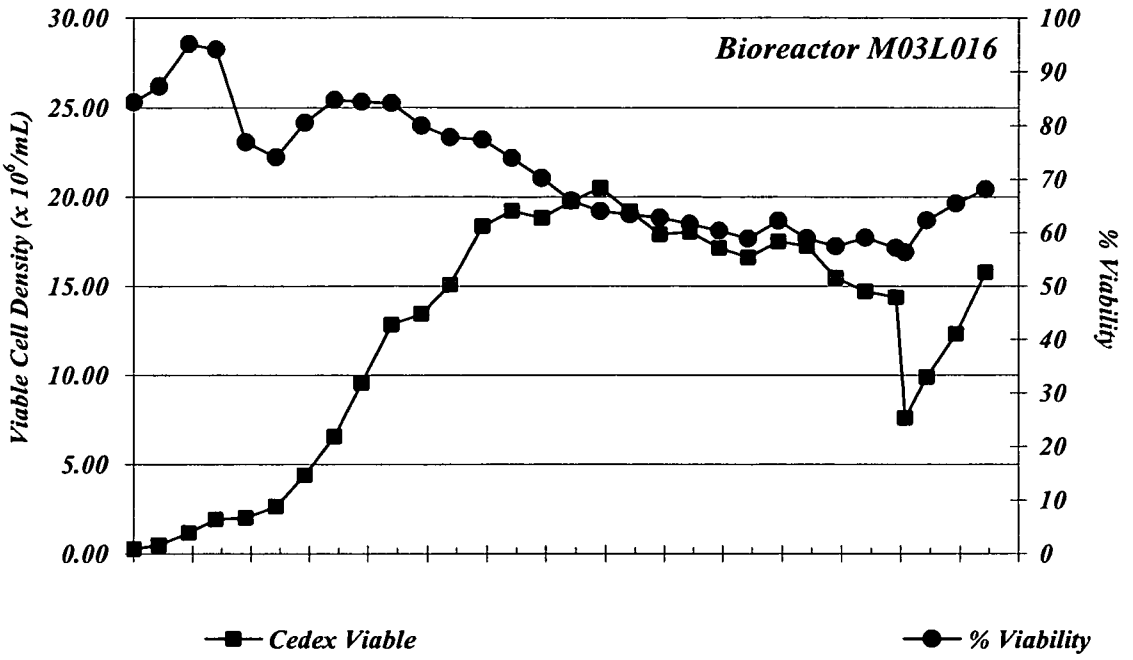

Bioreactor M03L016
Viable Cell Density (x 10^6/mL)
% Viability
30.00
25.00
20.00
15.00
10.00
5.00
0.00
100
90
80
70
60
50
40
30
20
10
0
Cedex Viable
% Viability

Fig. 2

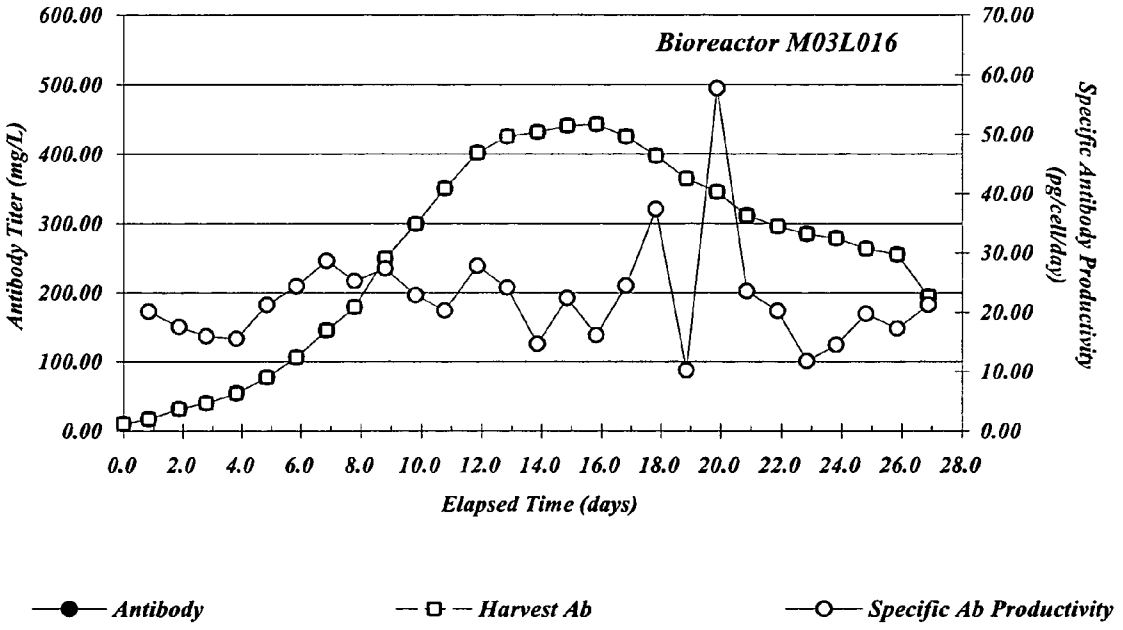
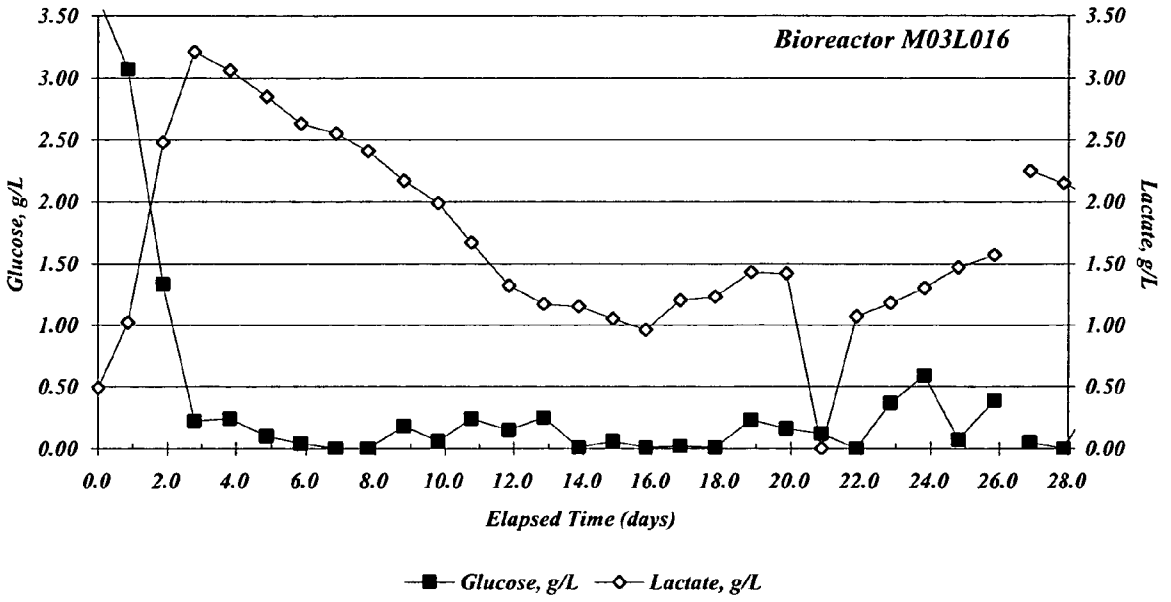

Bioreactor M03L016
Antibody Titer (mg/L)
600.00
500.00
400.00
300.00
200.00
100.00
0.00
Specific Antibody Productivity (pg/cell/day)
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
0.0 2.0 4.0 6.0 8.0 10.0 12.0 14.0 16.0 18.0 20.0 22.0 24.0 26.0 28.0
Elapsed Time (days)
Antibody
Harvest Ab
Specific Ab Productivity

Fig. 3


Bioreactor M03L016
Glucose, g/L
Lactate, g/L
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0.0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
16.0
18.0
20.0
22.0
24.0
26.0
28.0
Elapsed Time (days)
Glucose, g/L
Lactate, g/L

CHEMICALLY DEFINED MEDIA COMPOSITIONS

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/623,718, filed 29 Oct. 2004, the entire contents of which is incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to chemically defined media compositions for the culture of eukaryotic cells.

BACKGROUND OF THE INVENTION

Contamination of conventional eukaryotic cell culture media with "adventitious particles" such as bacterial, virus or prion particles is a serious potential problem in the industrial preparation of biopharmaceuticals such as antibodies or therapeutic proteins. Such contaminants in a biopharmaceutical are capable of causing patient infections and disease and may limit yields due to increased metabolic burdens on the host productioncell line.

Variant Creutzfeldt-Jakob disease (vCJD) is one example of a patient disease that could be caused by adventitious particle contamination. This disease is prion mediated in humans and is characterized by fatal neurodegeneration. vCJD has been strongly linked with exposure to the Bovine Spongiform Encephalopathy (BSE) prion which causes fatal, neurodegenerative "Mad Cow Disease" in cattle.

Adventitious particle contamination of conventional eukaryotic cell culture media can result from the incorporation of animal-derived components and protein growth factors into conventional media. Such contamination can occur when animal-derived media components are harvested from an animal harboring disease-causing bacteria, viruses, or prions. For example, bovine serum harvested from a cow with BSE may be contaminated with prions capable of causing human vCJD. The ultimate result of such adventitious particle contamination can be the contamination of eukaryotic cell cultures and the biopharmaceuticals prepared from such cultures.

Adventitious particle contamination can be avoided by culturing eukaryotic cells in animal component free cell culture media. Ideally, such media are "chemically defined" such that the media compositions contain only known chemical compounds, and are free of all proteins—even those not of animal origin such as recombinant proteins.

Chemically defined media compositions optimal for production of biopharmaceuticals, such as antibodies, must satisfy several different criteria. First, such compositions must limit eukaryotic cell damage resulting from shear forces and other cell-damaging processes that occur in the bioreactor vessels typically used for biopharmaceutical production. Second, such compositions must enable eukaryotic cell cultures to have high viable cell densities (i.e., number viable cells/ml media) and high percentages of viable cells. Third, such compositions must permit high titers of secreted biopharmaceutical products (i.e., antibody mg/L media) and high specific productivities (i.e., pg antibody/viable cell/day). Lastly, such compositions must limit the production of lactic acid by cultured eukaryotic cells to permit the most efficient cellular use of glucose.

Thus, a need exists for chemically defined media compositions which satisfy these criteria and are optimized for biopharmaceutical production.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1**. Eukaryotic cell viability in MET 1.5 cell culture media.

FIG. **2**. Antibody titer and specific productivity in MET 1.5 cell culture media.

FIG. **3**. Decreased lactate production in MET 1.5 cell culture media.

SUMMARY OF THE INVENTION

One aspect of the invention is a soluble composition, suitable for producing a cell culture media, wherein the media comprises the following components in the following amounts per liter:

anhydrous $CaCl_2$, 5-200 mg;
anhydrous $MgCl_2$, 15-50 mg;
anhydrous $MgSO_4$, 20-80 mg;
$FeSO_4.7H_2O$, 0.05-0.50 mg;
$Fe(NO_3)_3.9H_2O$, 0.01-0.08 mg;
$ZnSO_4.7H_2O$, 0.40-1.20 mg;
ferric ammonium citrate, 0.04-200 mg;
KCl, 280-500 mg;
NaCl, 5000-7500 mg;
NaH_2PO_4—H_2O, 30-100 mg;
Na_2HPO_4, 30-100 mg;
$CuSO_4.5H_2O$, 0.001-0.005 mg;
$CoCl_2.6H_2O$, 0.001-0.10 mg;
$(NH_4)_6Mo_7O_{24}$ $4H_2O$, 0.001-0.005 mg;
$MnSO_4$—H_2O, 0.000070-0.0080 mg;
$NiSO_4.6H_2O$, 0.000025-0.0005 mg;
Na_2SeO_3, 0.004-0.07 mg;
$Na_2SiO_3.9H_2O$, 0.02-0.4 mg;
$SnCl_2.2H_2O$, 0.000025-0.0005 mg;
NH_4VO_3, 0.0001-0.0025 mg;
D-Glucose, 500-8000 mg;
sodium pyruvate, 0.0-1000 mg;
sodium hypoxanthine, 0.0-20.0 mg;
glycine, 0.0-150 mg;
L-alanine, 0.0-150 mg;
L-arginine.HCl, 200-5000 mg;
L-asparagine.H_2O, 40-250 mg;
L-aspartic acid, 20-1000 mg;
L-cysteine.HCl H_2O, 25.0-250 mg;
L-cystine.2HCl, 15-150 mg;
L-glutamic acid, 0-1000 mg;
L-histidine.HCl.H_2O, 100-500 mg;
L-isoleucine, 50-1000 mg;
L-leucine, 50-1000 mg;
L-lysine.HCl, 100-1000 mg;
L-methionine, 50-500 mg;
L-ornithine.HCl, 0-100 mg;
L-phenylalanine, 25-1000 mg;
L-proline, 0-1000 mg;
L-serine, 50-500 mg;
L-taurine, 0-1000 mg;
L-threonine, 50-600 mg;
L-tryptophan, 2-500 mg;
L-tyrosine.2Na.2H_2O, 25-250 mg;
L-valine, 100-1000 mg;
d-biotin, 0.04-1.0 mg;
D-calcium pantothenate, 0.1-5.0 mg;
choline chloride, 1-100 mg;
folic acid, 1-10 mg;
i-Inositol, 10-1000 mg;
nicotinamide, 0.5-30 mg;
p-aminobenzoic acid, 0.1-20 mg;

riboflavin, 0.05-5.0 mg;
thiamine.HCl, 0.5-20 mg;
thymidine, 0-3.0 mg;
vitamin B_{12}, 0.05-5.0 mg;
linoleic acid, 0.01-2.0 mg;
DL-α-lipoic acid, 0.03-1.0 mg;
pyridoxine.HCl, 0.5-30 mg;
putrescine.2HCl, 0.025-0.25 mg; and
ethanolamine.HCl, 2-100 mg.

Another aspect of the invention is a soluble composition, suitable for producing a cell culture media, wherein the media comprises the following components in the following amounts per liter:
$CaCl_2$, 100.95 mg;
$MgCl_2$, 24.77 mg;
$MgSO_4$, 42.24 mg;
$FeSO_4.7H_2O$, 0.3607 mg;
$Fe(NO_3)_3.9H_2O$, 0.0432 mg;
$ZnSO_4.7H_2O$, 0.6225 mg;
ferric ammonium citrate, 43.25 mg;
KCl, 386.9 mg;
NaCl, 5866.0 mg;
NaH_2PO_4—H_2O, 54.07 mg;
Na_2HPO_4, 61.44 mg;
$CuSO_4.5H_2O$, 0.003287 mg;
$CoCl_2.6H_2O$, 0.0020606 mg;
$(NH_4)_6Mo_7O_{24}.4H_2O$, 0.000535 mg;
$MnSO_4.H_2O$, 0.00008571 mg;
$NiSO_4.6H_2O$, 0.0000514 mg;
Na_2SeO_3, 0.007489 mg;
$Na_2SiO_3.9H_2O$, 0.03671 mg;
$SnCl_2.2H_2O$, 0.0000488 mg;
NH_4VO_3, 0.0002530 mg;
D-Glucose, 3680.52 mg;
sodium pyruvate, 100 mg;
sodium hypoxanthine, 2.069 mg;
glycine, 16.23 mg;
L-alanine, 79.31 mg;
L-arginine.HCl, 674.89 mg;
L-asparagine.H_2O, 182.25 mg;
L-aspartic acid, 67.23 mg;
L-cysteine.HCl.H_2O, 57.63 mg;
L-cystine.2HCl, 106.70 mg;
L-glutamic acid, 6.36 mg;
L-histidine.HCl.H_2O, 250.55 mg;
L-isoleucine, 245.43 mg;
L-leucine, 263.42 mg;
L-lysine.HCl, 276.41 mg;
L-methionine, 85.40 mg;
L-ornithine.HCl, 2.44 mg;
L-phenylalanine, 104.23 mg;
L-proline, 14.94 mg;
L-serine, 146.36 mg;
L-taurine, 3.64 mg;
L-threonine, 199.09 mg;
L-tryptophan, 70.71 mg;
L-tyrosine.2Na.2H_2O, 195.58 mg;
L-valine, 174.34 mg;
d-biotin, 0.4359 mg;
D-calcium pantothenate, 1.9394 mg;
choline chloride, 10.8009 mg;
folic acid, 3.4329 mg;
i-inositol, 81.7965 mg;
nicotinamide, 3.1342 mg;
p-aminobenzoic acid, 2.1645 mg;
riboflavin, 0.5359 mg;
thiamine.HCl, 2.3377 mg;
thymidine, 0.316 mg;
vitamin B_{12}, 0.5887 mg;
linoleic acid, 0.0364 mg;
DL-α-lipoic acid, 0.0909 mg;
pyridoxine.HCl, 3.0442 mg;
putrescine.2HCl, 0.0701 mg; and
ethanolamine.HCl, 14.37 mg.

The invention also provides compositions comprising cell culture media which can be made from the soluble compositions of the invention.

DETAILED DESCRIPTION OF THE INVENTION

All publications, including but not limited to patents and patent applications, cited in this specification are herein incorporated by reference as though fully set forth.

The term "buffering molecule" as used herein and in the claims means a molecule that has a buffering range suitable for maintaining a pH between 5.9 and 7.8.

The term "pK_a" as used herein and in the claims means the negative logarithm of the acid dissociation constant (K_a) of a buffering molecule in an aqueous solution. pK_a is, in part, a function of the temperature of the aqueous solution in which a buffering molecule is solubilized.

