



(12)

Oversættelse af europæisk patent

Patent- og
Varemærkestyrelsen

(51) Int.Cl.: **C 07 K 16/00 (2006.01)** **A 61 P 37/08 (2006.01)** **C 07 K 16/42 (2006.01)**

(45) Oversættelsen bekendtgjort den: **2016-03-21**

(80) Dato for Den Europæiske Patentmyndigheds
bekendtgørelse om meddelelse af patentet: **2016-01-27**

(86) Europæisk ansøgning nr.: **11170255.1**

(86) Europæisk indleveringsdag: **2004-02-02**

(87) Den europæiske ansøgnings publiceringsdag: **2012-01-18**

(30) Prioritet: **2003-02-01 US 444229 P**

(62) Stamansøgningsnr: **08104978.5**

(84) Designerede stater: **AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IT LI LU MC NL PT RO SE SI SK TR**

(73) Patenthaver: **Tanox, Inc., 10301 Stella Link Road, Houston, TX 77025, USA**

(72) Opfinder: **Foster, Catherine, 20 Golf View Drive, Dumont, NJ 07628, USA**
SINGH, Sanjaya, 4 Owl Ridge Lane, Sandy Hook, CT 06482, USA
WU, Herren, 14405 Harvest Moon Road, Boyds, MD 20841, USA

(74) Fuldmægtig i Danmark: **Zacco Denmark A/S, Arne Jacobsens Allé 15, 2300 København S, Danmark**

(54) Benævnelse: **Anti-human-IgE-antistoffer med høj affinitet**

(56) Fremdragne publikationer:
WO-A-92/17207
WO-A-99/38531
US-A- 5 958 708
CHANG T W ET AL: "MONOCLONAL ANTIBODIES SPECIFIC FOR HUMAN IGE-PRODUCING B CELLS A POTENTIAL THERAPEUTIC FOR IGE-MEDIATED ALLERGIC DISEASES", BIO/TECHNOLOGY, NATURE PUBLISHING CO. NEW YORK, US, vol. 8, no. 2, February 1990 (1990-02), pages 122-126, XP009084134, ISSN: 0733-222X
KOLBINGER F ET AL: "HUMANIZATION OF A MOUSE ANTI-HUMAN IGE ANTIBODY: A POTENTIAL THERAPEUTIC FOR IGE-MEDIATED ALLERGIES", PROTEIN ENGINEERING, OXFORD UNIVERSITY PRESS, SURREY, GB, vol. 6, no. 8, November 1993 (1993-11), pages 971-980, XP000983997, ISSN: 0269-2139
RACINE-POON AMY ET AL: "Efficacy, pharmacodynamics, and pharmacokinetics of CGP 51901, an anti-immunoglobulin E chimeric monoclonal antibody, in patients with seasonal allergic rhinitis", CLINICAL PHARMACOLOGY AND THERAPEUTICS, vol. 62, no. 6, December 1997 (1997-12), pages 675-690, XP009099497, ISSN: 0009-9236
CORNE JONATHAN ET AL: "The effect of intravenous administration of a chimeric anti-IgE antibody on serum IgE levels in atopic subjects: Efficacy, safety, and pharmacokinetics", JOURNAL OF CLINICAL INVESTIGATION, NEW YORK, NY, US, vol. 99, no. 5, March 1997 (1997-03), pages 879-887, XP002164091, ISSN: 0021-9738
BACA M ET AL: "Antibody humanization using monovalent phage display", JOURNAL OF BIOLOGICAL

Fortsættes ...

CHEMISTRY, AMERICAN SOCIETY OF BIOLOCHEMICAL BIOLOGISTS, BIRMINGHAM,, US, vol. 272, no. 16, 1997, pages 10678-10684, XP002308888, ISSN: 0021-9258
HAWKINS R E ET AL: "SELECTION OF PHAGE ANTIBODIES BY BINDING AFFINITY MIMICKING AFFINITY MATURATION", JOURNAL OF MOLECULAR BIOLOGY, LONDON, GB, vol. 226, no. 3, 1992, pages 889-896, XP000940994, ISSN: 0022-2836

DESCRIPTION

Background of the Invention

[0001] Allergy is a hypersensitive state induced by an exaggerated immune response to a foreign agent, such as an allergen. Immediate (type I) hypersensitivity, characterized by allergic reactions immediately following contact with the allergen, is mediated via B cells and is based on antigen-antibody reactions. Delayed hypersensitivity is mediated via T cells and based on mechanisms of cellular immunity. In recent years, the term "allergy" has become more and more synonymous with type I hypersensitivity.