The term "cell protectant" as used herein and in the claims means a substance that protects eukaryotic cells from damage. Such damage may be caused, for example, by shear forces or the effects of gas bubble sparging in a bioreactor vessel.

The present invention provides chemically defined compositions useful in the culture of eukaryotic cells. Such eukaryotic cells may have insect, avian, mammalian, or other origins. These cells may secrete a protein, such as an antibody, or produce other useful products or results. These proteins, products, or results may be constituatively produced by a cell or produced as the result of transfection with a nucleic acid sequence. The cells may be cultured in liquid media as suspension cultures or as adherent cultures. Cells may also be cultured by suspension in semi-solid media comprising the compositions of the invention.

Cells may be cultured in a variety of vessels including, for example, perfusion bioreactors, cell bags, culture plates, flasks and other vessels well known to those of ordinary skill in the art. Ambient conditions suitable for cell culture, such as temperature and atmospheric composition, are also well known to those skilled in the art. Methods for the culture of cells are also well known to those skilled in the art.

The compositions of the invention are particularly useful in the culture of mammalian cells. Examples of mammalian cells include myeloma derived cells, non-immortalized cells of the B cell lineage, and immortalized cells of the B cell lineage such as hybridomas. Examples of myeloma derived cell lines include the SP2/0 (American Type Culture Collection (ATCC), Manasas, Va., CRL-1581), NSO (European Collection of Cell Cultures (ECACC), Salisbury, Wiltshire, UK, ECACC No. 85110503), FO (ATCC CRL-1646), and Ag653 (ATCC CRL-1580) cell lines which were obtained from mice. The C743B cell line is an example of a SP2/0 derived cell line that produces a fully human, anti-IL-12 mAb as the result of stable transfection. The YB2/0 cell line (ATCC CRL-1662) is an example of a myeloma derived cell line obtained from rats (*Rattus norvegicus*). An example of a myeloma derived cell line obtained from humans is the U266 cell line (ATTC CRL-TIB-196). Some myeloma derived cell lines, such as NSO, YB2/0, and Ag653 cells and related cell lines may require chemically defined lipid concentrates or other supplements for successful culture. Those skilled in the

art will recognize other myeloma cell lines and myeloma derived cell lines as well as any supplements required for the successful culture of such cells.

In one aspect the invention provides a soluble composition, suitable for producing a cell culture media, wherein the media comprises the following components in the following amounts per liter:

anhydrous $CaCl_2$, 5-200 mg;
anhydrous $MgCl_2$, 15-50 mg;
anhydrous $MgSO_4$, 20-80 mg;
$FeSO_4.7H_2O$, 0.05-0.50 mg;
$Fe(NO_3)_3.9H_2O$, 0.01-0.08 mg;
$ZnSO_4.7H_2O$, 0.40-1.20 mg;
ferric ammonium citrate, 0.04-200 mg;
KCl, 280-500 mg;
NaCl, 5000-7500 mg;
$NaH_2PO_4.H_2O$, 30-100 mg;
Na_2HPO_4, 30-100 mg;
$CuSO_4.5H_2O$, 0.001-0.005 mg;
$CoCl_2.6H_2O$, 0.001-0.10 mg;
$(NH_4)_6Mo_7O_{24}$ $4H_2O$, 0.001-0.005 mg;
$MnSO_4.H_2O$, 0.000070-0.0080 mg;
$NiSO_4.6H_2O$, 0.000025-0.0005 mg;
Na_2SeO_3, 0.004-0.07 mg;
$Na_2SiO_3.9H_2O$, 0.02-0.4 mg;
$SnCl_2.2H_2O$, 0.000025-0.0005 mg;
NH_4VO_3, 0.0001-0.0025 mg;
D-Glucose, 500-8000 mg;
sodium pyruvate, 0.0-1000 mg;
sodium hypoxanthine, 0.0-20.0 mg;
glycine, 0.0-150 mg;
L-alanine, 0.0-150 mg;
L-arginine.HCl, 200-5000 mg;
L-asparagine.H_2O, 40-250 mg;
L-aspartic acid, 20-1000 mg;
L-cysteine.HCl H_2O, 25.0-250 mg;
L-cystine.2HCl, 15-150 mg;
L-glutamic acid, 0-1000 mg;
L-histidine.HCl.H_2O, 100-500 mg;
L-isoleucine, 50-1000 mg;
L-leucine, 50-1000 mg;
L-lysine.HCl, 100-1000 mg;
L-methionine, 50-500 mg;
L-ornithine.HCl, 0-100 mg;
L-phenylalanine, 25-1000 mg;
L-proline, 0-1000 mg;
L-serine, 50-500 mg;
L-taurine, 0-1000 mg;
L-threonine, 50-600 mg;
L-tryptophan, 2-500 mg;
L-tyrosine-2Na-$2H_2O$, 25-250 mg;
L-valine, 100-1000 mg;
d-biotin, 0.04-1.0 mg;
D-calcium pantothenate, 0.1-5.0 mg; choline chloride, 1-100 mg;
folic acid, 1-10 mg;
i-Inositol, 10-1000 mg;
nicotinamide, 0.5-30 mg;
p-aminobenzoic acid, 0.1-20 mg;
riboflavin, 0.05-5.0 mg;
thiamine.HCl, 0.5-20 mg;
thymidine, 0-3.0 mg;
vitamin B_{12}, 0.05-5.0 mg;
linoleic acid, 0.01-2.0 mg;
DL-α-lipoic acid, 0.03-1.0 mg;
pyridoxine.HCl, 0.5-30 mg;
putrescine.2HCl, 0.025-0.25 mg; and
ethanolamine.HCl, 2-100 mg.

This type of soluble composition has been named "MET" and typically is a powder.

In another aspect the invention provides a soluble composition, suitable for producing a cell culture media, wherein the media comprises the following components in the following amounts per liter:

$CaCl_2$, 100.95 mg;
$MgCl_2$, 24.77 mg;
$MgSO_4$, 42.24 mg;
$FeSO_4.7H_2O$, 0.3607 mg;
$Fe(NO_3)_3.9H_2O$, 0.0432 mg;
$ZnSO_4.7H_2O$, 0.6225 mg;
ferric ammonium citrate, 43.25 mg;
KCl, 386.9 mg;
NaCl, 5866.0 mg;
$NaH_2PO_4.H_2O$, 54.07 mg;
Na_2HPO_4, 61.44 mg;
$CuSO_4.5H_2O$, 0.003287 mg;
$CoCl_2.6H_2O$, 0.0020606 mg;
$(NH_4)_6Mo_7O_{24}.4H_2O$, 0.000535 mg;
$MnSO_4.H_2O$, 0.00008571 mg;
$NiSO_4.6H_2O$, 0.0000514 mg;
Na_2SeO_3, 0.007489 mg;
$Na_2SiO_3.9H_2O$, 0.03671 mg;
$SnCl_2.2H_2O$, 0.0000488 mg;
NH_4VO_3, 0.0002530 mg;
D-Glucose, 3680.52 mg;
sodium pyruvate, 100 mg;
sodium hypoxanthine, 2.069 mg;
glycine, 16.23 mg;
L-alanine, 79.31 mg;
L-arginine.HCl, 674.89 mg;
L-asparagine.H_2O, 182.25 mg;
L-aspartic acid, 67.23 mg;
L-cysteine.HCl.H_2O, 57.63 mg;
L-cystine.2HCl, 106.70 mg;
L-glutamic acid, 6.36 mg;
L-histidine.HCl.H_2O, 250.55 mg;
L-isoleucine, 245.43 mg;
L-leucine, 263.42 mg;
L-lysine-HCl, 276.41 mg;
L-methionine, 85.40 mg;
L-ornithine-HCl, 2.44 mg;
L-phenylalanine, 104.23 mg;
L-proline, 14.94 mg;
L-serine, 146.36 mg;
L-taurine, 3.64 mg;
L-threonine, 199.09 mg;
L-tryptophan, 70.71 mg;
L-tyrosine.2Na.$2H_2O$, 195.58 mg;
L-valine, 174.34 mg;
d-biotin, 0.4359 mg;
D-calcium pantothenate, 1.9394 mg;
choline chloride, 10.8009 mg;
folic acid, 3.4329 mg;
i-inositol, 81.7965 mg;
nicotinamide, 3.1342 mg;
p-aminobenzoic acid, 2.1645 mg;
riboflavin, 0.5359 mg;
thiamine.HCl, 2.3377 mg;
thymidine, 0.316 mg;
vitamin B_{12}, 0.5887 mg;
linoleic acid, 0.0364 mg;
DL-α-lipoic acid, 0.0909 mg;

pyridoxine.HCl, 3.0442 mg;
putrescine.2HCl, 0.0701 mg; and
ethanolamine.HCl, 14.37 mg.

This soluble composition has been named "MET 1.5" and typically is a powder.

In one embodiment the soluble MET and MET 1.5 compositions of the invention comprise a buffering molecule with a pK_a of between 5.9 and 7.8; and a cell protectant. Examples of buffering molecules with a pK_a of between 5.9 and 7.8 include MOPS (pK_a 7.20 at 25° C.; pK_a 7.02 at 37° C.), TES (2-[tris (hydroxymothyl) methyl]amino ethanesulphonic acid; pK_a 7.40 at 25° C.; pK_a 7.16 at 37° C.), and imidazole (pK_a 6.95 at 25° C.). Examples of cell protectants are non-ionic surfactants such as Pluronic-F68, polyvinyl alcohol (PVA), polyethylene glycol (PEG), and dextran sulfate. Those skilled in the art will recognize other buffering molecules with a pK_a of between 5.9 and 7.8 and cell protectants.

In another embodiment of the soluble MET compositions of the invention the buffering molecule consists of MOPS in the amount of 1047-5230 mg per liter of media volume, and the cell protectant consists of Pluronic-F68 in the amount of 250-1500 mg per liter of media volume.

In another embodiment of the soluble MET1.5 compositions of the invention the buffering molecule consists of MOPS in the amount of 2709.66 mg per liter of media volume, and the cell protectant consists of Pluronic-F68 in the amount of 865.80 mg per liter of media volume.

The soluble compositions of the invention may be prepared in a variety of forms. It is preferred that the soluble compositions of the invention are prepared in the form of a powder. The powdered forms of the soluble compositions of the invention are suitable for cell culture for at least 3 years from the date the soluble composition is prepared. The soluble compositions of the invention may also be prepared, for example, in the form of one or more pellets or tablets.

The soluble compositions of the invention can be solubilized in water. Typically, the water used to solubilize the soluble compositions of the invention has a resistivity of 18.2 MΩ·cm at 25° C., a total organic content of less than 20 ppb, a total microorganism content of less than 10 colony forming units per ml, a total heavy metal content of less than 0.01 ppm, a total silicates content of less than 0.01 ppb, and a total dissolved solids content of less than 0.03 ppm. Water with these properties can be prepared using a Super-Q™ Plus Water Purification System (Millipore Corp., Billerica, Mass., USA). The water used to solubilize the soluble compositions of the invention may also be filtered through a filter suitable for the removal of microorganisms. A filter with a 0.22 μM pore size is an example of such a filter. Microorganisms and other adventitious particles may also be removed or inactivated by other means well known in the art.

In one embodiment the invention provides a composition comprising a cell culture media made by the steps comprising selecting a final media volume, providing a soluble MET composition, solubilizing the soluble composition in a volume of water less than the final media volume, adding 1.022 g of L-glutamine per liter of final media volume, adding a bicarbonate ion providing substance sufficient to a produce a bicarbonate ion concentration of between 0.020 M and 0.030 M in the final media volume, optionally adding at least one substance selected from the group consisting of mycophenolic acid, hypoxanthine, xanthine, or soy hydrosylate, adding a quantity of base sufficient to adjust the pH of the solution to between pH 5.9 and pH 7.8, and adding water sufficient to bring the volume of the composition to the selected final media volume. In this embodiment of the invention the media composition that is the product of this process has been named "MET media." Typically MET media is a liquid media.

In another embodiment the invention provides a composition comprising a cell culture media made by the steps comprising selecting a final media volume; providing a soluble MET1.5 composition, solubilizing the soluble composition in a volume of water less than the final media volume, adding 1.022 g of L-glutamine per liter of final media volume, adding a bicarbonate ion providing substance sufficient to a produce a bicarbonate ion concentration of between 0.020 M and 0.030 M in the final media volume, optionally adding at least one substance selected from the group consisting of mycophenolic acid, hypoxanthine, xanthine or soy hydrosylate, adding a quantity of base sufficient to adjust the pH of the solution to between pH 5.9 and pH 7.8, and adding water sufficient to bring the volume of the composition to the selected final media volume. In this embodiment of the invention the media composition that is the product of this process has been named "MET 1.5 media." Typically MET 1.5 media is a liquid media.

In one embodiment of the invention the bicarbonate ion providing substance sufficient to a produce a bicarbonate ion concentration of between 0.020 M and 0.030 M in the final media volume is 2.1 g of $NaHCO_3$ per liter of final media volume. Adding this amount of $NaHCO_3$ per liter of final media volume produces a bicarbonate ion concentration of 0.025 M in the final media volume.

In one embodiment of the invention MET 1.5 media comprises the following components added in the following amounts per liter:
0.5 mg mycophenolic acid;
2.5 mg hypoxanthine; and
50 mg xanthine.

The MET media and MET 1.5 media compositions of the invention are typically provided to cells as a liquid media. The pH of the MET media and MET 1.5 media compositions of the invention is between pH 5.9 and pH 7.8. The pH of a liquid is a function of the temperature of the liquid. It is preferred that the pH of each media composition be between 7.1 and 7.25 at the temperature at which eukaryotic cell culture is being performed. Eukaryotic cell culture may be performed at temperatures higher or lower than 37° C., but is typically performed at 37° C.

In some applications liquid MET media and liquid MET 1.5 media may be used in the preparation of semi-solid cell culture media. For example, methylcellulose may be used to generate a semi-solid media incorporating the liquid MET media and liquid MET 1.5 media compositions of the invention. Such semi-solid media may be prepared by methods well known to those skilled in the art. Eukaryotic cells may be suspended in such semi-solid media and cultured by methods well known to those skilled in the art.

Other substances that can enhance cell growth or productivity may also be added to the soluble MET, MET media, soluble MET 1.5 and MET 1.5 media compositions of the invention. These substances may be lipids, nucleosides, peptide chains, corticosteroids, steroids, and the like. Such substance may be, for example:
adenosine preferably 0-20 μM;
guanosine preferably 0-20 μM;
cytidine preferably 0-20 μM;
uridine preferably 0-20 μM;
deoxyadenosine preferably 0-20 μM;
deoxyguanosine preferably 0-20 μM;
deoxycytidine preferably 0-20 μM;
thymidine preferably 0-20 μM;

dexamethasone preferably 10-150 nM;
hydrocortisone preferably 0-150 μM;
L-glycine-L-Lysine-L-glycine (GKG) peptide chain preferably 0-200 μm;
N-acetyl cysteine preferably 0-500 mg/L;
betaine preferably 0-500 mg/L;
L-malic acid preferably 0-500 mg/L;
oxaloacetic acid preferably 0-500 mg/L;
glycyrrhizic acid preferably 0-500 mg/L;
glycyrrhizic acid ammonium salt preferably 0-500 mg/L;
α-ketoglutarate preferably 0-500 mg/L;
L-leucine preferably 245-490 mg/L;
L-isoleucine preferably 220-440 mg/L;
L-lysine-HCl preferably 187-360 mg/L;
L-valine preferably 155-310 mg/L;
L-methionine preferably 57-114 mg/L;
L-phenylalanine preferably 76-152 mg/L;
L-serine preferably 37-74 mg/L;
L-threonine preferably 107-214 mg/L;
L-arginine.HCl preferably 200-300 mg/L;
L-asparagine preferably 114-170 mg/L;
L-aspartic acid (10-25 mg/L);
L-cysteine.HCl.H_2O preferably 46-75 mg/L;
Histidine.HCl.H_2O preferably 75-150 mg/L;
L-tyrosine preferably 40-80 mg/L;
L-tryptophan preferably 41-82 mg/L;
nicotinamide preferably 0.9-1.8 mg/L; and
ethanolamine HCl preferably 14-20 mg/L.

The quantities of each substance added to the compositions of the invention are those necessary to achieve the preferred molar concentration or mass per unit media volume prepared shown above.

The present invention is further described with reference to the following examples. These examples are merely to illustrate various aspects of the present invention and are not intended as limitations of this invention.

EXAMPLE 1

Eukaryotic Cell Viability in MET 1.5 Cell Culture Media

Chemically defined MET 1.5 cell culture media can sustain high cell growth and viability (FIG. **1**). To examine viable cell numbers, MET 1.5 media was supplied to 3 L perfusion bioreactors. Bioreactors were then inoculated with C743B cells such that the initial cell density was 3×10^6 cells/ml of MET 1.5 media. The C743B cell line produces a fully human, anti-IL-12 mAb and is a chemically adapted cell line derived from SP2/0 myeloma cells. C743B cells were grown for 29 days in the bioreactor and viable cell densities were monitored. Cell culture media was neutralized with a 0.2 M Na_2CO_3 (aq) solution for the first 9 days of culture and with 0.2 M Na_2CO_3, 0.0054 M K_2CO_3 (aq). Excessive cell density in the bioreactor was prevented by the removed of biomass from the bioreactor; cell removal began on day 15 and was gradually increased until day 26. The bioreactor was perfused with one volume of MET 1.5 media per day. Viable cell numbers were determined via a standard trypan blue dye exclusion assay using a CEDEX cell counter (Innovatis AG, Bielefeld, Del.). Total cell numbers for calculation of the percentage of viable cells were determined with the CEDEX instrument. For each determination the CEDEX instrument was used according to the manufacturer's instructions. O_2 and CO_2 were supplied to the bioreactor as a gas stream sparged into the bioreactor vessel. Data presented in Example 1, 2, and 3 are all from the same bioreactor run.

EXAMPLE 2

Antibody Titer and Specific Productivity in MET 1.5 Cell Culture Media

Chemically defined MET 1.5 cell culture media can sustain high monoclonal antibody titers and specific productivity (FIG. **2**). Cell culture and bioreactor operation was as described above. Fully human, anti-IL-12 mAb titers (mg/L) were determined by standard nephelometry techniques using a Beckman Array Analyzer. A purified fully human, anti-IL-12 mAb of known concentration was used to generate a standard curve for the determination of mAb titers by nephelometry. Viable cell numbers for calculation of specific productivity were determined as described above. Data presented in Example 1, 2, and 3 are all from the same bioreactor run.

EXAMPLE 3

Decreased Lactate Production in MET 1.5 Cell Culture Media

Lactate concentrations in MET 1.5 media decrease (FIG. **3**) as viable cell density increases (FIG. **1**). Cell culture and bioreactor operation was as described above. Lactate concentrations and glucose concentrations in the bioreactor culture media were determined using standard assays. Data presented in Example 1, 2, and 3 are all from the same bioreactor run.

As FIG. **3** indicates, lactate concentrations in MET 1.5 media gradually decreased until day 16 when biomass removal to decrease total cell density in the bioreactor began. During the same period glucose concentrations remained comparatively constant (FIG. **3**). Comparison of FIG. **3** to FIG. **1** reveals that viable C743B cell numbers in the same bioreactor were increasing until day 16. Together this data indicates a decrease in lactate production by C743B cells cultured in MET 1.5 media and more efficient metabolism of D-glucose by cells cultured in MET 1.5 media.

The present invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

The invention claimed is:

1. A soluble composition, suitable for producing a final volume of cell culture media, wherein the composition comprises the following components in the following amounts per liter of the final volume of cell culture media:
anhydrous $CaCl_2$, 5-200 mg;
anhydrous $MgCl_2$, 15-50 mg;
anhydrous $MgSO_4$, 20-80 mg;
$FeSO_4.7H_2O$, 0.05-0.50 mg;
$Fe(NO_3)_3.9H_2O$, 0.01-0.08 mg;
$ZnSO_4.7H_2O$, 0.40-1.20 mg;
ferric ammonium citrate, 0.04-200 mg;
KCl, 280-500 mg;
NaCl, 5000-7500 mg;
$NaH_2PO_4.H_2O$, 30-100 mg;
Na_2HPO_4, 30-100 mg;
$CuSO_4.5H_2O$, 0.001-0.005 mg;
$CoCl_2.6H_2O$, 0.001-0.10 mg;
$(NH_4)_6Mo_7O_{24}$ $4H_2O$, 0.001-0.005 mg;
$MnSO_4.H_2O$, 0.000070-0.0080 mg;

$NiSO_4.6H_2O$, 0.000025-0.0005 mg;
Na_2SeO_3, 0.004-0.07 mg;
$Na_2SiO_3.9H_2O$, 0.02-0.4 mg;
$SnCl_2.2H_2O$, 0.000025-0.0005 mg;
NH_4VO_3, 0.0001-0.0025 mg;
D-Glucose, 500-8000 mg;
sodium pyruvate, 0.0-1000 mg;
sodium hypoxanthine, 0.0-20.0 mg;
glycine, 0.0-150 mg;
L-alanine, 0.0-150 mg;
L-arginine.HCl, 200-5000 mg;
L-asparagine.H_2O, 40-250 mg;
L-aspartic acid, 20-1000 mg;
L-cysteine.HCl H_2O, 25.0-250 mg;
L-cystine.2HCl, 15-150 mg;
L-glutamic acid, 0-1000 mg;
L-histidine.HCl.H_2O, 100-500 mg;
L-isoleucine, 50-1000 mg;
L-leucine, 50-1000 mg;
L-lysine.HCl, 100-1000 mg;
L-methionine, 50-500 mg;
L-ornithine.HCl, 0-100 mg;
L-phenylalanine, 25-1000 mg;
L-proline, 0-1000 mg;
L-serine, 50-500 mg;
L-taurine, 0-1000 mg;
L-threonine, 50-600 mg;
L-tryptophan, 2-500 mg;
L-tyrosine.2Na.2H_2O, 25-250 mg;
L-valine, 100-1000 mg;
d-biotin, 0.04-1.0 mg;
D-calcium pantothenate, 0.1-5.0 mg;
choline chloride, 1-100 mg;
folic acid, 1-10 mg;
i-Inositol, 10-1000 mg;
nicotinamide, 0.5-30 mg;
p-aminobenzoic acid, 0.1-20 mg;
riboflavin, 0.05-5.0 mg;
thiamine.HCl, 0.5-20 mg;
thymidine, 0-3.0 mg;
vitamin B_{12}, 0.05-5.0 mg;
linoleic acid, 0.01-2.0 mg;
DL-α-lipoic acid, 0.03-1.0 mg;
pyridoxine.HCl, 0.5-30 mg;
putrescine.2HCl, 0.025-0.25 mg; and
ethanolamine.HCl, 2-100 mg.

2. The soluble composition of claim **1** further comprising a buffering molecule with a pK_a between 5.9 and 7.8 and a cell protectant.

3. The soluble composition of claim **2** wherein the buffering molecule consists of MOPS in the amount of 1047-5230 mg per liter of final media volume and the cell protectant consists of Pluronic-F68 in the amount of 250-1500 mg per liter of final media volume.

4. A composition comprising a cell culture media made by the steps comprising:

a) selecting a final media volume;
b) providing the soluble composition of claim **2** or claim **3**;
c) solubilizing the soluble composition in a volume of water less than the final media volume;
d) adding 1.022 g of L-glutamine per liter of final media volume;
e) adding a bicarbonate ion providing substance sufficient to produce a bicarbonate ion concentration of between 0.020 M and 0.030 M in the final media volume;

f) optionally adding at least one substance selected from the group consisting of mycophenolic acid, hypoxanthine, xanthine, and soy hydrolysate;
g) adding a quantity of base sufficient to adjust the pH of the solution to between pH 5.9 and pH 7.8; and
h) adding water sufficient to bring the volume of the composition to the selected final media volume.

5. The composition of claim **4** where the bicarbonate ion providing substance sufficient to a produce a bicarbonate ion concentration of between 0.020 M and 0.030 M in the final media volume is 2.1 g of $NaHCO_3$ per liter of final media volume.

6. A soluble composition, suitable for producing a final volume of cell culture media, wherein the composition comprises the following components in the following amounts per liter of the final volume of cell culture media:

$CaCl_2$, 100.95 mg;
$MgCl_2$, 24.77 mg;
$MgSO_4$, 42.24 mg;
$FeSO_4.7H_2O$, 0.3607 mg;
$Fe(NO_3)_3.9H_2O$, 0.0432 mg;
$ZnSO_4.7H_2O$, 0.6225 mg;
ferric ammonium citrate, 43.25 mg;
KCl, 386.9 mg;
NaCl, 5866.0 mg;
$NaH_2PO_4.H_2O$, 54.07 mg;
Na_2HPO_4, 61.44 mg;
$CuSO_4.5H_2O$, 0.003287 mg;
$CoCl_2.6H_2O$, 0.0020606 mg;
$(NH_4)_6Mo_7O_{24}.4H_2O$, 0.000535 mg;
$MnSO_4.H_2O$, 0.00008571 mg;
$NiSO_4.6H_2O$, 0.0000514 mg;
Na_2SeO_3, 0.007489 mg;
$Na_2SiO_3.9H_2O$, 0.03671 mg;
$SnCl_2.2H_2O$, 0.0000488 mg;
NH_4VO_3, 0.0002530 mg;
D-Glucose, 3680.52 mg;
sodium pyruvate, 100 mg;
sodium hypoxanthine, 2.069 mg;
glycine, 16.23 mg;
L-alanine, 79.31 mg;
L-arginine.HCl, 674.89 mg;
L-asparagine.H_2O, 182.25 mg;
L-aspartic acid, 67.23 mg;
L-cysteine.HCl.H_2O, 57.63 mg;
L-cystine.2HCl, 106.70 mg;
L-glutamic acid, 6.36 mg;
L-histidine.HCl.H_2O, 250.55 mg;
L-isoleucine, 245.43 mg;
L-leucine, 263.42 mg;
L-lysine.HCl, 276.41 mg;
L-methionine, 85.40 mg;
L-ornithine.HCl, 2.44 mg;
L-phenylalanine, 104.23 mg;
L-proline, 14.94 mg;
L-serine, 146.36 mg;
L-taurine, 3.64 mg;
L-threonine, 199.09 mg;
L-tryptophan, 70.71 mg;
L-tyrosine.2Na.2H_2O, 195.58 mg;
L-valine, 174.34 mg;
d-biotin, 0.4359 mg;
D-calcium pantothenate, 1.9394 mg;
choline chloride, 10.8009 mg;
folic acid, 3.4329 mg;
i-inositol, 81.7965 mg;
nicotinamide, 3.1342 mg;

p-aminobenzoic acid, 2.1645 mg;
riboflavin, 0.5359 mg;
thiamine.HCl, 2.3377 mg;
thymidine, 0.316 mg;
vitamin B_{12}, 0.5887 mg;
linoleic acid, 0.0364 mg;
DL-α-lipoic acid, 0.0909 mg;
pyridoxine.HCl, 3.0442 mg;
putrescine.2HCl, 0.0701 mg; and
ethanolamine.HCl, 14.37 mg.

7. The soluble composition of claim **6** further comprising a buffering molecule with a pK_a of between 5.9 and 7.8 and a cell protectant.

8. The soluble composition of claim **7** wherein the buffering molecule consists of MOPS in the amount of 2709.66 mg per liter of final media volume, and the cell protectant consists of Pluronic-F68 in the amount of 865.80 mg per liter of final media volume.

9. The soluble composition of claim **7** further comprising the following components in the following amounts per liter of final media volume:

0.5 mg mycophenolic acid;
2.5 mg hypoxanthine; and
50 mg xanthine.

10. A composition comprising a cell culture media made by the steps comprising:

a) selecting a final media volume;
b) providing the soluble composition of claim **7** or claim **8**;
c) solubilizing the soluble composition in a volume of water less than the final media volume;
d) adding 1.022 g of L-glutamine per liter of final media volume;
e) adding a bicarbonate ion providing substance sufficient to produce a bicarbonate ion concentration of between 0.020 M and 0.030 M in the final media volume;
f) optionally adding at least one substance selected from the group consisting of mycophenolic acid, hypoxanthine, xanthine and soy hydrolysate;
g) adding a quantity of base sufficient to adjust the pH of the solution to between pH 5.9 and pH 7.8; and
h) adding water sufficient to bring the volume of the composition to the selected final media volume.

11. The composition of claim **10** where the bicarbonate ion providing substance sufficient to a produce a bicarbonate ion concentration of between 0.020 M and 0.030 M in the final media volume is 2.1 g of $NaHCO_3$ per liter of final media volume.

* * * * *

EXHIBIT E

US006900056B2

(12) **United States Patent**
Lee et al.

(10) **Patent No.: US 6,900,056 B2**
(45) **Date of Patent: May 31, 2005**

(54) **CHEMICALLY DEFINED MEDIUM FOR CULTURED MAMMALIAN CELLS**

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(73) Assignee: **Centocor, Inc.**, Malvern, PA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 242 days.

(21) Appl. No.: **10/067,382**

(22) Filed: **Feb. 5, 2002**

(65) **Prior Publication Data**

US 2003/0096402 A1 May 22, 2003

Related U.S. Application Data

(60) Provisional application No. 60/268,849, filed on Feb. 15, 2001.

(51) **Int. Cl.**[7] **C12N 5/00**
(52) **U.S. Cl.** **435/404**; 435/405; 435/406
(58) **Field of Search** 435/404, 405, 435/406

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Primary Examiner—Leon B. Lankford, Jr.

(57) **ABSTRACT**

The present invention relates to methods and compositions for chemically defined media for growth of mammalian cells for production of commercially useful amounts of expressed proteins.

3 Claims, 2 Drawing Sheets

FIG. 1

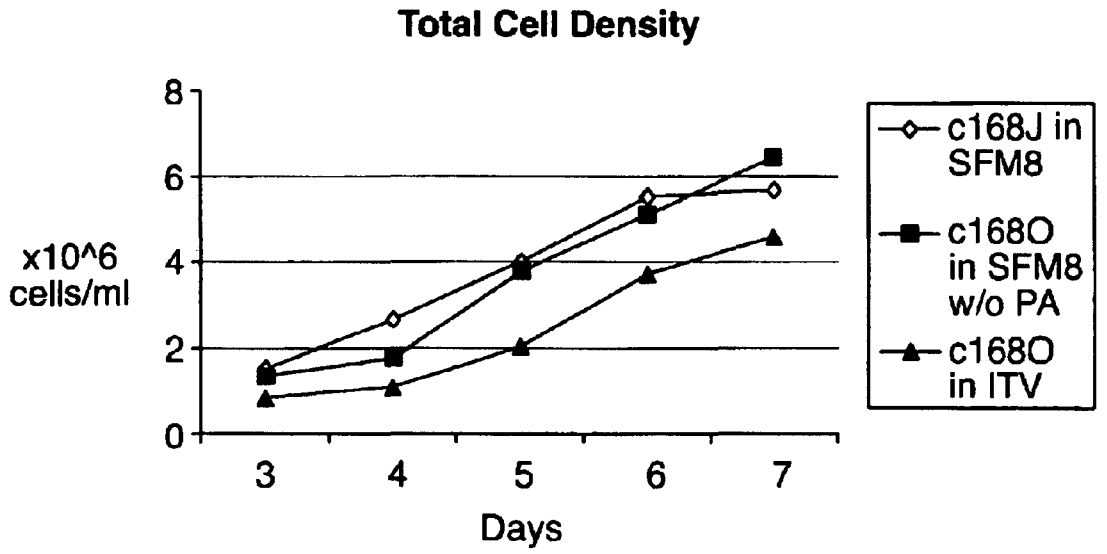

Total Cell Density
x10^6 cells/ml
8
6
4
2
0
3
4
5
6
7
Days
c168J in SFM8
c168O in SFM8 w/o PA
c168O in ITV

FIG. 2

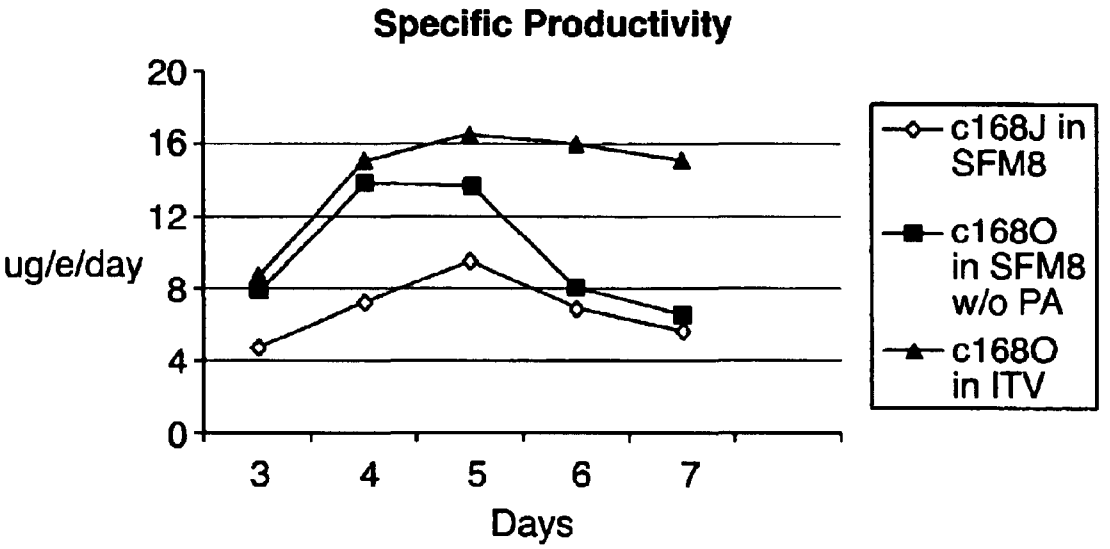

Specific Productivity
ug/e/day
20
16
12
8
4
0
3
4
5
6
7
Days
c168J in SFM8
c168O in SFM8 w/o PA
c168O in ITV