[0002] Immediate hypersensitivity is a response based on the production of antibodies of the immunoglobulin class E (IgE antibodies) by B cells which upon exposure to an allergen differentiate into antibody secreting plasma cells. The IgE induced reaction is a local event occurring at the site of the allergen's entry into the body, i.e. at mucosal surfaces and/or at local lymph nodes. Locally produced IgE will first sensitize local mast cells, i.e. IgE antibodies bind with their constant regions to Fc ϵ receptors on the surface of the mast cells, and then "spill-over" IgE enters the circulation and binds to receptors on both circulating basophils and tissue-fixed mast cells throughout the body. When the bound IgE is subsequently contacted with the allergen, the Fc ϵ receptors are crosslinked by binding of the allergen causing the cells to degranulate and release a number of anaphylactic mediators such as histamine, prostaglandins, leukotrienes, etc. It is the release of these substances which is responsible for the clinical symptoms typical of Immediate hypersensitivity, namely contraction of smooth muscle in the respiratory tract or the intestine, the dilation of small blood vessels and the increase in their permeability to water and plasma proteins, the secretion of mucus resulting, e.g. in allergic rhinitis, atopic eczema and asthma, and the stimulation of nerve endings in the skin resulting in itching and pain. In addition, the reaction upon second contact with the allergen is intensified because some B cells form a "memory pool" of surface IgE positive B cells (sIgE $^+$ B cells) after the first contact with the allergen by expressing IgE on the cell surface.

[0003] There are two major receptors for IgE, the high affinity receptor Fc ϵ RI and the low-affinity receptor Fc ϵ RII. Fc ϵ RI is predominantly expressed on the surface of mast cells and basophils, but low levels of Fc ϵ RI can also be found on human Langerhan's cells, dendritic cells, and monocytes, where it functions in IgE-mediated allergen presentation. In addition, Fc ϵ RI has been reported on human eosinophils and platelets (Hasegawa, S. et. al., *Hematopoiesis*, 1999, 93:2543-2551). Fc ϵ RI is not found on the surface of B cells, T cells, or neutrophils. The expression of Fc ϵ RI on Langerhan's cells and dermal dendritic cells is functionally and biologically important for IgE-bound antigen presentation in allergic individuals (Klubal R. et. al., *J. Invest. Dermatol.* 1997, 108 (3):336-42).

[0004] The low-affinity receptor, Fc ϵ RII (CD23) is a lectin-like molecule comprising three identical subunits with head structures extending from a long α -helical coiled stalk from the cellular plasma membrane (Dierks, A.E. et. al., *J. Immunol.* 1993, 150:2372-2382). Upon binding to IgE, Fc ϵ RII associates with CD21 on B cells involved in the regulation of synthesis of IgE (Sanon, A. et. al., *J. Allergy Clin. Immunol.* 1990, 86:333-344, Bonnefoy, J. et. al., *Eur. Resp. J.* 1996, 9:63s-66s). Fc ϵ RII has long been recognized for allergen presentation (Sutton and Gould, 1993, *Nature*, 366:421-428). IgE bound to Fc ϵ RII on epithelial cells is responsible for specific and rapid allergen presentation (Yang, P.P., *J. Clin. Invest.*, 2000, 106:879-886). Fc ϵ RII is present on several cell types, including B-cells, eosinophils, platelets, natural killer cells, T-cells, follicular dendritic cells, and Langerhan's cells.

[0005] The structural entities on the IgE molecule that interact with Fc ϵ RI and Fc ϵ RII have also been identified. Mutagenesis studies have indicated that the CH3 domain mediates IgE interaction with both Fc ϵ RI (Presta et. al., *J. Biol. Chem.* 1994, 269:26368-26373; Henry A.J. et. al., *Biochemistry*, 1997, 36:15568-15578) and Fc ϵ RII (Sutton and Gould, *Nature*, 1993, 366: 421-428; Shi, J. et. al., *Biochemistry*, 1997, 36:2112-2122). The binding sites for both high- and low-affinity receptors are located symmetrically along a central rotational axis through the two CH3 domains. The Fc ϵ RI-binding site is located in a CH3 domain on the outward side near the junction of the CH2 domain, whereas the Fc ϵ RII-binding site is on the carboxyl-terminus of CH3.

[0006] A promising concept for the treatment of allergy involves the application of monoclonal antibodies, which are IgE isotype-specific and are thus capable of binding IgE. This approach is based on the inhibition of allergic reactions by downregulating the IgE immune response, which is the earliest event in the induction of allergy and provides for the maintenance of the allergic state. As the response of other antibody classes is not affected, both an immediate and a long lasting effect on allergic symptoms is achieved. Early studies of human basophil density showed a correlation between the level of IgE in the plasma of a patient and the number of Fc ϵ RI receptors per basophil (Malveaux et. al., *J. Clin. Invest.*, 1978, 62:176). They noted that the Fc ϵ RI densities in allergic and non-allergic persons range from 10⁴ to 10⁶ receptors per basophil. Later it was shown that treatment of allergic diseases with anti-IgE decreased the amount of circulating IgE to 1% of pretreatment levels (MacGlashan et. al., *J. Immunol.*, 1997,

158:1438-1445). MacGlashan analyzed serum obtained from patients treated with whole anti-IgE antibody, which binds free IgE circulating in the serum of the patient. They reported that lowering the level of circulating IgE in a patient resulted in a lower number of receptors present on the surface of basophils. Thus, they hypothesized that Fc ϵ RI density on the surface of basophils and mast cells is directly or indirectly regulated by the level of circulating IgE antibody.