FIG. 3

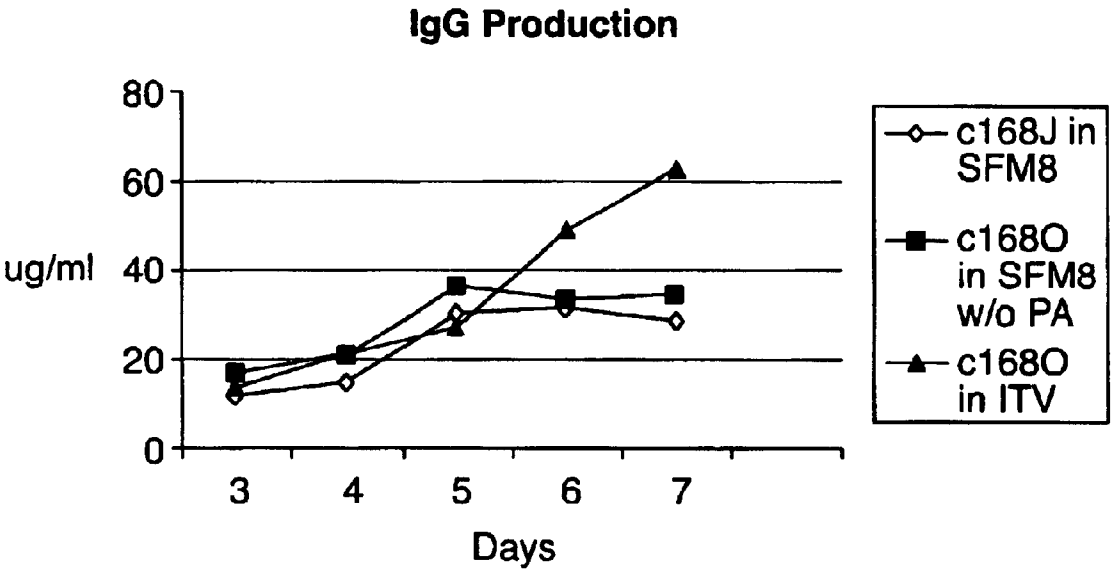
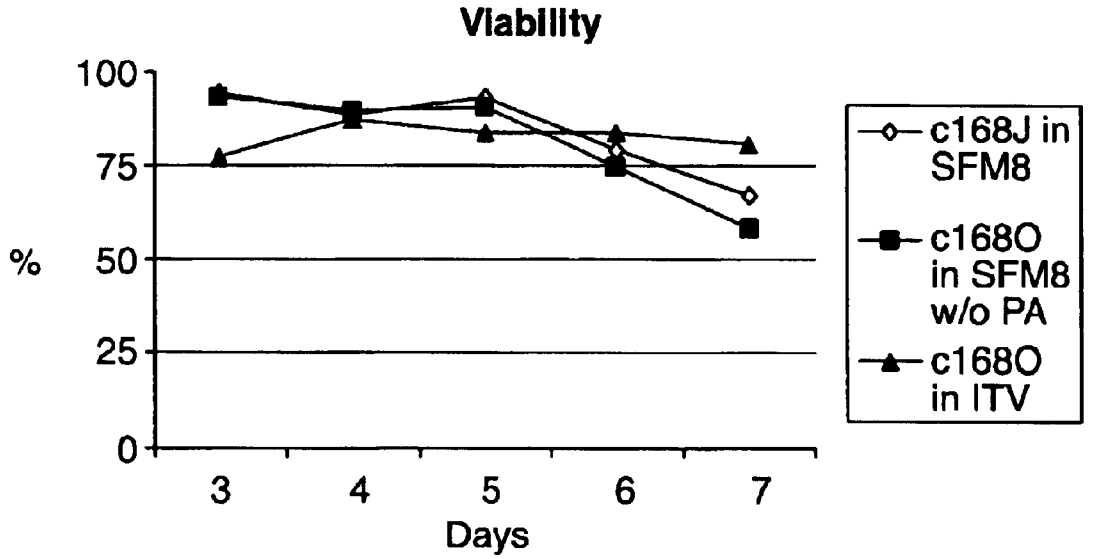

IgG Production
80
60
40
20
0
ug/ml
3
4
5
6
7
Days
c168J in SFM8
c168O in SFM8 w/o PA
c168O in ITV

FIG. 4


Viability
100
75
50
25
0
%
3
4
5
6
7
Days
c168J in SFM8
c168O in SFM8 w/o PA
c168O in ITV

CHEMICALLY DEFINED MEDIUM FOR CULTURED MAMMALIAN CELLS

FIELD OF THE INVENTION

This application is based in part on, and claims priority to, U.S. Provisional No. 60/268,849 filed Feb. 15, 2001, of which is entirely incorporated herein by reference.

The present invention in the field of biotechnology, relates to methods and compositions for providing chemically defined media for growth of cultured mammalian cells for production of commercially useful amounts of expressed proteins.

BACKGROUND OF THE INVENTION

Bovine serum is commonly used in mammalian cell culture to promote cell growth and protein production. Since serum is expensive, non-defined animal materials such as primatone and albumin have been used as serum replacements. However, the quality of these non-defined animal proteins varies from batch to batch and consistent cell growth in these media is difficult to achieve. Moreover, pathogens such as prions and viruses have been identified as potential infectious agents (Balter, M. 2000, Kozak et al. 1996) that may reside in those animal derived products. Many regulations now strongly address these concerns about using serum or non-defined animal proteins in mammalian cells.

To support the growth of animal cells, a variety of components are essential to be included in the culture media. For example, glutamine and glucose are basic energy sources that support animal cell growth. Breakdown of these compounds provides resources for energy-generating pathways, the TCA cycle and glycolysis. The byproducts of these pathways are also the building blocks or sources for bio polymer synthesis (Petch and Bulter 1994). In addition, vitamins, amino acids and growth factors are also essential for robust cell growth by either supressing the cascade of the suicide pathway known as apoptosis or by promoting the progression of the cell cycle so that cells may replicate (Franek F. 1994, Murakami et al. 1982, Mastrangelo et al. 1999, Xie and Wang, 1996, Muhamed Al-Rubeai 1998).

Trace elements are also important for the growth of animal cells. Ham and McKeehan (1979) noticed that adding trace elements, such as Zinc, iron, selenium, copper, molybdenum, and manganese, etc., was important for cloning and continuous passage of animal cells in stringent conditions of serum-free media. Regardless, the importance of supplementing trace elements in the media for animal cells has not been well addressed (Schneider 1989, Merten and Litwin 1991). This may be due to the assumption that trace elements existed as contaminated impurities within serum or non-defined animal derived materials already.

Accordingly, there is also a need to provide chemically defined media for cell culture and/or production of heterologous proteins in commercially useful amounts.

SUMMARY OF INVENTION

The present invention provides chemically defined media (CDF) formulations and methods that provide certain compounds, amino acids, lipids, carbohydrates, trace elements and/or vitamins that provide a chemically defined media that excludes the use of non-defined animal derived raw materials (e.g., but not limited to, primatone, albumin and Excyte™, as well as other similar materials derived from serum or other animal derived proteins or products).

Such media compositions and formulations of the present invention allow the growth of myeloma and other cell cultures to provide commercially useful amounts of the desired proteins expressed in such cell cultures. Accordingly the present invention provides specific media, formulations and methods of making and using thereof, as well as proteins provided therefrom. The present invention provides media that provide one or more advantages of being chemically defined, better protein producing, commercially suitable, cost-effective, and/or pose reduced regulatory concerns for proteins produced in cell lines grown therein.

DESCRIPTION OF THE DRAWINGS

FIG. **1** shows by graphical representation that CDM media of the present invention can support high cell density up to 4.5×10^6 cells/mL on Day 7.

FIG. **2** shows by graphical representation that specific productivity for CDM culture is at 16 $\mu g/10^6$ cells/day.

FIG. **3** shows that at high cell density between $4–5\times10^6$ cells/mL, IgG production reached above 60 μg/mL.

FIG. **4** shows by graphical representation that Viability of the CDM spinner culture remained above 75% throughout the experiment.

Also included in FIGS. **1–4** are data of C168O in SFM8 without primatone, albumin and C168J in SFM8 as references for comparison.

DETAILED DESCRIPTION

The present invention provides media formulations and methods that provide a chemically defined media that provides advantages over known media, and which can be used for commercial production of mammalian cell-cultured proteins. The present invention also provides a chemically defined media (CDM) comprising novel components, as well as, or optionally further comprising, at least one of specified amino acids, lipids, carbohydrates, trace elements, vitamins, compounds and/or proteins, as described and enabled herein, in combination with what is known in the art.

The present invention avoids of one or more problems associated with media that contains animal derived, or non-defined animal derived, components (e.g., but not limited to, primatone, albumin and excyte, as well as other similar materials derived from serum or other animal proteins in recombinant, synthetic or purified form).

Accordingly, chemically defined media (CDM) compositions and formulations of the present invention allow the growth of myeloma and other cell cultures to provide commercially useful amounts of the desired proteins expressed in such cell cultures. The present invention thus provides specific media formulations that are chemically defined, cost-effective, and pose reduced regulatory concerns compared to known media that comprise animal-derived materials that are not completely defined, or known chemically defined media.

Media of the present invention includes the substitution of specified components, without the use of animal derived proteins. In a preferred embodiment, media of the present invention comprises specified components, e.g., but not limited to, trace elements and vitamins, the media termed "chemically defined media" (CDM). Media of the present invention provides utility and improvements, including, but not limited to, at least one of suitable high cell density growth, improved growth rate, improved growth in scale up, improved viability, improved viability in scale up, improved protein yield, improved protein yield in scale up, and the like.

Suitable cell lines that can be used according to the present invention include any transformed or immortalized mammalian cell line. Such cell lines can include myeloma cell lines, such as Sp2/0, NSO, NSl, CHO, BHK, Ag653, P3X63Ag8.653 cells (ATCC Accession Number CRL-1580) and SP2/0-Ag14 cells (ATCC Accession Number CRL-1851), COS-1 (e.g., ATCC CRL 1650), COS-7 (e.g., ATCC CRL-1651), HEK293, BHK21 (e.g., ATCC CRL-10), CHO (e.g., ATCC CRL 1610, CHO DXB-11, CHO DG44), BSC-1 (e.g., ATCC CRL-26) cell lines, HepG2 cells, P3X63Ag8.653, 293 cells, HeLa cells, NIH 3T3, COS-1, COS-7, NIH 273, and the like, or any cells derived therefrom, including cell fusions of the above, such as to protein producing cells, such as B-cells, antibody producing cells, isolated or cloned spleen or lymph node cells, and the like. A preferred cell line is derived from Sp2/0 and is designated C463A, as described herein.

Cell lines, such as those presented herein, can be adapted to a chemically defined media according to the present invention, using known techniques and/or as described herein. Such methods can take from 1–30 days, or up to several months, depending on the particular cell line and media formulation used. However, adaption of mammalian cells to grow in chemically defined media of the present invention is unexpectedly found to occur in significantly shorter times that known defined or undefined media.

At least one formulation of media of the present invention is unexpectedly discovered to provide at least one of several advantages over known media, including at least one of: (1) supporting improved or robust growth and protein or antibody production from various mammalian cell lines; (2) facilitated adaptation for protein producing cell lines; (3) cost-effective media components, as compared to known components, such as bovine serum and excyte, which do not need to be and are not included; and/or (4) better suitability for regulatory approval as the media components are at least one of better defined, do not include animal derived proteins or other products, and do not contain or potentially contain infectious agents.

The use of this medium in cell culture technologies, such as but not limited to culture dishes, culture plates, culture bottles, suspension culture, spin filter suspension culture, bioreactors, perfusion type bioreactors, mammalian cell fermentation culture, or any other suitable type of cell culture, is also included in the present invention.

A media formulation of the present invention includes at least one of specified buffers, salts, carbohydrates, vitamins, proteins, amino acids, lipids, trace elements, minerals, and the like as described herein in combination with what is known in the art.

The media preferably comprises, in addition to known mammalian or hybridoma cell culture components without undefined protein- or animal-derived components, at least one or more of ammonium metavanadate, cadmium chloride, chromic potassium sulfate, ferric citrate, germanium dioxide, molybdic acid, salt or ammonium salt, nickel sulfate, zirconium chloride and/or hydrocortisone, or any suitable form, salt, halide, hydrate, solution, suspension, emulsion, or colloid thereof, powder and the like. In preferred embodiments, the media comprises, in addition to known components, at least one, two, three, four, five, six, seven, eight, or nine of the above components.

Non-limiting examples of such buffers and include at least one of MOPS, sodium phosphate, potassium phosphate, HEPES, and other known buffers. Salts included in such buffers include, but are not limited to sodium chloride, potassium chloride, and the like. Non-limiting examples are presented as one or more of the salts, as hydrous, anhydrous or other salt form, in the following table:

Component	g/L
INORGANIC SALTS	
AlCl3.6H2O	0.0000001–0.00001
$NH4Vo_3$	0.00000006–0.000001
$BaCl_2$	0.0000002–0.000001
CaCl2.2H2O	0.004–0.09
CoCl2.6H2O	0.0000002–0.00001
$CrK(So4)_2$	0.0000001–0.00001
CuSo4.5H2O	0.0000005–0.00001
FeSo4.7H2O	0.000001–0.0001
Geo_2	0.00000005–0.000001
LiCl	0.001–0.1
MgCl.6H2O	0.01–1.0
MnCl(anhyd)	0.00000001–0.000001
Na2Moo4.2H2O	0.00000001–0.000001
NiNo3.6H2O	0.00000002–0.000001
KBr	0.00000001–0.000001
KCl	0.01–1.0
KI	0.00000001–0.000001
RbCl	0.000000001–0.0000001
AgCl	0.0000000004–0.0000001
$NaHCo_3$	0.0000001–0.00001
NaCl	O.1–50
NaF	0.0000004–0.00001
$Na2HPo_4$(anhyd)	0.01–5
$Na2Seo_3$	0.000003–0.0001
SnCl2.2H2O	0.00000001–0.000001
Tio_2	0.0000001–0.0001
ZnSo4.7H2O	0.000008–0.0001

Such carbohydrates include, but are not limited to, glucose (dextrose), fructose, mannose, galactose, and any other suitable monosaccharide, disaccharide, polysaccharide, polymer, carbohydrate and the like. Non-limiting examples of amounts include 0.0000001–100 g/L for one or more carbohydrate components.

Such vitamins and co-factors can include, but are not limited to, biotin, ascorbic acid, pantothenate, choline, folate, inositol, niacin, niacinamide, pyridoxal, riboflavin, thamine, cyanocbalamin, L-abscorbic acid and salts, D-biotin, calciferol, choline, cocarboxylase, coenzyme A, 2-deoxyadenosine, 2-deoxyguanosine, 2-deoxycytidine, ergocalciferol, flavin adenosine dinucleotide, FAD, folic acid, D-glucoronic acid, lactone, D-glucoronic acid, glutathione, myo-inositol, mammalian recombinant insulin, menadione, 5'methylcytosine, niacinamide, NADP, NAD, nicotinic acid, oxalacetic acid, p-amino benzoic acid, D-pantothenic acid, pyroxidal, pyroxidine, retinol acetate, riboflavin, α-tocopherol, thiamine, thymidine, UMP, UDP, UTP, AMP, ADP., ATP, GMP, GDP, GTP, CMP, CDP, CTP, TMP, TDP, TTP, vitamin B12, and the like, in any suitable form, such as salt, acid, base, and the like.

Such proteins or amino acids include, but are not limited to, alanine, arginine, asparagine, aspartate, cysteine, cystine, glutamine, glutamate, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, and salts or other derivatives thereof. Alternatively, such amino acids include at least one of L-α-amino-n-butyric acid, L-alanine, L-arginine, L-aspartic acid, L-asparagine, L-cysteine, L-citrulline,

L-cysteine, D-glucosamine, L-glutamic acid, L-glutamine, L-glycine, L-histidine, hydroxy-L-Proline, L-isoleucine, L-leucine, L-lysine, L-methionine, L-ornithine, L-omithine, L-phenylalanine, L-proline, L-serine, taurine, L-threonine, L-tryptophan, L-tyrosine, L-valine, and the like, as well as salts, hydrates, hydrides, acids, bases thereof and the like.

Such trace elements and minerals include, but are not limited to, salts (e.g., chlorides, iodides, bromides, fluorides, sodium or potassium salts, phosphates, salts, and the like), acids (e.g., acetates, sulfates, sulfides, nitrates, nitrides, dioxides, and the like), bases (e.g., NaOH, KOH, and the like), of magnesium, potassium, sodium, calcium, and the like, such as sodium acetate, sodium chloride, sodium phosphate, selenium, aluminum, ammonium metavanadate, barium, cadmium, cobalt chloride, chromic potassium sulfate, cupric sulfate, ferric citrate, germanium dioxide, lithium chloride, magnesium chloride, manganese chloride, molybdic acid, nickel nitrate, potassium bromide, potassium iodide, rubidium chloride, silver chloride, sodium fluoride, stannous chloride, sodium silicate, tin chloride tin chloride, titanium chloride, zinc sulfate, zirconium oxychloride, and the like, and salts thereof.