[0007] More recently, WO 99/62550 disclosed the use of IgE molecules and fragments, which bind to Fc ϵ RI and Fc ϵ RII IgE binding sites to block IgE binding to receptors. However, effective therapies that lack deleterious side effects for the management of these allergic diseases are limited. One therapeutic approach to treating allergic diseases involved using humanized anti-IgE antibody to treat allergic rhinitis and asthma (Come, J. et al., J. Clin. Invest. 1997, 99:879-887; Racine-Poon, A. et al., Clin. Pharmacol. Ther. 1997, 62:675-690; Fahy, J.V. et al., Am. J. Resp. Crit. Care Med. 1997, 155:1824-1834; Boulet, L. P. et al., Am. J. Resp. Crit. Care Med., 1997, 155:1835-1840; Milgrom, E. et al., N. Engl. J. Med., 1999, 341:1966-1973). These clinical data demonstrate that inhibition of IgE binding to its receptors is an effective approach to treating allergic diseases.

[0008] Kolbinger et al., Protein Engineering 1993, 6, no. 8, 971-980 and US5958708 describe humanized antibodies obtained by humanization of the murine anti-IgE antibody TES-C21.

[0009] Antibodies suitable as anti-allergic agents should react with surface IgE positive B cells which differentiate into IgE producing plasma cells, so that they can be used to functionally eliminate those B cells. However, antibodies to IgE in principle may also induce mediator release from IgE sensitized mast cells by crosslinking the Fc ϵ receptors, thus antagonizing the beneficial effect exerted on the serum IgE and sIgE $^+$ B cell level. Therefore, antibodies applicable for therapy of allergy must not be capable of reacting with IgE bound on sensitized mast cells and basophils, but should retain the capability to recognize sIgE $^+$ B cells.

[0010] Such IgE isotype-specific antibodies have been described e.g. by Chang et al. (Biotechnology 8, 122-128 (1990)), in European Patent No. EP0407392, and several U.S. Patents, e.g., U.S. Patent No. 5,449,760. However, as the disclosed antibodies are not of human origin they are less suitable for application to humans due to their immunogenicity as foreign proteins. This drawback may potentially be reduced by transforming, e.g., a rodent anti-IgE monoclonal antibody into a chimeric antibody which combines the variable domains of the rodent antibody with human antibody constant domains. This approach conserves the antigen-binding site of the rodent parent anti-IgE antibody, while conferring the human isotype and effector functions. The immunogenicity of a chimeric antibody can be further reduced by grafting rodent hypervariable regions, also termed complementarity determining regions (CDRs), into the frameworks of human light and heavy chain variable region domains resulting in reshaped human antibodies. The technique involves the substitution or recombinant grafting of antigen-specific rodent CDR sequences for those existent within "generic" human heavy and light chain variable domains (U.S. Patent No. 6,180,370).

[0011] Natural intact immunoglobulins or antibodies comprise a generally Y-shaped tetrameric molecule having an antigen binding-site at the end of each upper arm. An antigen binding site consists of the variable domain of a heavy chain associated with the variable domain of a light chain. More specifically, the antigen binding site of an antibody is essentially formed by the 3 CDRs of the variable domain of a heavy chain (V_H) and the 3 CDRs of the variable domain of the light chain (V_L). In both V_L and V_H the CDRs alternate with 4 framework regions (FRs) forming a polypeptide chain of the general formula

(i) FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4 (I),

wherein the polypeptide chain is described as starting at the N-terminal extremity and ending at the C-terminal extremity. The CDRs of V_H and V_L are also referred to as H1, H2, H3, and L1, L2, L3, respectively. The determination as to what constitutes an FR or a CDR is usually made by comparing the amino acid sequences of a number of antibodies raised in the same species and general rules for identification are known in the art ("Sequences of proteins of immunological interest", Kabat, E. A. et al., US department of health and human service, Public health service, National Institute of Health).

[0012] The contribution made by a light chain variable domain to the energetics of binding is small as compared with that made by the associated heavy chain variable domain, and isolated heavy chain variable domains have an antigen binding activity on their own. Such molecules are commonly referred to as single domain antibodies (Ward, E. S. et al., Nature 341, 544-546 (1989)).

[0013] The CDRs form loops which, within the domains, are connected to a β -sheet framework. The relationship between amino acid sequence and structure of a loop can be described by a canonical structure model (Chothia et al., Nature 342, 887-883 (1989)). According to this model, antibodies have only a few main-chain conformations or "canonical structures" for each

hypervariable region. The conformations are determined by the presence of a few key amino acid residues at specific sites in the CDRs and, for certain loops, in the framework regions. Hypervariable regions that have the same conformations in different immunoglobulins have the same or very similar amino acid residues at these sites.

[0014] CDR grafting has been carried out for monoclonal antibodies yielding humanized human antibodies with a binding affinity significantly lower than that of the rodent CDR-donor antibody. Findings have indicated that, in addition to the transfer of CDRs, changes within the framework of the human sequence may be necessary in some instances to provide satisfactory antigen binding activity in the CDR-grafted product.

[0015] Queen et al. (Proc. Natl. Acad. Sci. USA 86, 10029-10033 (1989)) disclosed that the CDRs from a murine anti-Tac monoclonal antibody could be grafted into a human framework. The human frameworks were chosen to maximize homology with the murine sequence. The authors used a computer model of the murine parent antibody to identify amino acid residues located within the FRs that are close enough to interact with the CDRs or antigen. These residues were mutated to the residue found in the murine sequence. The humanized anti-Tac antibody had an affinity that was only about 1/3 that of the murine anti-Tac antibody and maintenance of the human character of this antibody was problematic.