As a further non-limiting example, a formulation of CDM media of the present invention comprises: sodium chloride, 3–5 g/L; potassium chloride, 0.2–0.4 g/L; , HEPES, 5–7 g/L; glucose (dextrose), 3.5–5.5 g/L; biotin, 0.000005–0.000025 g/L; ascorbic acid, 0.002–0.004; pantothenate, 0.002–0.006 g/L; choline, 0.002–0.006 g/L; folate, 0.002–0.006 g/L; inositol, 0.005–0.02 g/L; niacinamide, 0.002–0.006 g/L; pyridoxal, 0.002–0.006 g/L; riboflavin, 0.0002–0.0006 g/L; thiamine, 0.002–0.006 g/L; cyanocbalamin, 0.000005–0.000025 g/L; oxaloacetic acid, 0.1–0.4 g/L; alanine, 0.015–0.035 g/L; asparagine, 0.01–0.035 g/L; arginie, 0.06–0.10 g/L; aspartate, 0.02–0.04 g/L; cysteine, 0.3–0.5 g/L; cystine, 0.05–0.2 g/L; glutamine, 0.8–1.5 g/L; glutamate, 0.06–0.09 g/L; glycine, 0.02–0.04 g/L; histidine, 0.03–0.05 g/L; isoleucine, 0.05–0.25 g/L; leucine, 0.05–0.25 g/L: lysine, 0.05–0.25 g/L; methionine, 0.02–0.04 g/L; phenylalanine, 0.055–0.075. proline, 0.03–0.05 g/L; serine, 0.03–0.055 g/L; threonine, 0.07–0.15 g/L; tryptophan, 0.005–0.025 g/L; tyrosine, 0.05–0.15 g/L; valine, sodium selenate, 0.0000005–0.000060; magnesium sulfate, 0.05–0.2 g/L; potassium chloride, 0.15–0.45 g/L; sodium phosphate, 0.075–0.2 g/L; potassium nitrate, 0.00005–0.00009 g/L; calcium chloride, 0.08–0.25 g/L; sodium pyruvate 0.05–0.4 g/L; insulin, 0.05–2 g/L; hydrocortisone, 20–80 μg/L; linoleic acid, 1–100 mg/L; ethanolamine, 5–25 μg/L; sodium bicarbonate, 1–5 g/L; APO transferrin or ferric citrate, 1–10 mg/L; Pluronic F68, 0.2–2 g/L; sodium hydroxide, 0.3–0.9 g/L; mycophenolic acid, 0.1–2 mg/L; hypoxanthine, 2–5 mg/L; xanthine; 10–200 mg/L; sodium bicarbonate 1.5–4.5 g/L.

Known serum free hybridoma media that can be modified to provide the media of the present invention include, but are not limited to, e.g., Sigma/Aldrich product numbers S2772, S2897 and S8284 (www.sigma-aldrich.com); similar known serum free media include those from Life Technologies, Rockville, Md. (www.lifetech.com) and JRH Biosciences, Lenexa, Kans. (www jrhbio.com). For example, known serum free hybridoma cell cultures can include HEPES or MOPS, sodium bicarbonate, L-glutamine, cholesterol, insulin, BSA, transferrin or ferric citrate, in addition to other serum free mammalian cell culture components. See, e.g., SIGMA catalog, 1998, pp 1776–1777, 1677–1704, 1715–1755, 1795–1847, entirely incorporated herein by reference. Non-limiting examples of known serum free media that can be modified to provide CDM of the present invention include, but are not limited to, sigma media product numbers S2772, S2897 and S8284, as follows:

SIGMA Prod. # Component	S 2897 g/L	S 8284 g/L	S 2772 g/L
INORGANIC SALTS			
AlCl3.6H2O	0.000001	0.000001	0.000001
$NH4Vo_3$	0.0000006	0.0000006	0.0000006
$BaCl_2$	0.000002	0.000002	0.000002
CaCl2.2H2O	0.0441	0.0441	0.0441
CoCl2.6H2O	0.000002	0.000002	0.000002
$CrK(So4)_2$	0.000001	0.000001	0.000001
CuSo4.5H2O	0.0000051	0.0000051	0.0000051
FeSo4.7H2O	0.000834	0.000834	0.000834
Geo_2	0.0000005	0.0000005	0.0000005
LiCl	0.01	0.01	0.01
MgCl.6H2O	0.123	0.123	0.123
MnCl(anhyd)	0.0000001	0.0000001	0.0000001
Na2Moo4.2H2O	0.0000001	0.0000001	0.0000001
NiNo3.6H2O	0.0000002	0.0000002	0.0000002
KBr	0.0000001	0.0000001	0.0000001
KCl	0.224	0.224	0.224
KI	0.0000001	0.0000001	0.0000001
RbCl	0.00000001	0.00000001	0.00000001
AgCl	0.0000000044	0.0000000044	0.0000000044
$NaHCo_3$	—	2.25	2.25
NaCl	7.599	7.599	7.599
NaF	0.000004	0.000004	0.000004
$Na2HPo_4$(anhyd)	0.39739	0.39739	0.39739
$Na2Seo_3$	0.00003	0.00003	0.00003
SnCl2.2H2O	0.0000001	0.0000001	0.0000001
Tio_2	0.000001	0.000001	0.000001
ZnSo4.7H2O	0.000863	0.000863	0.000863
AMINO ACIDS			
L-Alanine	0.009	0.009	0.009
L-Arginine	0.211	0.211	0.211
L-Asparagine.H2O	0.03401	0.03401	0.03401
L-Aspartic Acid	0.0133	0.0133	0.0133
L-Citrulline	0.005	0.005	0.005
L-Cysteine.HCl.H2O	0.035	0.035	0.035
L-Glutamic Acid	0.0147	0.0147	0.0147
L-Glutamine	0.396	0.396	0.396
Glycine	0.00751	0.00751	0.00751
L-Histidine.HCl.H2O	0.071	0.071	0.071
L-Isoleucine	0.164	0.164	0.164
L-Leucine	0.133	0.133	0.133
L-Lysine.HCl	0.109	0.109	0.109
L-Methionine	0.015	0.015	0.015
L-Ornithine	0.008	0.008	0.008
L-Phenylalanine	0.055	0.055	0.055

See, e.g., Ham et al., Proc. Natl. Acad. Sci. USA 53: 288–193 (1965); Myoken etal., In Vitro 25: 477–480 (1989).

More preferably, the media further comprises at least one selected from the group consisting of buffers, salts, carbohydrates, amino acids, lipids, vitamins, co-factors, and the like in suitable form. Suitable media that can be modified according to the present invention can include one or more or a combination of Iscove's modified media, Dulbecco's Modified Eagle Medium, Ham's F-12 media, e.g., as provided by SIGMA, LIFE TECHNOLOGIES OR JRH

BIOSCIENCES, as listed above. Non-limiting examples, include, but are not limited to:

Iscove's Modified Media: (Sigma I2510, I7633, I2762, I3390):

SIGMA Prod. Num. COMPONENT	I2510, I7633 g/L	I2762, I3390 g/L
INORG. SALTS		
CaCl2.2H2O	0.219	0.219
MgSO4 (anhyd)	0.09767	0.09767
KCl	0.33	0.33
KNO3	0.000076	0.000076
NaHCO3	—	3.024
KCl	4.505	4.505
NaH2PO4 (anhyd.)	0.109	0.109
Na2SeO3	0.000017	0.000017
AMINO ACIDS		
Alanine	0.025	0.025
L-Arginine.HCl	0.084	0.084
L-Asparagine.H_2O	0.0284	0.0284
L-Aspartic Acid	0.03	0.03
L-Cystine.2HCl	0.09124	0.09124
L-Glutamic Acid	0.075	0.075
L-Glutamine	0.584	—
Glycine	0.03	0.03
L-Histidine.HCl.H_2O	0.042	0.042
L-Isoleucine	0.105	0.105
L-Leucine	0.105	0.105
L-Lysine.HCl	0.146	0.146
L-Methionine	0.03	0.03
L-Phenylalanine	0.066	0.066
L-Proline	0.04	0.04
L-Serine	0.042	0.042
L-Threonine	0.095	0.095
L-Tryptophan	0.016	0.016
L-Tyrosine.2Na.2H2O	0.10379	0.10379
L-Valine	0.094	0.094
VITAMINS		
D-Biotin	0.000013	0.000013
Choline Chloride	0.004	0.004

-continued

Iscove's Modified Media: (Sigma I2510, I7633, I2762, I3390):

SIGMA Prod. Num. COMPONENT	I2510, I7633 g/L	I2762, I3390 g/L
Folic Acid	0.004	0.004
myo-Inositol	0.0072	0.0072
Niacinamide	0.004	0.004
D-Pantothenic Acid.½Ca	0.0004	0.004
Pyridoxal.HCl	0.004	0.004
Riboflavin	0.0004	0.0004
Thiamine.HCl	0.004	0.004
Vitamin B12	0.000013	0.000013
OTHER		
D-Glucose	4.5	4.5
HEPES	5.958	5.958
Phenol Red.Na	0.016	0.016
Pyruvic Acid.Na	0.11	0.11
ADD		
NaHCO3	3.024	—
L-Glutamine	—	0.584
Grams of powder required to prepare 1 L	17.7	N/A

See, e.g., Iscove et al., J. Exp. Med. 147: 923–933 (1978); Iscove, et al., Exp. Cell Res. 126: 121–126 (1980).

Dulbecco's Modified Eagle's Medium (e.g., Sigma D0422, D1152, D2429, D2554, D2902, D3656, D5030, D5280, D5523).

SIGMA Prod # COMPONENT	D0422 g/L	D1152 g/L	D2429 g/L	D2554 g/L	D2902 g/L	D3656 g/L	D5030 g/L	D5280 g/L	D5523 g/L
INORGANIC SALTS									
CaCl2.2H2O	0.265	0.265	2.65	2.65	0.265	0.265	0.265	0.265	0.265
Fe(NO3)3.9H2O	0.0001	0.0001	0.001	0.001	0.0001	0.0001	0.0001	0.0001	0.0001
MgSO4	0.09767	0.09767	0.9767	0.9767	0.09767	0.09767	0.09767	0.09767	0.09767
KCl	0.4	0.4	4.0	4.0	0.4	0.4	0.4	0.4	0.4
NaHCO3	3.7	—	—	—	—	—			
NaCl	6.4	4.4	64.0	64.0	6.4	6.4	6.4	6.4	6.4
NaH2PO4	0.109	0.109	1.09	1.09	0.109	—	0.109	0.109	0.109
Succinic Acid		—	—	—	—	—	0.075	—	
Sodium Succinate		—	—	—	—	—	0.1	—	
AMINO ACIDS									
L-Arginine.HCl	0.84	0.084	0.84	0.84	0.084	0.084	0.084	0.084	0.084
L-Cystine.2HCl	—	0.0626	0.626	0.626	0.0626	0.0626	0.0626	0.0626	0.0626
L-Glutamine	0.03	0.584	0.30	0.30	0.584	0.584	—	—	0.584
Glycine	0.042	0.030	0.42	0.42	0.030	0.030	0.030	0.030	0.030
L-Histidine.HCl.H2O	0.105	0.042	1.05	1.05	0.042	0.042	0.042	0.042	0.042
L-Isoleucine	0.105	0.105	1.05	1.05	0.105	0.105	0.105	0.105	0.105
L-Leucine	1.46	0.105	1.46	1.46	0.105	0.105	0.105	0.105	0.105

-continued

Dulbecco's Modified Eagle's Medium (e.g., Sigma D0422, D1152, D2429, D2554, D2902, D3656, D5030, D5280, D5523).

SIGMA Prod # COMPONENT	D0422 g/L	D1152 g/L	D2429 g/L	D2554 g/L	D2902 g/L	D3656 g/L	D5030 g/L	D5280 g/L	D5523 g/L
L-Lysine.HCl	—	0.146	0.30	0.30	0.146	0.146	0.146	0.146	0.146
L-Methionine	0.066	0.030	0.66	0.66	0.030	0.030	0.030	0.030	0.030
L-Phenylalanine	0.042	0.066	0.42	0.42	0.066	0.066	0.066	0.066	0.066
L-Serine	0.095	0.042	0.95	0.95	0.042	0.042	0.042	0.042	0.042
L-Threonine	0.016	0.095	0.16	0.16	0.095	0.095	0.095	0.095	0.095
L-Tryptophan	0.016	—	—	0.016	0.016	0.016	0.016	0.016	
L-Tyrosine (free base)	0.10379	—	—	1.0379	1.0379	—	—	0.072	—
L-Tyrosine.2Na.2 H2O	0.10379	0.10379	0.10379	0.10379	—	0.10379			
L-Valine	0.094	0.094	0.94	0.94	0.094	0.094	0.094	0.094	0.094
VITAMINS									
Choline Bitartrate	0.004	—	0.04	0.04	—	—	—	0.0072	—
Choline Chloride	0.004	0.004	—	—	0.004	0.004	0.004	—	0.004
Folic Acid	0.0072	0.004	0.072	0.072	0.004	0.004	0.004	0.004	0.004
myo-Inositol	0.004	0.0072	0.04	0.04	0.0072	0.0072	0.0072	0.0072	0.0072
Niacinamide	0.004	0.004	0.04	0.04	0.004	0.004	0.004	0.004	0.004
D-Pantothenic Acid.½Ca	0.004	0.004	—	—	0.004	0.004	0.004	0.004	0.004
Pyridoxal.HCl	—	0.004	0.04	0.04	0.004	0.004	0.004	0.004	0.004
Pyridoxine.HCl	0.0004	—	0.004	0.004	—	—	—	—	—
Riboflavin	0.004	0.0004	0.04	0.04	0.0004	0.0004	0.0004	0.0004	0.0004
Thiamine.HCl	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004
OTHER									
D-Glucose	4.5	4.5	10.0	45.0	1.0	4.5	—	1.0	—
HEPES	—	5.958	—	—	—	—	—	—	0.0159
Phenol Red.Na	0.0159	0.0159	0.159	0.159	—	0.0159	—	0.0093	0.11
Pyruvic Acid.Na	0.11	—	1.1	1.1	0.11	—	—	0.11	—
ADD									
Glucose	—	—	—	—	—	1.0	—	—	—
L-Glutamine	0.584	—	0.584	0.584	—	—	0.584	0.584	—
L-Cystine.2HCl	—	—	—	—	—	—	—	—	—
L-Leucine	—	—	—	—	—	—	—	—	—
L-Lysine.HCl	—	—	—	—	—	—	—	—	—
L-Methionine	—	—	—	—	—	—	—	—	—
NaHCO3	—	3.7	3.7	3.7	3.7	3.7	3.7	3.7	3.7
NaH2PO4	—	—	—	—	—	0.109	—	—	—
Phenol Red.Na	—	—	—	—	—	—	—	—	—
Pyruvic Acid.Na	—	—	—	—	—	—	—	—	—
Grams of powder to prepare 1 L	N/A	17.4	N/A	N/A	N/A	N/A	N/A	N/A	10.0

See, e.g., Dulbecco and Freeman, Virology 8: 396–397 (1959); Smith et al.,, J. D. Freeman, G., Vogt, M. and Dulbecco, R. (1960). Virology 12: 185–196 (1960); Morton, In Vitro 6: 89 (1970); Rutzky and Pumper, In Vitro 9: 468 (1974).

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Ham's F-12/Dulbecco's Modified Eagle's Medium (e.g., Sigma D6905, D8900, D2906, D9785, D6421)

SIGMA Prod.# COMPONENT	D6905, D8900 g/L	D2906 g/L	D9785 g/L	D6421 g/L
INORGANIC SALTS				
$CaCl_2.2H_2O$	0.1545	0.1545	—	0.1545
$CuSO4.5H_2O$	0.0000013	0.0000013	0.0000013	0.0000013
$Fe(NO3)3.9H_2O$	0.00005	0.00005	0.00005	0.00005
$FeSO_4.7H_2O$	0.000417	0.000417	0.000417	0.000417
$MgCl.6H_2O$	0.06120	0.0612	—	0.0612
$MgSO_4$	0.04884	0.04884	—	0.04884
KCl	0.3118	0.3118	0.3118	0.3118
$NaHCO_3$	—	—	—	1.2
NaCl	6.996	6.996	6.996	6.996
Na2HPO4	0.07102	0.07102	0.07102	0.07102
NaH2PO4	0.0543	0.0543	0.0543	0.0543

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Ham's F-12/Dulbecco's Modified Eagle's Medium (e.g., Sigma D6905, D8900, D2906, D9785, D6421)				
SIGMA Prod.# COMPONENT	D6905, D8900 g/L	D2906 g/L	D9785 g/L	D6421 g/L
ZnSO4.7H2O	0.000432	0.000432	0.000432	0.000432
AMINO ACIDS				
L-Alanine	0.00445	0.00445	0.00445	0.0045
L-Arginine.HCl	0.1475	0.1475	0.1475	0.1475
L-Asparagine.H2O	0.0075	0.0075	0.0075	0.0075
L-Aspartic Acid	0.00665	0.00665	0.00665	0.00665
L-Cystine.HCl.H2O	0.01756	0.01756	0.01756	0.01756
L-Cysteine.2HCl	0.03129	0.03129	0.03129	0.03129
L-Glutamic Acid	0.00735	0.00735	0.00735	0.00735
L-Glutamine	0.365	0.365	—	—
Glycine	0.01875	0.01875	0.01875	0.01875
L-Histidine.HCl.H_2O	0.03148	0.03148	0.03148	0.03148
L-Isoleucine	0.05447	0.05447	0.05447	0.5447
L-Leucine	0.05905	0.05905	—	0.05905
L-Lysine.HCl	0.09125	0.09125	—	0.09125
L-Methionine	0.01724	0.01724	—	0.01724
L-Phenylalanine	0.03548	0.03548	0.03548	0.03548
L-Proline	0.01725	0.01725	0.01725	0.01725
L-Serine	0.02625	0.02625	0.02625	0.02625
L-Threonine	0.05345	0.05345	0.05345	0.05345
L-Tryptophan	0.00902	0.00902	0.00902	0.00902
L-Tyrosine.2Na.$2H_2O$	0.05579	0.05579	0.05579	0.05579
L-Valine	0.05285	0.05285	0.05285	0.05285
VITAMINS				
D-Biotin	0.0000035	0.0000035	0.0000035	0.0000035
Choline Chloride	0.00898	0.00898	0.00898	0.00898
Folic Acid	0.00265	0.00266	0.00266	0.00266
myo-Inositol	0.0126	0.0126	0.0126	0.0126
Niacinamide	0.00202	0.00202	0.00202	0.00202
D-Pantothenic Acid.½Ca	0.00224	0.00224	0.00224	0.00224
Pyridoxal.HCl	0.002	0.002	0.002	—
Pyridoxine.HCl	0.000031	0.000031	0.000031	0.002031
Riboflavin	0.000219	0.000219	0.000219	0.000219
Thiamine.HCl	0.00217	0.00217	0.00217	0.00217
Vitamin B-12	0.00068	0.00068	0.00068	0.00068

See, e.g., Barnes and Sato, Analyt. Biochem. 102:255–270 (1980).