[0016] Treatment of diseases with very high levels of IgE may require an antibody with higher affinity to reduce the risk of immunogenicity, and to expand the clinical indications to diseases with very high levels of IgE, e.g., atopic dermatitis. Thus, it is desirable to have an anti-IgE antibody with greater level of humanization and much higher affinity for IgE. The antibodies in this invention are anti-human IgE antibodies with ultra high affinities and a higher degree of human sequence homology lowering the risk of immunogenicity.

[0017] Thus, there is a need for higher affinity humanized antibodies that will allow lowering the amount of antibody necessary to treat disease, thereby lowering the potential side-effects from immunogenicity of the drug and the cost to the patient. Moreover, the present invention improves the probability that high affinity antibodies will be identified.

Summary of the invention

[0018] The present invention relates to a high affinity anti-IgE antibody as defined in the appended claims.

[0019] The present invention provides an isolated antibody or an antigen binding fragment thereof, comprising a variable light chain region comprising CDRL1, CDRL2, and CDRL3 and a variable heavy chain region comprising CDRH1, CDRH2, and CDRH3, wherein CDRL1 consists of the amino acid sequence set forth in SEQ ID NO:5, CDRL2 consists of the amino acid sequence set forth in SEQ ID NO:8, CDRL3 consists of the amino acid sequence QQSWSWPTT, CDRH1 consists of the amino acid sequence set forth in SEQ ID NO: 15, CDRH2 consists of the amino acid sequence set forth in SEQ ID NO:25, and CDRH3 consists of the amino acid sequence set forth in SEQ ID NO:26, wherein the antibody or fragment thereof binds specifically to IgE. In one embodiment, variable light chain region of said antibody comprises the amino acid sequence set forth in SEQ ID NO:63, or the variable heavy chain region comprises the amino acid sequence set forth in SEQ ID NO:64. In another embodiment, the variable light chain region has the amino acid sequence set forth in SEQ ID NO:63 and the variable heavy chain region has the amino acid sequence set forth in SEQ ID NO:64. In another embodiment, the antibody or the antigen binding fragment thereof further comprises a constant light chain region and a constant heavy chain region. In another embodiment, the constant light chain region has the amino acid sequence set forth in SEQ ID NO:58 and the constant heavy chain region has the amino acid sequence set forth in SEQ ID NO:60. In yet another embodiment, the antibody of the invention or the antigen binding fragment thereof is further linked to a label.

[0020] The invention further provides a composition comprising a therapeutically effective amount of the antibody comprising a nucleic acid encoding the antibody of the invention or the antigen binding fragment thereof or the antigen binding fragment thereof and a pharmaceutically acceptable carrier.

[0021] The invention further provides a diagnostic kit comprising the antibody of the invention or the antigen binding fragment thereof.

[0022] The invention further provides a method for diagnosing a disorder associated with an abnormal level of IgE in a subject, comprising contacting a sample of the subject, which sample comprises IgE molecules, with the antibody of the invention or the antigen binding fragment; and determining the level of retention of the antibody or the antigen binding fragment by the sample relative to a control sample of a control subject, wherein a higher or lower level of retention of the antibody or the antigen binding

fragment by the sample of the subject relative to the control sample indicates that the subject has a disorder associated with an abnormal level of IgE.

[0023] The present invention further provides the antibody of the invention or antigen binding fragment thereof for use in a method of treating a disorder associated with an abnormally high level of IgE in a subject. In a particular embodiment, the disorder is asthma, allergic rhinitis, eczema, urticaria, atopic dermatitis, or a food allergy.

[0024] The invention further provides a method for measuring the level of IgE in a subject, comprising contacting a sample of the subject, which sample comprises IgE molecules, with the antibody of the invention or the antigen binding fragment thereof; and determining the level of retention of the antibody or the antigen binding fragment by the sample relative to a control sample of a control subject, wherein a higher or lower level of retention of the antibody or the antigen binding fragment by the sample of the subject relative to the control sample indicates that the subject has a higher or lower level of IgE molecules relative to that in the control subject.

[0025] The invention further provides a vector comprising a nucleic acid encoding the antibody of the invention or the antigen binding fragment thereof.

[0026] The invention further provides a cell comprising the vector comprising a nucleic acid encoding the antibody of the invention or the antigen binding fragment thereof. In a preferred embodiment, the cell is ATCC Deposit number PTA-5680.

[0027] Also described herein is a method of making such high affinity antibodies from a parent antibody molecule, combining the humanization and affinity maturation of a non-human antibody in a rapid and efficient method that increases binding affinity significantly over other methods. This method involves the simultaneous or sequential modification of the CDRs and framework regions of the parent antibody molecule by generating a library of randomly substituted CDRs and/or framework regions, and screening for high affinity molecules.

Brief Description of the Figures

[0028]

Figure 1 is a schematic representation of the phage vector used in antibody cloning and screening.

Figure 2 is a schematic representation of oligonucleotides useful in generating antibody variants.