Any suitable or desired protein for mammalian cell expression can be used in cell culture using media according to the present invention. Non-limiting examples of such proteins include, but are not limited to therapeutic or diagnostic proteins, such as eukaryotic or prokaryotic proteins. Preferred proteins can include, but art not limited to, cytokines, receptors, soluble receptors, interleukins, growth factors, and the like.

Citations

All publications or patents cited herein are entirely incorporated herein by reference as they show the state of the art at the time of the present invention and/or to provide description and enablement of the present invention. Publications refer to scientific or patent publications, or any other information available in any media format, including all recorded, electronic or printed formats. The following references are entirely incorporated herein by reference: Ausubel, et al., ed., Current Protocols in Molecular Biology, John Wiley & Sons, Inc., NY, N.Y. (1987–1999); Sambrook, et al., Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor, N.Y. (1989); Harlow and Lane, Antibodies, a Laboratory Manual, Cold Spring Harbor, N.Y. (1989); Colligan, et al., eds., Current Protocols in Immunology, John Wiley & Sons, Inc., N.Y. (1994–1998); Colligan et al., Current Protocols in Protein Science, John Wiley & Sons, NY, N.Y., (1997–1999).

EXAMPLES

Example 1

Production of Protein in Chemically Defined Media of the Present Invention Using Adapted Cell lines

An IgG protein producing myeloma cell line named C168O was not ideally suited for commercial production of IgG or for suitable regulatory approval, due to need for media components of known serum free media containing animal protein derived preparations that were not sufficiently defined or characterized, such as Excyte and others. This Excyte dependency was not able to be alleviated by adding chemically defined lipids or other components. However, when Excyte was removed and trace elements/vitamins were supplemented, a robust growth of C168O was achieved. This medium without primatone, albumin and excyte but supplemented with trace elements and vitamins is now called "CDM". A semi-batch culture of C168O in CDM medium showed that CDM medium was able to support both high cell density growth and high IgG production.

Another myeloma cell line called C463A is capable of growing in various commercial defined media. However, this growth was not ideally suited for commercial production of IgG or for suitable regulatory approval. C463A is derived from Sp2/0 and potentially can be used as a transfection host to develop commerically suitable cell lines. In semi-batch cultures, the cell density of C463A in CDM medium of the present invention routinely reached 6 to 7 million cells per milliliter (ml) compared with 3 to 4 million per ml in other tested defined media. The viability is similar amongst all tested media (80% to 90%). Apparently, CDM has the capability to support cell growth at a higher density than other chemically defined media.

To adapt cell lines derived from Sp2/0 cells in chemically defined media is a lengthy process. It usually takes several months to one year to obtain one. When CDM medium is used, we noticed that the length of time for adaptation was much shorter than that in other defined media. In one case, it took only a few weeks to obtain CDM culture compared to several months from previous experiences.

In summary, we find that trace elements and vitamins are essential for the growth of myeloma cells in the absence of bovine serum and non-defined animal derived materials. A chemically defined formulation was generated based on the addition of trace elements and vitamins to a suitable serum free media system. This formulation provides several advantages: 1. Supports robust growth and IgG or other protein production of various myeloma and other cell lines, 2. Easy adaptation for mammalian cells, e.g., Sp2/0-derived IgG or protein producing cell lines, 3. Cost-effective since expensive components, such as bovine serum and excyte, are excluded and 4. Regulatory-friendly since potentially infectious agents are eliminated.

The use of this medium in perfusion type bioreactors is or other types of cell culture can also be used according to the present invention.

Formulation of CDM Medium:

The formulation of a CDM media of the present invention is provided as follows, e.g., Tables A-B. Table A1 shows the components added to make the media. Tables A2–A3 and B1–B4 show the listing of components for the additional formulations used in Table A1. The components are avail-

able from commercial sources, as individual components, or as custom formulations that can be ordered, e.g., from Sigma (St. Louis, Mo., USA), Aldrich (St. Louis, Mo., USA), JRH Biosciences (Lenexa, Kans., USA), and the like.

Tables A1–A3:

TABLE A1

NON-LIMITING EXAMPLE OF CDM MEDIA OF THE INVENTION

Components	Final Concentration In Media	Physical Property	Storage Temp.
CM-2 (Part A, Table A2)	18.8 g/l	Powder	+2–8° C.
CH-2 (Part B; Table A3)	10 ml/l	Liquid	−20° C.
$NaHCO_3$	3.02 g/l	Powder	Ambient
Bovine APO	5 mg/l	Stock solution	+2–8° C.
Transferrin or ferric citrate		Powder	+2–8° C.
Pluronic F68	0.8 g/l	Stock Solution	+2–8° C.
		Powder	Ambient
		Powder	+2–8° C.
NaOH	0.7 g/l	Stock Solution	Ambient
		Pellets	Ambient
Ethanolamine	10 or 20 mg/l	Stock Solution	−20° C.
		Liquid	Ambient
Glutamine	0.29 g/l	Powder	Ambient
Mycophenolic acid	0.5 mg/l	Stock Solution WSS	+2–8° C.
Hypoxanthine	2.5 mg/l	2	
Xanthine (MHX)	50 mg/l		
Hydrocortisone	20 ug/l	WSS 9	+2–8° C.
Vitamins (Table B1)	1X	100X Liquid	+2–8° C.
Trace Minerals 1 (Table B1)	1X	1000X Liquid	+2–8° C.
Trace Minerals 2 (Table B2)	1X	1000X Liquid	+2–8° C.
Trace Minerals 3 (Table B3)	1X	1000X Liquid	+2–8° C.

Preparation Instructions: Add components in order listed above. The sodium hydroxide should be made the same day.
Note: Prior to pH adjustment, pH = 6.7–6.8. The density at liquid stock solutions are the same as water (p-1 g/ml). Therefore, volume or weight can be used alternatively.

S1 pH: 7.3–7.6
S1 Osm: 305–368

TABLE A2

CM-2 (Part A)

Component	Final Conc. gm/L (Dry, 18.8 g total/L)
Sodium Chloride	4.505
Potassium Chloride	0.330
Sodium Phosphate Monobasic H_2O	0.125
Magnesium Sulfate, Anhydrous	0.09767
Potassium Nitrate	0.000076
Sodium Selenite	0.0000173
Calcium Chloride, Anhydrous	0.165
L-Alanine	0.025
L-Asparagine H_2O	0.0284
L-Arginine HCl	0.084
L-Aspartic Acid	0.030
L-Cysteine HCl H_2O	0.4175
L-Cystine 2HCl	0.09124
L-Glutamic Acid	0.075
L-Glutamine	0.8763
Glycine	0.030
L-Histidine HCl H_2O	0.042
L-Isoleucine	0.105

TABLE A2-continued

CM-2 (Part A)

Component	Final Conc. gm/L (Dry, 18.8 g total/L)
L-Leucine	0.105
L-Lysine HCl	0.146
L-Methionine	0.030
L-Phenylalanine	0.066
L-Proline	0.040
L-Serine	0.042
L-Threonine	0.095
L-Tryptophan	0.016
L-Tyrosine 2Na $2H_2O$	0.10379
L-Valine	0.094
Dextrose	4.500
Sodium Pyruvate	0.220
Biotin	0.000013
Ascorbic Acid	0.003
D-Ca Pantothenate	0.004
Choline Chloride	0.004
Folic Acid	0.004
i-Inositol	0.0072
Niacinamide	0.004
Pyridoxal HCl	0.004
Riboflavin	0.0004
Thiamine HCl	0.004
Cyanocobalamin	0.000013
Oxalacetic Acid	0.300
HEPES	5.958
	18.7776193

TABLE A3

CH-2 (Part B) (100X)

Component	100X: Amount/L	Final Conc.
Insulin	1.0 g	10 mg/L
Hydrocortisone	200 μg	2 μg/L
Linoleic Acid	500 mg	5 mg/L
Ethanolamine (1.02 mg/μl)	1020 g	10 mg/L
NaCl	8.5 g	85 mg/L

CDM medium is prepared according to Table A1 by adding components CH-2, parts A (18.8 gm/L, Table A2) and B (10 ml/L (100×), Table A3), followed by $NaHCO_3$ (3.02 g/L), Bovine APO transferrin or ferric citrate (5 mg/L), Pluronic F68 (0.8 g/L), NaOH (0.7 g/L), Ethanolamine (10 μl/L), Glutamine (0.29 g/L), mycophenolic acid (0.5 mg/L), hypoxanthine (2.5 mg/L), xanthine (50 mg/L), hydrocortisone (20 μg/L), vitamins (100×, 10 ml/L, Table B 1), trace minerals 1 (1000×, 0.33–1.0 ml/L, Table B2), trace minerals 2 (1000×, 0.33–1.0 ml/L, Table B3), trace minerals 3 (1000×, 0.33–1.0 ml/L, Table B4). In this example of CDM medium of the present invention, the working concentration of trace elements is 0.33–1.00×, and 1× for vitamins.

Tables B1–B4:

TABLE B1

Vitamin Solution (100X)

Component	Final Concentration Added(1X) mg/L	Liquid 100X mg/L
NaCl	85.00	8500.00
D-Calcium Pantothenate	1.00	100.00
Choline Chloride	1.00	100.00
Folic Acid	1.00	100.00
i-Inositol	2.00	200.00
Nicotinamide	1.00	100.00
Pyridoxine-HCl	1.00	100.00
Riboflavin	0.1	10.00
Thiamine-HCl	1.00	100.00

TABLE B2

Trace Metals 1, 1000X

COMPONENT	Final Conc. Added 0.33X µg/L	Final Conc. Added 1X µg/L	Liquid 1000X mg/L
$CuSO_4$—$5H_2O$	0.53	1.59	1.60
$ZnSO_4$,—$7H_2O$	284.8	854.4	863.00
Selenite-2Na	5.7	17.1	17.30
Ferric Citrate	381.2	1143.6	1155.10

TABLE B3

Trace Metals 2, 1000X

COMPONENT	Final Concentration Added (0.33X) µg/L	Final Concentration Added (1X) µg/L	Liquid 1000X mg/L
$AlCl_4$, $6H_2O$	0.40	1.20	1.20
$AgNO_3$	0.056	0.168	0.17
Ba $(C_2H_4O_2)_2$	0.842	2.53	2.55

TABLE B3-continued

Trace Metals 2, 1000X

COMPONENT	Final Concentration Added (0.33X) µg/L	Final Concentration Added (1X) µg/L	Liquid 1000X mg/L
KBr	0.040	0.12	0.12
$CdCl_2$	0.75	2.25	2.28
$CoCl_26H_2O$	0.785	2.355	2.38
$CrCl_2$, (anhydeous)	0.015	0.045	0.32
NaF	1.39	4.17	4.20
GeO_2	0.175	0.525	0.53
K1	0.056	0.168	0.17
RbCl	0.400	1.20	1.21
$ZrOCl_2$ $8H_2O$	1.06	3.18	3.22

TABLE B4

Trace Metals 3, 1000X

COMPONENT	Final Concentration Added (0.33X) µg/L	Final Concentration Added (1X) µg/L	Liquid 1000X mg/L
$MnSO_4$ H_2O	0.056	0.168	0.17
$NaSiO_3$ $9H_2O$	46.2	138.6	140.00
Molybdic Acid, Ammonium Salts	0.409	1.227	1.24
NH_4 VO_3	0.21	0.63	0.65
$NiSO_4$ $6H_2O$	0.043	0.129	0.13
$SnCl_2$ (anhydrous)	0.040	0.120	0.12

In this experiment, a chemically defined commercial medium, CD-hybridoma, from Gibco/Life Technology was used as a reference medium. A semi-batch growth profile (a 75% media change was performed daily after Day 3 of the experiment) was initiated to determine the effects of various additives on CDM media. Data at Day 5 were used for this comparison.

TABLE I

Comparing the effects of various additives on CDM w/o PAE in a Semi-Batch Growth Profile of C463A.

Cultures	Media Identification	Viable Cell Density (e/mL)	Total Cell Density (e/mL)	% Viability
A	CDM w/o OPI (oxaloacetate, pyruvate and insulin), bovine transferrin or ferric citrate and defined lipids, trace elements and vitamins	3.24	5.39	60
B	CDM in A above + OPI (oxaloacetate, pyruvate and insulin)	1.14	1.90	60
C	CDM in A above + bovine transferrin or ferric citrate and defined lipids	3.32	4.68	71
D	CDM in A above + trace elements and vitamins (CDM)	5.22	6.54	80
E	CDM in A above + OPI + transferrin or ferric citrate and defined lipids	1.68	2.26	74

TABLE I-continued

Comparing the effects of various additives on CDM w/o PAE in a Semi-Batch Growth Profile of C463A.

Cultures	Media Identification	Viable Cell Density (e/mL)	Total Cell Density (e/mL)	% Viability
F	CDM in A above + OPI + transferrin or ferric citrate + lipids + trace elements + vitamins	2.74	3.74	73
G	CDM in A above + 1% Sigma PFSF	3.6	4.72	76
H	LIT's CD Hybridoma media-modified	2.64	3.84	69
I	LIT's CD Hybridoma media-modified	3.34	5.04	66

CDM Medium Performs Best Compared to Other Tested Chemically Defined Media

Another semi-batch growth profile experiment was initiated to compare the growth performance of C463A in CDM medium to other commercial chemically defined media. Day 3 and subsequent media change is similar to that stated in Table I.

Table II shows the results collected on Day 5 of the semi-batch experiment. The CDM culture reached the highest viable and total densities among the group. C463A viability in CDM medium was also the highest of the four cultures at 82%. The outcome of this experiment reveals that CDM medium still provides the best support for C463A growth.

TABLE II

Comparing CDM to other chemically defined commercial media in a semi-batch growth profile of C463A

Cultures	Media Identification	Viable Cell Density (e/mL)	Total Cell Density (e/mL)	% Viability
A	CDHY + Sigma S8284 PFSF (50:50)	2.47	3.45	72
B	CDHY + trace elements + vitamins	1.58	3.45	46
C	CDM including + trace elements + vitamins (CDM)	3.86	4.71	82
D	LIT's CD Hybridoma media-modified	1.52	4.03	38

CDM Medium supports high cell density growth and IgG production of C168O, a Remicade producing cell line.

Once CDM medium was determined to enhance growth in our new host cell line, a semi-batch experiment in spinners was initiated for C168O, a Remicade producing cell line derived from C168J (see, e.g.,. FIG. A shows that CDM can support high cell density up to 4.5×10^6 cells/mL on Day 7. In FIG. B, specific productivity for CDM culture is at 16 ug/10^6 cells/day. FIG. C shows that at high cell density between 4–5×10^6 cells/mL, IgG production reached above 60 ug/mL. Viability of the CDM spinner culture remained above 75% throughout the experiment as seen in FIG. D.

Quick Adaptation in CDM Medium

Previously, the adaptation of myeloma cell lines to defined media has been difficult and may take up to 1 year to complete. With CDM medium, the adaptation period has decreased to several weeks. Below, Table III shows another IgG-producing cell line, C380C, adapted to CDM medium in a short period of about 4–5 weeks. After C380C sustained stability in CDM medium, viability was maintained above 90% and doubling time remained within 30–35 hrs. The specific productivity and overgrown IgG titer of C380C in CDM are above that when grown in IMDM w/ 5% FBS medium.

TABLE III

Adaptation of C380C culture in CDM medium

Media	Viability	Mean Doubling Time	Specific Productivity (ug/e/day)	Spent culture IgG titer (ug/mL)
IMDM w/ 5% FBS	>90%	~22–24 hrs.	12–13	55–95

TABLE III-continued

Adaptation of C380C culture in CDM medium

Media	Viability	Mean Doubling Time	Specific Productivity (ug/e/day)	Spent culture IgG titer (ug/mL)
CDM	>90%	Now: 30–40 hrs.	17–22	75–140

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It will be clear that the invention can be practiced otherwise than as particularly described in the foregoing description and examples. Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, are within the scope of the appended claims.