Figure 3A depicts the comparison of the light chains of the murine anti-IgE antibody TES-C21 and the combined human template of L16 and JK4.

Figure 3B depicts the comparison of the heavy chains of TES-C21 and the combined human template DP88 and JH4b.

Figure 4 presents a table of the framework residue variants having high affinity as compared to the parent TES-C21.

Figure 5A and B depict the ELISA titration curves for clones 4, 49, 72, 78, and 136, as compared to the parent Fab of TES-C21 and negative control (5D12).

Figure 6 depicts an inhibition assay of clones 2C, 5A, and 5I, as compared to the parent TES-C21 and a negative control antibody.

Figure 7A depicts the sequences of clones having a combination of beneficial mutations which resulted in even greater affinity for IgE.

Figure 8A & 8B depict the framework sequences of the entire light chain variable region for clones 136, 1, 2, 4, 8, 13, 15, 21, 30, 31, 35, 43, 44, 53, 81, 90, and 113.

Figure 9A & 9B depict the framework sequences of the entire heavy chain variable region for 35 clones.

Figures 10A-F depict the complete heavy and light chain sequences for clones 136, 2C, 5I, 5A, 2B, and 1136-2C.

Detailed Description of the Invention

Definitions

[0029] Terms used throughout this application are to be construed with ordinary and typical meaning to those of ordinary skill in the art. However, Applicants desire that the following terms be given the particular definition as defined below.

[0030] The phrase "substantially identical" with respect to an antibody chain polypeptide sequence may be construed as an antibody chain exhibiting at least 70%, or 80%, or 90% or 95% sequence identity to the reference polypeptide sequence. The term with respect to a nucleic acid sequence may be construed as a sequence of nucleotides exhibiting at least about 85%, or 90%, or 95% or 97% sequence identity to the reference nucleic acid sequence.

[0031] The term "identity" or "homology" shall be construed to mean the percentage of amino acid residues in the candidate sequence that are identical with the residue of a corresponding sequence to which it is compared, after aligning the sequences and introducing gaps, if necessary to achieve the maximum percent identity for the entire sequence, and not considering any conservative substitutions as part of the sequence identity. Neither N- or C-terminal extensions nor insertions shall be construed as reducing identity or homology. Methods and computer programs for the alignment are well known in the art. Sequence identity may be measured using sequence analysis software.

[0032] The term "antibody" is used in the broadest sense, and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, and multispecific antibodies (e.g., bispecific antibodies). Antibodies (Abs) and immunoglobulins (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific target, immunoglobulins include both antibodies and other antibody-like molecules which lack target specificity. Native antibodies and immunoglobulins are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end.

[0033] As used herein, "anti-human IgE antibody" means an antibody which binds to human IgE in such a manner so as to inhibit or substantially reduce the binding of such IgE to the high affinity receptor, $Fc\epsilon R I$.

[0034] The term "variable" in the context of variable domain of antibodies, refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular target. However, the variability is not evenly distributed through the variable domains of antibodies. It is concentrated in three segments called complementarity determining regions (CDRs) also known as hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of variable domains are called the framework (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a β -sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the β -sheet structure. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the target binding site of antibodies (see Kabat et al.) As used herein, numbering of immunoglobulin amino acid residues is done according to the immunoglobulin amino acid residue numbering system of Kabat et al., (Sequences of Proteins of Immunological Interest, National Institute of Health, Bethesda, Md. 1987), unless otherwise indicated.

[0035] The term "antibody fragment" refers to a portion of a full-length antibody, generally the target binding or variable region. Examples of antibody fragments include Fab, Fab', F(ab')₂ and Fv fragments. The phrase "functional fragment or analog" of an antibody is a compound having qualitative biological activity in common with a full-length antibody. For example, a functional fragment or analog of an anti-IgE antibody is one which can bind to an IgE immunoglobulin in such a manner so as to prevent or substantially reduce the ability of such molecule from having the ability to bind to the high affinity receptor, $Fc\epsilon R I$. As used herein, "functional fragment" with respect to antibodies, refers to Fv, F(ab) and F(ab')₂ fragments. An "Fv" fragment is the minimum antibody fragment which contains a complete target recognition and binding site. This region consists of a dimer of one heavy and one light chain variable domain in a tight, non-covalent association (V_H - V_L dimer). It is in this configuration that the three CDRs of each variable domain interact to define an target binding site on the surface of the V_H - V_L dimer. Collectively, the six CDRs confer target binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an target) has the ability to recognize and bind target, although at a lower affinity than the entire binding site. "Single-chain Fv" or "sFv" antibody fragments comprise the V_H and V_L domains of an antibody, wherein these domains are

present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for target binding.

[0036] The Fab fragment contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxyl terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. F(ab') fragments are produced by cleavage of the disulfide bond at the hinge cysteines of the F(ab')₂ pepsin digestion product. Additional chemical couplings of antibody fragments are known to those of ordinary skill in the art.

[0037] The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single target site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the target. In addition to their specificity, monoclonal antibodies are advantageous in that they may be synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies for use with the present invention may be isolated from phage antibody libraries using the well known techniques. The parent monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler and Milstein, *Nature* 256,495 (1975), or may be made by recombinant methods.