What is claimed is:

1. A chemically defined medium, said medium suitable for adaptation and growth of immortalized mammalian cells in culture to high cell densities, said medium comprising, sodium chloride, 3–5 g/L; potassium chloride, 0.2–0.4 g/L; , HEPES, 5–7 g/L; glucose (dextrose), 3.5–5.5 g/L; biotin, 0.000005–0.000025 g/L; ascorbic acid, 0.002–0.004 g/L; pantothenate, 0.002–0.006 g/L; choline, 0.002–0.006 g/L; folate, 0.002–0.006 g/L; inositol, 0.005–0.02 g/L; niacinamide, 0.002–0.006 g/L; pyridoxal, 0.002–0.006 g/L; riboflavin, 0.0002–0.0006 g/L; thiamine, 0.002–0.006 g/L; cyanocbalamin, 0.000005–0.000025 g/L; oxaloacetic acid, 0.1–0.4 g/L; alanine, 0.015–0.035 g/L; asparagine, 0.01–0.035 g/L; arginine, 0.06–0.10 g/L; aspartate, 0.02–0.04 g/L; cysteine, 0.3–0.5 g/L; cystine, 0.05–0.2 g/L; glutamine, 0.8–1.5 g/L; glutamate, 0.06–0.09 g/L; glycine, 0.02–0.04 g/L; histidine, 0.03–0.05 g/L; isoleucine, 0.05–0.25 g/L; leucine, 0.05–0.25 g/L; lysine, 0.05–0.25 g/L; methionine, 0.02–0.04 g/L; phenylalanine, 0.055–0.075 g/L; proline, 0.03–0.05 g/L; serine, 0.03–0.55 g/L; threonine, 0.07–0.15 g/L; tryptophan, 0.005–0.025 g/L; tyrosine, 0.05–0.15 g/L; valine, 0.094 g/L; sodium selenate, 0.0000005–0.000060 g/L; magnesium sulfate, 0.05–0.2 g/L; potassium chloride, 0.15–0.45 g/L; sodium phosphate, 0.075–0.2 g/L; potassium nitrate, 0.00005–0.00009 g/L; calcium chloride, 0.08–0.25 g/L; sodium pyruvate 0.05–0.4 g/L; insulin, 0.05–2 g/L; hydrocortisone, 20–80 μg/L; linoleic acid, 1–100 mg/L; ethanolamine, 5–25 μg/L; sodium bicarbonate, 1–5 g/L; APO transferrin or ferric citrate, 1–10 mg/L; Pluronic F68, 0.2–2 g/L; sodium hydroxide, 0.3–0.9 g/L; mycophenolic acid, 0.1–2 mg/L; hypoxanthine, 2–5 mg/L; xanthine; 10–200 mg/L; sodium bicarbonate 1.5–4.5 g/L.

2. The chemically defined medium of claim **1** wherein the medium is suitable for growth of immortalized mammalian cells in culture to cell densities of at least 4.5×10^6 cells/ml.

3. The chemically defined medium of claim **1** wherein the immortalized mammalian cells are the myeloma cell line Sp2/0 or derivatives thereof.

* * * * *

EXHIBIT F

US006773600B2

(12) **United States Patent**
Rosenblatt et al.

(10) **Patent No.: US 6,773,600 B2**
(45) **Date of Patent: Aug. 10, 2004**

(54) **USE OF A CLATHRATE MODIFIER, TO PROMOTE PASSAGE OF PROTEINS DURING NANOFILTRATION**

(75) Inventors: **Barry P. Rosenblatt**, Morrisville, PA (US); **Richard C. Siegel**, Chester Springs, PA (US)

(73) Assignee: **Cantocor, Inc.**, Malvern, PA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **10/454,089**

(22) Filed: **Jun. 4, 2003**

(65) **Prior Publication Data**

US 2003/0230532 A1 Dec. 18, 2003

Related U.S. Application Data

(60) Provisional application No. 60/394,733, filed on Jun. 14, 2002.

(51) **Int. Cl.**[7] **B01D 61/20**; B01D 37/00; A61K 39/395; C07K 16/34

(52) **U.S. Cl.** **210/639**; 210/644; 210/651; 210/739; 210/743; 210/749; 424/176.1; 436/177; 436/178; 530/414

(58) **Field of Search** 210/639, 644, 210/650, 651, 652, 739, 743, 749, 767; 424/176.1; 436/177, 178; 530/387.1, 390.1, 414

(56) **References Cited**

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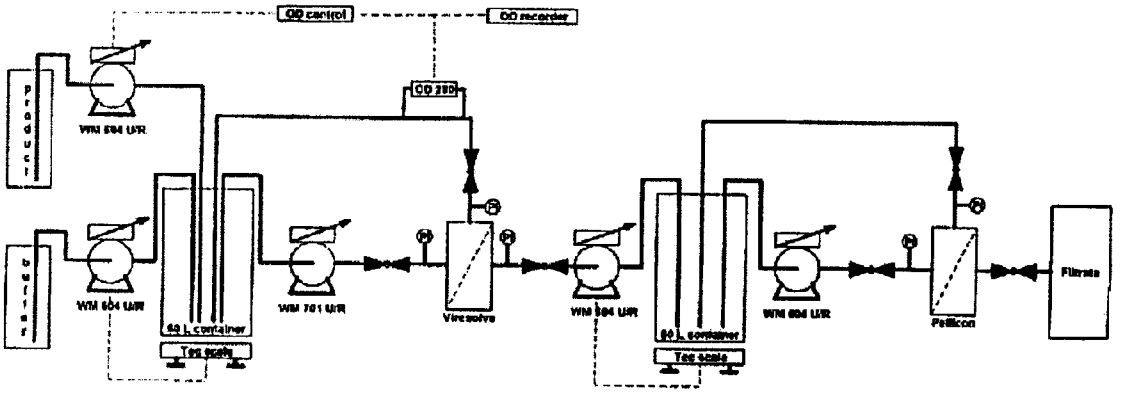
Primary Examiner—John Kim

(57) **ABSTRACT**

The invention relates to the field of protein purification and the recovery of large proteinaceous material through small, nanometer sized, pore exclusion filters for removal of contaminants such as viral pathogens.

7 Claims, 1 Drawing Sheet

Viresolve and Concentration / Diafiltration Process



Viresolve and Concentration / Diafiltration Process

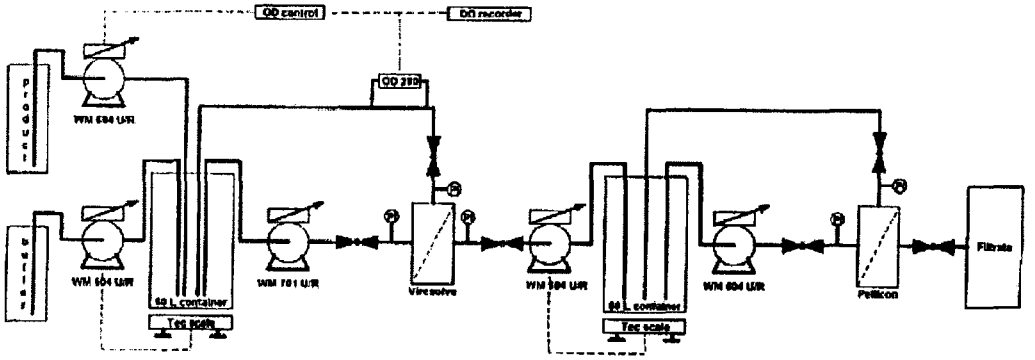


Figure 1

USE OF A CLATHRATE MODIFIER, TO PROMOTE PASSAGE OF PROTEINS DURING NANOFILTRATION

CROSS REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application Serial No. 60/394,733, filed Jun. 14, 2002.

FIELD OF THE INVENTION

The invention relates to the field of protein purification and the recovery of large proteinaceous material through small, nanometer sized, pore exclusion filters for removal of contaminants such as viral pathogens. The invention relates to the use of additives to promote solubility of proteins in solutions being filtered for the purpose of removing pathogens, particularly viral pathogens, and has particular applicability to the purification of large proteinaceous biomolecules such as immunoglobulins.

BACKGROUND OF THE INVENTION

Liquid and gas separation processes are well known in the art. Most common separation processes involve a phase change, which increases the cost of the processes and often requires excessive temperature changes which can alter the product. Membrane separations, however, can achieve desired levels of separation without a change in the substances' phase. In essence, membrane separation selectively forces one or more substances through pores of a filter, leaving one or more larger substances behind. This process is often repeated with diminishing filter pore sizes until a satisfactory level of separation is achieved.

The use of nanofiltration to remove contaminants such as virus particles from parenteral protein products is based upon the ability of a filter of defined pore size to allow a soluble protein to pass through while denying passage of the larger viral particles (DiLeo, A J, et al, BioTechnology 1992, 10: 182,188.) Removal of virus from large biomolecules such as immunoglobulins (monoclonal or polyclonal antibodies), by size exclusion, is hindered by the difficulty of passing the large biomolecules through pore sizes of nanometer size, typically 12–15 nm. While a protein in solution, even one as large as an immunoglobulin, is expected to have a molecular radius much smaller than a viral particle, several factors can lead to an effective reduction in pore size and sieving coefficient. Some of these factors are due to interactions between the protein and the filter surface resulting in build up on the membrane surface known as a gelation or polarization layer. Other factors, such as protein self-association or aggregation, cause the protein to be trapped by the filter due to formation of masses too large to pass through the filter pores or that have surface characteristics that exhibit affinity for the membrane surface or pore surfaces causing them to adhere to the membrane instead of passing through.

International patent application, WO 9600237, describes methods for successful nanofiltration using pore sizes as small as 15 nm to filter purified proteins of molecular weight less than 150 kDa. WO 9600237 discloses the use of salt concentrations lying in the range from about 0.2 M up to saturation of the solution in virus-filtering of proteins, polysaccharides, and polypeptides to increase sieving coefficients. The advantage of the salt is stated by the applicants to be because the "protein contracts" and more easily passes through the filter. The use of a high salt content according to this method is also suggested to enable the use of "dead-end" filtering with membranes having pore sizes of 5–30 nm. Dead-end filtering refers to the practice of using a single pump to force fluid through the membrane from the surface. Dead-end filtration is simpler and more cost effective than tangential filtering process wherein a first pump maintains constant flow rate at the surface of the membrane and a second pump draws the protein through the filter by creating a negative pressure (suction) at the back of the membrane.

U.S. Pat. No. 6,096,872 recognized the utility of adding surfactants along with high ionic strength buffering during nanofiltration to remove viruses from immunoglobulin containing solutions in order to reduce protein dimerization, trimerization and aggregation, the teachings of which are hereby incorporated herein by reference.

It is also generally known that in order to reduce the interaction of a substance with the membrane surface, the "zeta-" or "z-"potential of the membrane surface should not be electrically attractive to that substance and altering the charge properties of the membrane can minimize surface precipitation. For example, U.S. Pat. No. 6,177,011 teaches that the neutralization of surface charges measured as zeta potential can reduce surface adsorption of membrane-fouling substances during reverse osmosis filtration processes where the substance carries a charged group. Changes in pH and salt concentration are other means of altering the z-potential of both the solutes and the membrane surface. In some cases, however, the manipulation of the z-potential by the addition of salt is counter-productive, resulting in an increase in soluble aggregation and an increase in the hydrophobic character of the membrane surface which may promote interaction with hydrophobic protein regions. Pall, et al (Colloids and Surfaces 1 (1980), 235–256.), reported that the phenomenon of removal of particles smaller than the pores of a filter is due to adherence of the particles to the pore walls under conditions wherein the particles and the pore walls are oppositely charged or alternatively wherein the zeta potential of the particles and the pore walls of the membrane are both low. Zierdt (Applied and Environmental Microbiology, (1979) 38:1166–1172) attributed the aforementioned phenomenon to electrostatic forces. Furthermore, these modifications do not address the effects of molecular geometry or protein aggregation in solution on membrane filtration.

In addition to the considerations of buffer components and their concentrations, care must be take to maintain the protein to be filtered in a concentration appropriate to maintaining good flow and minimal transmembrane pressure across the filter. WO 9837086 teaches the addition of buffer to the retentate in order to maintain transmembrane pressure during tangential flow of a pretreatment step to remove proteins having a molecular weight greater than that of the product protein(s). WO 9837086 further notes that nanofiltration is limited to therapeutic proteins having a molecular weight up to 150 kDa. Immunoglobulin G molecules are composed of two heavy chains and two light chain polypetides all covalently linked and have an average molecular weight of about 180 kDa. U.S. Pat. No. 6,096,872 seeks to address the problem of how to filter viruses from IgG products by including a non-ionic excipient with relatively high (physiological which is about 300 mOsm) ionic strength buffer. The use of high ionic strength buffers, however, may lead to protein aggregation or create the problem of salt removal from the product formulation. U.S. Pat. No. 6,096,872 teaches and claims a second nanofiltration step to concentrate the immunoglobulin and collect it in a low ionic strength buffer.

These methods suffer from various disadvantages, particularly in their efficiency. It is therefore the object of the present invention to overcome the short-comings of the prior art, particularly in developing a system for efficiently filtering pathogenic viruses from immunoglobulin products, thereby providing virally cleared, pure immunoglobulin for injection.

The molecular configuration or size of a protein species has been predicted by changes in the partial specific volume and self-association of proteins. The change in partial specific volume of proteins so modified has been demonstrated by the independent measurements of sedimentation coefficients using analytical centrifugation. The method described herein uses the addition of a clathrate modifying substance to modify the molecular configuration of the protein to minimize specific volume and aggregation thereby enhancing passage of the protein through the membrane in a nanofiltration process.

SUMMARY OF THE INVENTION

The method of the invention maximizes protein passage during membrane filtration by using buffer additives aimed to increase the hydrophobicity of the membrane surface and decrease the hydrodynamic radius of the protein as well as reduce the tendency for the self-association of the protein desired to be filtered. The method of the invention first maximizes protein passage by decreasing the pH and the salt of the buffer which increases the hydrophobicity of the membrane surface and decreases the hydrodynamic radius of the protein. Secondly, a clathrate modifer is included in the buffer which modifier decreases the hydrodynamic radius of the protein while minimizing the tendencies for the protein to associate with either itself or the membrane filter. Thirdly, the process optionally includes continuous in-line monitoring of the filtration in order to maintain the above parameters of pH and clathrate modifier constant while maintaining low local levels of soluble protein. The use of the methods of the invention result in an increase in sieving coefficient and the ability to maintain reduced transmembrane pressure during virus particle filtration. The process is applicable to the purification of any large proteinaceous biomolecule, particularly immunoglobulins. The immunoglobulins may be a monoclonal or polyclonal immunoglobulin.

The clathrate modifier is perferably a polyol sugar or sugar alcohol having from 4 to 8 hydroxyl groups. Examples of preferred polyols are sugars, including mono-saccharides and disaccharides preferably sucrose. The concentration of the polyol used as a clathrate modifier will generally be 5% w/v or greater. The use of sucrose causes a decrease in the size of the molecule and a reduction in the tendency for self-association of the protein desired to be freed from virus particles.

Thus, the invention contemplates a method for purifying a proteinacious material such as an immunoglobulin comprising the steps of:

(a) admixing the proteinaceous material with:
  (i) a low pH, low conductivity buffer solution formulated to reduce the pH between 5.0 and 6.0, and to achieve an ionic strength of less than 30 mS/cm;
  (ii) a non-ionic surfactant; and
  (iii) a clathrate modifier;
(b) performing nanofiltration on the proteinaceous material to obtain a purified material substantially free of viral particles.

Preferably, the clathrate modifier is a polyol sugar or sugar alcohol having from 4 to 8 hydroxyl groups.

The method of the invention may also include conducting an in-line pre-filtering step and monitoring the concentration of the material by installing an in-line concentration controlling monitor to maintain the parameters of pH, and protein concentration within pre-set ranges optimal for the material being purified.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1**. Is a schematic representation of the vessels and monitoring equipment used in nanofiltration and the direction of fluid flow.

DETAILED DESCRIPTION

The instant invention uses a combination of selection of buffer, non-ionic surfactant and the use of a clathrate modifier as processing aids during viral reduction or viral clearances using size exclusion nanofiltration for purification of large proteinaceous biomolecules. The invention allows a small pore size exclusion nanofilter to be used with a globular protein molecule such as an immunoglobulin in a manner which allows for efficient flowthrough, minimal yield loss and no significant change in the immunoglobulin characterization aggregate level or stability.

Virus removed from the proteinaceous material by the nanofiltration method of the invention include all potential categories of virus, both enveloped (for example HIV, Hepatitis B) and non-enveloped (for example Hepatitis A, Parvovirus B19).

The advantages of the use of the processing aids and the method of the present invention include:

(1) the reduction of processing time and increased yield since the conditions employed increase the hydrophobicity of the membrane surface and reduce the specific volume and aggregation of the proteinaceous material;
(2) the ability to use smaller pore size nanofilters, thereby ensuring removal of smaller size viral particles;
(3) the process can be automated for continuous monitoring to allow for maximum efficiency and highest product yield per filter area;
(4) the essential characteristics of the proteinaceous material are unaffected by the process maintaining the integrity and quality of the end product.

In a broad sense, a clathrate is a molecular association in which the result may form a particle. Clathrates are included among those complexes in which one component (the host) forms a cavity or, in the case of a crystal, a crystal lattice containing spaces in the shape of long tunnels or channels in which molecular entities of a second chemical species (the guest) are located. There is no covalent bonding between guest and host, the attraction being generally due to van der Waals forces. If the spaces in the host lattice are enclosed on all sides so that the guest species is "trapped" as in a cage, such compounds are known as "clathrates" or "cage" compounds". van der Waals forces and hydrophobic interactions bind the guest to the host molecule in clathrates and inclusion compounds. Examples of hydrogen-bonded molecules that form clathrates are hydroquinone and water, and host molecules of inclusion compounds, urea or thiourea.

In the present case, the term "clathrate modifier" means a substance that is capable of modifying the clathrate structure of a protein in an aqueous environment and reducing its overall specific volume. Substance such as large globular proteins are good candidates for clathrate modifiers because of their capability of forming hydrogen bonds in an aqueous environment. The polyol clathrate modifier of the present

invention, modifies the clathrate complex of the proteinaceous material thereby reducing its specific volume and allowing for a reduction in processing time and greater flowthrough in the nanofiltration process.

In this specification by "polyol sugars and sugar alcohols" is meant a group of polyols having from 4 to 8 hydroxyl groups. Examples of preferred polyols are sugars, including monosaccharides and disaccharides, and sugar alcohols as well as derivatives thereof having from 4 to 8 hydroxyl groups.

Examples of monosaccharides having 4 hydroxyl groups are arabinose, ribose and xylose. An example of a sugar alcohol having 4 hydroxyl groups is the sugar alcohol derived from erythrose, i.e. erythritol.