[0038] "Humanized" forms of non-human (e.g. murine) antibodies are chimeric Immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')₂ or other target-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody may also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin template chosen.

[0039] The terms "cell", "cell line" and "cell culture" include progeny. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Variant progeny that have the same function or biological property, as screened for in the originally transformed cell, are included. The "host cells" used in the present invention generally are prokaryotic or eukaryotic hosts.

[0040] "Transformation" of a cellular organism with DNA means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integration. "Transfection" of a cellular organism with DNA refers to the taking up of DNA, e.g., an expression vector, by the cell or organism whether or not any coding sequences are in fact expressed. The terms "transfected host cell" and "transformed" refer to a cell in which DNA was introduced. The cell is termed "host cell" and it may be either prokaryotic or eukaryotic. Typical prokaryotic host cells include various strains of *E. coli*. Typical eukaryotic host cells are mammalian, such as Chinese hamster ovary or cells of human origin. The introduced DNA sequence may be from the same species as the host cell of a different species from the host cell, or it may be a hybrid DNA sequence, containing some foreign and some homologous DNA.

[0041] The term "vector" means a DNA construct containing a DNA sequence which is operably linked to a suitable control sequence capable of effecting the expression of the DNA in a suitable host. Such control sequences include a promoter to effect transcription, an optional operator sequence to control such transcription, a sequence encoding suitable mRNA ribosome binding sites, and sequences which control the termination of transcription and translation. The vector may be a plasmid, a phage particle, or simply a potential genomic insert. Once transformed into a suitable host, the vector may replicate and function independently of the host genome, or may in some instances, integrate into the genome itself. In the present specification, "plasmid" and "vector" are sometimes used interchangeably, as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of vectors which serve equivalent function as and which are, or become, known in the art.

[0042] The expression "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation

signals, and enhancers. DNA for a presequence or secretory leader may be operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous.

[0043] "Mammal" for purposes of treatment refers to any animal classified as a mammal, including human, domestic and farm animals, nonhuman primates, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc.

[0044] The term "epitope tagged" when used herein in the context of a polypeptide refers to a polypeptide fused to an "epitope tag". The epitope tag polypeptide has enough residues to provide an epitope against which an antibody can be made, yet is short enough such that it does not interfere with activity of the polypeptide. The epitope tag preferably also is fairly unique so that the antibody does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least 6 amino acid residues and usually between about 8-50 amino acid residues (preferably between about 9-30 residues). Examples include the flu HA tag polypeptide and its antibody 12CA5 (Field et al, Mol Cell. Biol. 8: 2159-2165 (1988)); the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereagainst (Evan et al., Mol Cell. Biol. 5(12): 3610-3616 (1985)); and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody (Paborsky et al., Protein Engineering 3(6): 547-553 (1990)). In certain embodiments, the epitope tag may be an epitope of the Fc region of an IgG molecule (e.g., IgG1, IgG2, IgG3 or IgG4) that is responsible for increasing the *in vivo* serum half-life of the IgG molecule.

[0045] The word "label" when used herein refers to a detectable compound or composition which can be conjugated directly or indirectly to a molecule or protein, e.g., an antibody. The label may itself be detectable (e.g., radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable.

[0046] As used herein, "solid phase" means a non-aqueous matrix to which the antibody of the present invention, can adhere. Examples of solid phases encompassed herein include those formed partially or entirely of glass (e.g. controlled pore glass), polysaccharides (e.g., agarose), polyacrylamides, polystyrene, polyvinyl alcohol and silicones. In certain embodiments, depending on the context, the solid phase can comprise the well of an assay plate; in others it is a purification column (e.g. an affinity chromatography column).

[0047] As used herein, the term "IgE-modulated disorder" means a condition or disease which is characterized by the overproduction and/or hypersensitivity to the immunoglobulin IgE. Specifically it would be construed to include conditions associated with anaphylactic hypersensitivity and atopic allergies, including for example: asthma, allergic rhinitis & conjunctivitis (hay fever), eczema, urticaria, atopic dermatitis, and food allergies. The serious physiological condition of anaphylactic shock caused by, e.g., bee stings, snake bites, food or medication, is also encompassed under the scope of this term.

Generation of Antibodies

[0048] The starting or "parent" antibody may be prepared using techniques available in the art for generating such antibodies. These techniques are well known. Exemplary methods for generating the starting antibody are described in more detail in the following sections.

[0049] The antibody's binding affinity is determined prior to generating a high affinity antibody of the present invention. Also, the antibody may be subjected to other biological activity assays, e.g., in order to evaluate effectiveness as a therapeutic. Such assays are known in the art and depend on the target target and intended use for the antibody.

[0050] To screen for antibodies which bind to a particular epitope (e.g., those which block binding of IgE to its high affinity receptor), a routine cross-blocking assay such as that described in "Antibodies: A Laboratory Manual" (Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988)) can be performed. Alternatively, epitope mapping can be performed to determine where the antibody binds an epitope of interest. Optionally, the binding affinity of the antibody for a homolog of the target used to generate the antibody (where the homolog is from a different species) may be assessed using techniques known in the art. As described herein, the other species is a nonhuman mammal to which the antibody will be administered in preclinical studies. Accordingly, the species may be a nonhuman primate, such as rhesus, cynomolgus, baboon, chimpanzee and macaque. Specifically, the species may be a rodent, cat or dog, for example.

[0051] The parent antibody is altered according to the present invention so as to generate an antibody which has a higher or stronger binding affinity for the target than the parent antibody. The resulting high affinity antibody preferably has a binding affinity for the target which is at least about 10 fold higher, or at least about 20 fold higher, or at least about 500 fold higher or may be 1000 to 5000 fold higher, than the binding affinity of the parent antibody for the target. The degree of enhancement in binding affinity necessary or desired will depend on the initial binding affinity of the parent antibody.

[0052] In general, the method for making high affinity antibodies from a parent antibody involves the following steps:

1. 1. Obtaining or selecting a parent antibody which binds the target of interest, which comprises heavy and light chain variable domains. This may be done by traditional hybridoma techniques, phage-display techniques, or any other method of generating a target specific antibody.
2. 2. Selecting a framework sequence which is close in sequence to the parent framework, preferably a human template sequence. This template may be chosen based on, e.g., its comparative overall length, the size of the CDRs, the amino acid residues located at the junction between the framework and the CDRs, overall homology, etc. The template chosen can be a mixture of more than one sequence or may be a consensus template.
3. 3. Generating a library of clones by making random amino acid substitutions at each and every possible CDR position. One may also randomly substitute the amino acids in the human framework template that are, e.g., adjacent to the CDRs or that may affect binding or folding, with all possible amino acids, generating a library of framework substitutions. These framework substitutions can be assessed for their potential effect on target binding and antibody folding. The substitution of amino acids in the framework may be done either simultaneously or sequentially with the substitution of the amino acids in the CDRs. One method for generating the library of variants by oligonucleotide synthesis.
4. 4. Constructing an expression vector comprising the heavy and/or light chain variants generated in step (3) which may comprise the formulas: FRH1-CDRH1-FRH2-CDRH2-FRH3-CDRH3-FRH4(I) and FRL1-CDRL9-FRL2-CDRL2-FRL3-CDRL3-FRL4 (II), wherein FRL1, FRL2, FRL3, FRL4, FRH1, FRH2, FRH3 and FRH4 represent the variants of the framework template light chain and heavy chain sequences chosen in step 3 and the CDRs represent the variant CDRs of the parent antibody CDRs. An example of a vector containing such light and heavy chain sequences is depicted in Figure 1.
5. 5. Screening the library of clones against the specific target and those clones that bind the target are screened for improved binding affinity. Those clones that bind with greater affinity than the parent molecule may be selected. The optimal high affinity candidate will have the greatest binding affinity possible compared to the parent antibody, preferably greater than 20 fold, 100 fold, 1000 fold or 5000 fold. If the chosen variant contains certain amino acids that are undesirable, such as a glycosylation site that has been introduced or a potentially immunogenic site, those amino acids may be replaced with more beneficial amino acid residues and the binding affinity reassessed.

[0053] One may also use this method to generate high affinity antibodies from a fully human parent antibody by randomly substituting only the CDR regions, leaving the human framework intact.

[0054] Due to improved high throughput screening techniques and vectors such as the one depicted in Figure 1, an artisan can rapidly and efficiently screen a comprehensive library of substitutions at all sites in a given CDR and/or framework region. By randomly substituting all amino acids at all positions simultaneously, one is able to screen possible combinations that significantly increase affinity that would not have been anticipated or identified by individual substitution due to, e.g., synergy.

PARENT ANTIBODY PREPARATION

A. Target Preparation

[0055] Soluble targets or fragments thereof can be used as immunogens for generating antibodies. The antibody is directed against the target of interest. Preferably, the target is a biologically important polypeptide and administration of the antibody to a mammal suffering from a disease or disorder can result in a therapeutic benefit in that mammal. However, antibodies may be directed against nonpolypeptide targets. Where the target is a polypeptide, it may be a transmembrane molecule (e.g. receptor) or ligand such as a growth factor. The target of the present invention is IgE. Whole cells may be used as the immunogen for making antibodies. The target may be produced recombinantly or made using synthetic methods. The target may also be isolated from a natural source.

[0056] For transmembrane molecules, such as receptors, fragments of these (e.g. the extracellular domain of a receptor) can be used as the immunogen. Alternatively, cells expressing the transmembrane molecule can be used as the immunogen. Such cells can be derived from a natural source (e.g. mast cell lines) or may be cells which have been transformed by recombinant techniques to express the transmembrane molecule. Other targets and forms thereof useful for preparing antibodies will be apparent to those in the art.

B. Polyclonal Antibodies

[0057] Polyclonal antibodies are usually generated in non-human mammals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant target in combination with an adjuvant. It may be useful to conjugate the relevant target to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin. Numerous agents capable of eliciting an immunological response are well known in the art.

[0058] Animals are immunized against the target, immunogenic conjugates, or derivatives by combining the protein or conjugate (for rabbits or mice, respectively) with Freund's complete adjuvant and injecting the solution intradermally. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's incomplete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus.