Examples of monosaccharides having 5 hydroxyl groups are galactose, fructose, glucose and sorbose. An example of a sugar alcohol having 5 hydroxyl groups is the sugar alcohol derived from xylose, i.e. xylitol.

Examples of sugar alcohols having 6 hydroxyl groups are those derived from glucose and sorbose as well as from the hydrolysis products of sucrose, e.g. sorbitol and mannitol. Examples of disaccharides are maltose, lactose and sucrose, the latter being preferred, all of which contain 8 hydroxyl groups.

The large proteinaceous material which may be processed in accordance with the present invention include large globular proteins such as immunoglobulins (for example IgG) and fragments thereof, blood coagulation factors, growth hormones, apolipoproteins, enzymes and similar protein biomolecules, whether naturally occurring or genetically engineered.

The term "z-potential," as used herein, means surface charge. The surface charge of a particle is sometimes referred to as its z-potential, a measurement of charge which falls off with distance. The z-potential is directly correlated with the polarity or net charge of a compound.

As used herein, the term "nanofiltration" refers to filtration using size exclusion means where the pore size is of nanometer size. In general, the pore size of the nanofiltering units, also referred to as UF filters, employed in the production of substantially pure, virus-free immunoglobulin products of the instant invention is less than about 30 nm, most preferably less than about 15 nm. However, any membrane having the filter cutoff rating sufficient to reduce or eliminate non-enveloped virus from a proteinaceous solution can be employed in the processing methods of the invention. For example, the VIRESOLVE® 180 SYSTEM Ultrafiltration System (Millipore Corporation, Bedford, Mass.) unit may be employed, such unit having a molecular weight pore size rating of less than about 180 KD molecular weight or about 12 nm.

The nonionic surfactant or detergents which may be used in the present invention include the nonionic polyoxyethylene detergents for example the polysorbates, TWEENS; vinyl polymers, PLURONICS; polyoxyethylene-polypropylene polymers or co-polymers; Brij, Sterox-AJ, and Tritons. Most preferred is polyoxyethylene sorbitan monooleate, polysorbate 80 (TWEEN 80).

The buffer employed in the invention is selected from any suitable low pH, low conductivity buffer such as phosphate buffers, citrate buffers, borate buffers, acetate buffers and glycine buffers at a pH of about 5.0. The buffer is employed to maintain the pH below 6 and reduce aggregation of the protein thereby allowing more efficient flow, through the nanofilter. Preferably a buffer with a low ionic strength of 50 mM±/−20% is employed, preferably a sodium acetate buffer, pH 5.0.

The method involves transferring the protein of interest into a low pH (pH 5.0–6.0), low conductivity buffer (10–20 mS/cm), containing a non-ionic detergent such as TWEEN 80 at a concentration of 0.01% and sucrose at a concentration of between 5 and 10% w/v. The tangential flow apparatus is in fluid communication with several other vessels: a product tank, a buffer tank, and a feed/recirculation tank equipped with an agitator. The relationship of these vessels and the fluid flow between is shown in FIG. **1**.

The protein concentration used in the processing of the instant invention will be in the range of about 0.1% to about 1% by weight. Up to about 1% can be used when the protein is monomeric or monoclonal. For immunoglobulins such as a chimeric monoclonal IgG1, the initial protein concentration used for processing is about 1 to 10 mg/ml.

During processing and filtration, the protein concentration is preferably monitored to maintain optimal levels. As shown in FIG. **1**, this can be accomplished by the installation of an in line concentration monitor. A dead-end prefilter may be placed in the line between the feed/recirculation tank and the UF filter. A UV monitor is placed in-line between the UF filter and recirculation tank, on the retentate line. to provide a feed-back to the feed and buffer addition tanks to allow maintenance of the target protein concentration. Adjustment of the prefiltered product containing solution is achieved by the addition of buffer into the feed/recirculation tank to achieve the desired pH, conductivity, detergent concentration, and sucrose concentration. FIG. **1** shows the fluid flow from the feed/recirculation tank. During the filtration, the concentration of the retentate is kept constant by the addition of buffer in order to minimize protein-protein interaction. In the example shown, this is accomplished by control of the pumps supplying the product into the recirculation tank. By increasing/decreasing the speed of the pump, the concentration can be kept within a narrow specified range. A load cell under the recirculation tank is used as an addition feedback to the buffer pump to avoid overflowing the tank.

During filtration, the transmembrane pressure is preferably in the range of 0.2 to about 2.0 bar, most preferably maintained at less then about 1.0 bar. The sieving coefficient will preferably be in the range of 75–95% with excursions no lower than 60%.

EXAMPLE

A working example of this invention is demonstrated in the production of a chimeric human/mouse IgG1. The protein, after elution from a cation exchange column at pH 5.0, is placed in the product tank. The buffer tank is filled with 50 mM Sodium acetate, 6% sucrose, 0.01% polysorbate (tween) 80. The protein and buffer are mixed to achieve a final protein concentration of 2.0 ∀0.2 mg/mL in the feed tank. The filtration is started with a cross flow rate of xx mL/min/cm^2 and a permeate rate of no greater than yy mL/min/cm^2. Transmembrane pressure and retentate concentration is monitored to ensure that the process remains within the prescribed limits. Once the product tank is empty, the filters are rinsed with 3× the hold-up volume of the system to maximize the yield.

What is claimed is:

1. A method for purifying a proteinacious material comprising the steps of:

(a) admixing the proteinaceous material with:

(i) a low pH, low conductivity buffer solution formulated to reduce the pH between 5.0 and 6.0, and to achieve an ionic strength of less than 30 mS/cm;

(ii) a non-ionic surfactant; and

(iii) a clathrate modifier;

(b) performing nanofiltration on the proteinaceous material to obtain a purified material substantially free of viral particles.

2. The method of claim **1** wherein the proteinaceous material is an immunoglobulin.

3. The method of claim **1** wherein the clathrate modifier is a polyol sugar or sugar alcohol having from 4 to 8 hydroxyl groups.

4. The method of claim **3** wherein the polyol is a monosaccharides or disaccharides.

5. The method of claim **4** wherein the polyol is sucrose.

6. The method of claim **1** wherein the concentration of the polyol used as a clathrate modifier is about 5% w/v or greater.

7. A method for purifying a proteinacious material comprising the steps of:

a) admixing the proteinaceous material with a buffer solution:

adjusting the pH and the ionic strength of the buffer such that the pH is 5.0–6.0 and the ionic strength is less than 30 mS/cm;

b) adding a surfactant to the buffer to minimize protein-protein and protein-membrane interactions,

c) adding a clathrate modifier to the buffer, which clathrate modifier

i) Reduces the hydrodynamic radius of the protein and

ii) Minimizes the self-association of the protein;

d) installing an in-line prefilter to the system;

e) installing an in-line concentration controlling monitor to the system; and

f) using information from the in-line concentration controlling monitor to maintain the buffer parameter of pH and protein concentration within the range of pH of 5.0–6.0 and the ionic strength is less than 30 mS/cm.

* * * * *

JS 44 (Rev. 12/12)

CIVIL COVER SHEET

The JS 44 civil cover sheet and the information contained herein neither replace nor supplement the filing and service of pleadings or other papers as required by law, except as provided by local rules of court. This form, approved by the Judicial Conference of the United States in September 1974, is required for the use of the Clerk of Court for the purpose of initiating the civil docket sheet. *(SEE INSTRUCTIONS ON NEXT PAGE OF THIS FORM.)*

I. (a) PLAINTIFFS
JANSSEN BIOTECH, INC., and NEW YORK UNIVERSITY

(b) County of Residence of First Listed Plaintiff Montgomery County, PA
(EXCEPT IN U.S. PLAINTIFF CASES)

(c) Attorneys *(Firm Name, Address, and Telephone Number)*
Heather B. Repicky, Nutter McClennen & Fish, LLP, 155 Seaport, Boston, MA 02210 (617)439-2000

DEFENDANTS
CELLTRION HEALTHCARE CO., LTD., CELLTRION, INC., and HOSPIRA, INC.

County of Residence of First Listed Defendant Middlesex County, MA
(IN U.S. PLAINTIFF CASES ONLY)

NOTE: IN LAND CONDEMNATION CASES, USE THE LOCATION OF THE TRACT OF LAND INVOLVED.

Attorneys *(If Known)*

II. BASIS OF JURISDICTION *(Place an "X" in One Box Only)*

- ☐ 1 U.S. Government Plaintiff
- ☒ 3 Federal Question *(U.S. Government Not a Party)*
- ☐ 2 U.S. Government Defendant
- ☐ 4 Diversity *(Indicate Citizenship of Parties in Item III)*

III. CITIZENSHIP OF PRINCIPAL PARTIES *(Place an "X" in One Box for Plaintiff and One Box for Defendant)*
(For Diversity Cases Only)

	PTF	DEF		PTF	DEF
Citizen of This State	☐ 1	☐ 1	Incorporated *or* Principal Place of Business In This State	☐ 4	☐ 4
Citizen of Another State	☐ 2	☐ 2	Incorporated *and* Principal Place of Business In Another State	☐ 5	☐ 5
Citizen or Subject of a Foreign Country	☐ 3	☐ 3	Foreign Nation	☐ 6	☐ 6

IV. NATURE OF SUIT *(Place an "X" in One Box Only)*

CONTRACT
- ☐ 110 Insurance
- ☐ 120 Marine
- ☐ 130 Miller Act
- ☐ 140 Negotiable Instrument
- ☐ 150 Recovery of Overpayment & Enforcement of Judgment
- ☐ 151 Medicare Act
- ☐ 152 Recovery of Defaulted Student Loans (Excludes Veterans)
- ☐ 153 Recovery of Overpayment of Veteran's Benefits
- ☐ 160 Stockholders' Suits
- ☐ 190 Other Contract
- ☐ 195 Contract Product Liability
- ☐ 196 Franchise

REAL PROPERTY
- ☐ 210 Land Condemnation
- ☐ 220 Foreclosure
- ☐ 230 Rent Lease & Ejectment
- ☐ 240 Torts to Land
- ☐ 245 Tort Product Liability
- ☐ 290 All Other Real Property

TORTS

PERSONAL INJURY
- ☐ 310 Airplane
- ☐ 315 Airplane Product Liability
- ☐ 320 Assault, Libel & Slander
- ☐ 330 Federal Employers' Liability
- ☐ 340 Marine
- ☐ 345 Marine Product Liability
- ☐ 350 Motor Vehicle
- ☐ 355 Motor Vehicle Product Liability
- ☐ 360 Other Personal Injury
- ☐ 362 Personal Injury - Medical Malpractice

PERSONAL INJURY
- ☐ 365 Personal Injury - Product Liability
- ☐ 367 Health Care/ Pharmaceutical Personal Injury Product Liability
- ☐ 368 Asbestos Personal Injury Product Liability

PERSONAL PROPERTY
- ☐ 370 Other Fraud
- ☐ 371 Truth in Lending
- ☐ 380 Other Personal Property Damage
- ☐ 385 Property Damage Product Liability

CIVIL RIGHTS
- ☐ 440 Other Civil Rights
- ☐ 441 Voting
- ☐ 442 Employment
- ☐ 443 Housing/ Accommodations
- ☐ 445 Amer. w/Disabilities - Employment
- ☐ 446 Amer. w/Disabilities - Other
- ☐ 448 Education

PRISONER PETITIONS

Habeas Corpus:
- ☐ 463 Alien Detainee
- ☐ 510 Motions to Vacate Sentence
- ☐ 530 General
- ☐ 535 Death Penalty

Other:
- ☐ 540 Mandamus & Other
- ☐ 550 Civil Rights
- ☐ 555 Prison Condition
- ☐ 560 Civil Detainee - Conditions of Confinement

FORFEITURE/PENALTY
- ☐ 625 Drug Related Seizure of Property 21 USC 881
- ☐ 690 Other

LABOR
- ☐ 710 Fair Labor Standards Act
- ☐ 720 Labor/Management Relations
- ☐ 740 Railway Labor Act
- ☐ 751 Family and Medical Leave Act
- ☐ 790 Other Labor Litigation
- ☐ 791 Employee Retirement Income Security Act

IMMIGRATION
- ☐ 462 Naturalization Application
- ☐ 465 Other Immigration Actions

BANKRUPTCY
- ☐ 422 Appeal 28 USC 158
- ☐ 423 Withdrawal 28 USC 157

PROPERTY RIGHTS
- ☐ 820 Copyrights
- ☒ 830 Patent
- ☐ 840 Trademark

SOCIAL SECURITY
- ☐ 861 HIA (1395ff)
- ☐ 862 Black Lung (923)
- ☐ 863 DIWC/DIWW (405(g))
- ☐ 864 SSID Title XVI
- ☐ 865 RSI (405(g))

FEDERAL TAX SUITS
- ☐ 870 Taxes (U.S. Plaintiff or Defendant)
- ☐ 871 IRS—Third Party 26 USC 7609

OTHER STATUTES
- ☐ 375 False Claims Act
- ☐ 400 State Reapportionment
- ☐ 410 Antitrust
- ☐ 430 Banks and Banking
- ☐ 450 Commerce
- ☐ 460 Deportation
- ☐ 470 Racketeer Influenced and Corrupt Organizations
- ☐ 480 Consumer Credit
- ☐ 490 Cable/Sat TV
- ☐ 850 Securities/Commodities/ Exchange
- ☐ 890 Other Statutory Actions
- ☐ 891 Agricultural Acts
- ☐ 893 Environmental Matters
- ☐ 895 Freedom of Information Act
- ☐ 896 Arbitration
- ☐ 899 Administrative Procedure Act/Review or Appeal of Agency Decision
- ☐ 950 Constitutionality of State Statutes

V. ORIGIN *(Place an "X" in One Box Only)*

☒ 1 Original Proceeding ☐ 2 Removed from State Court ☐ 3 Remanded from Appellate Court ☐ 4 Reinstated or Reopened ☐ 5 Transferred from Another District *(specify)* ☐ 6 Multidistrict Litigation

VI. CAUSE OF ACTION
Cite the U.S. Civil Statute under which you are filing *(Do not cite jurisdictional statutes unless diversity)*:
42 U.S.C. s 262(l) and 35 U.S.C. s 271
Brief description of cause:
Enforcement of 42 U.S.C. s 262(l) and patent infringement

VII. REQUESTED IN COMPLAINT: ☐ CHECK IF THIS IS A CLASS ACTION UNDER RULE 23, F.R.Cv.P. **DEMAND $** CHECK YES only if demanded in complaint: **JURY DEMAND:** ☐ Yes ☒ No

VIII. RELATED CASE(S) IF ANY *(See instructions)*: JUDGE _____ DOCKET NUMBER _____

DATE 3/6/15 SIGNATURE OF ATTORNEY OF RECORD Heather B. Repicky /acc

FOR OFFICE USE ONLY

RECEIPT # _____ AMOUNT _____ APPLYING IFP _____ JUDGE _____ MAG. JUDGE _____

UNITED STATES DISTRICT COURT
DISTRICT OF MASSACHUSETTS

1. **Title of case (name of first party on each side only)** Janssen Biotech, Inc. and New York University v. Celltrion Healthcare Co., Ltd., Celltrion, Inc., and Hospira, Inc.

2. **Category in which the case belongs based upon the numbered nature of suit code listed on the civil cover sheet. (See local rule 40.1(a)(1)).**

- [x] **I.** **410, 441, 470, 535, 830*, 891, 893, 895, R.23, REGARDLESS OF NATURE OF SUIT.**
- [ ] **II.** **110, 130, 140, 160, 190, 196, 230, 240, 290,320,362, 370, 371, 380, 430, 440, 442, 443, 445, 446, 448, 710, 720, 740, 790, 820*, 840*, 850, 870, 871.**
- [x] **III.** **120, 150, 151, 152, 153, 195, 210, 220, 245, 310, 315, 330, 340, 345, 350, 355, 360, 365, 367, 368, 375, 385, 400, 422, 423, 450, 460, 462, 463, 465, 480, 490, 510, 530, 540, 550, 555, 625, 690, 751, 791, 861-865, 890, 896, 899, 950.**

***Also complete AO 120 or AO 121. for patent, trademark or copyright cases.**

3. **Title and number, if any, of related cases. (See local rule 40.1(g)). If more than one prior related case has been filed in this district please indicate the title and number of the first filed case in this court.**

4. **Has a prior action between the same parties and based on the same claim ever been filed in this court?**

YES [x] **NO** [ ]

5. **Does the complaint in this case question the constitutionality of an act of congress affecting the public interest? (See 28 USC §2403)**

YES [ ] **NO** [x]

If so, is the U.S.A. or an officer, agent or employee of the U.S. a party?

YES [ ] **NO** [ ]

6. **Is this case required to be heard and determined by a district court of three judges pursuant to title 28 USC §2284?**

YES [ ] **NO** [x]

7. **Do all of the parties in this action, excluding governmental agencies of the United States and the Commonwealth of Massachusetts ("governmental agencies"), residing in Massachusetts reside in the same division? - (See Local Rule 40.1(d)).**

YES [ ] **NO** [x]

A. **If yes, in which division do all of the non-governmental parties reside?**

Eastern Division [ ] **Central Division** [ ] **Western Division** [ ]

B. **If no, in which division do the majority of the plaintiffs or the only parties, excluding governmental agencies, residing in Massachusetts reside?**

Eastern Division [x] **Central Division** [ ] **Western Division** [ ]

8. **If filing a Notice of Removal - are there any motions pending in the state court requiring the attention of this Court? (If yes, submit a separate sheet identifying the motions)**

YES [ ] **NO** [ ]

(PLEASE TYPE OR PRINT)

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