[0059] The mammalian antibody selected will normally have a sufficiently strong binding affinity for the target. For example, the antibody may bind the human anti-IgE target with a binding affinity (Kd) value of about 1×10^{-8} M. Antibody affinities may be determined by saturation binding; enzyme-linked immunoabsorbant assay (ELISA); and competition assays (e.g., radioimmunoassays).

[0060] To screen for antibodies that bind the target of interest, a routine crosslinking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988) can be performed. Alternatively, epitope mapping, e.g., as described in Champe, et al. *J. Biol. Chem.* 270: 1388-1394 (1995), can be performed to determine binding.

C. Monoclonal Antibodies

[0061] Monoclonal antibodies are antibodies which recognize a single antigenic site. Their uniform specificity makes monoclonal antibodies much more useful than polyclonal antibodies, which usually contain antibodies that recognize a variety of different antigenic sites. Monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature*, 256: 495 (1975), or may be made by recombinant DNA methods.

[0062] In the hybridoma method, a mouse or other appropriate host animal, such as a rodent, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 590-103 (Academic Press, 1986)).

[0063] The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), substances which prevent the growth of HGPRT-deficient cells. Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Human myeloma and mouse-human heteromyeloma cell lines have been described for the production of human monoclonal antibodies (Kozbar, *J. Immunol.* 133: 3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

[0064] After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and*

Practice, pp. 59-103, Academic Press, 1986)). Suitable culture media for this purpose include. The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

[0065] DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). The hybridoma cells serve as a source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transferred into host cells such as *E. coli* cells, NS0 cells, Chinese hamster ovary (CHO) cells, or myeloma cells to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; Morrison et al., Proc. Natl. Acad. Sci. USA 81: 6851 (1984)), or by covalently joining to the immunoglobulin polypeptide.

D. Humanized Antibodies

[0066] Humanization is a technique for making a chimeric antibody wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. A humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., Nature 321: 522-525 (1986); Riechman et al., Nature 332: 323-327 (1988); Verhoeyens et al., Science 239: 1534-1536 (1988)), by substituting non-human CDR's or CDR sequences for the corresponding sequences in a human antibody (See, e.g., U.S. Pat. No. 4,816,567). As practiced in the present invention, the humanized antibody may have some CDR residues and some FR residues substituted by residues from analogous sites in murine antibodies.

[0067] The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best fit" method, the sequence of the variable domain of a non-human antibody is compared with the library of known human variable-domain sequences. The human sequence which is closest to that of the non-human parent antibody is then accepted as the human framework for the humanized antibody (Sims et al., J. Immunol. 151: 2296 (1993); Chothia et al., J. Mol. Biol. 196: 901 (1987)). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., Proc. Natl. Acad. Sci. USA, 89: 4285 (1992); Presta et al., J. Immunol. 151: 2623 (1993)).

E. Antibody Fragments

[0068] Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., Journal of Biochemical and Biophysical Methods 24: 107-117 (1992) and Brennan et al., Science 229: 81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from an antibody phage library. Alternatively, F(ab')₂-SH fragments can be directly recovered from *E. coli* and chemically coupled to form F(ab')₂ fragments (Carter et al., Bio/Technology 10: 163-167 (1992)). According to another approach, F(ab')₂ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). (PCT patent application WO 93/16185).

PREPARATION OF HIGH AFFINITY ANTIBODIES

[0069] Once the parent antibody has been identified and isolated, one or more amino acid residues are altered in one or more of the variable regions of the parent antibody. Alternatively, or in addition, one or more substitutions of framework residues may be introduced in the parent antibody where these result in an improvement in the binding affinity of the antibody, for example, for human IgE. Examples of framework region residues to modify include those which non-covalently bind target directly (Amit et al.

Science 233: 747-753 (1986)); interact with/effect the conformation of CDR (Chothia et al. J. Mol. Biol. 196: 901-917 (1987)); and/or participate in the VL-VH Interface (EP 239 400.B1). In certain embodiments, modification of one or more of such framework region residues results in an enhancement of the binding affinity of the antibody for the target of interest.

[0070] Modifications in the antibodies' biological properties may be accomplished by selecting substitutions that differ significantly in their effect on maintaining, e.g., (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation; (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

[0071] Nucleic acid molecules encoding amino acid sequence variants are prepared by a variety of methods known in the art. These methods include, oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non variant version of the species-dependent antibody. The preferred method for generating variants is an oligonucleotide-mediated synthesis. As described herein, the antibody variant will only have a single hypervariable region residue substituted, e.g. from about two to about fifteen hypervariable region substitutions.

[0072] One method for generating the library of variants is by oligonucleotide mediated synthesis according to the scheme depicted in Figure 2. Three oligonucleotides of approximately 100 nucleotides each may be synthesized spanning the entire light chain or heavy chain variable region. Each oligonucleotide may comprise: (1) a 60 amino acid stretch generated by the triplet (NNK)₂₀ where N is any nucleotide and K is G or T, and (2) an approximately 15-30 nucleotide overlap with either the next oligo or with the vector sequence at each end. Upon annealing of these three oligonucleotides in a PCR reaction, the polymerase will fill in the opposite strand generating a complete double stranded heavy chain or light chain variable region sequence. The number of triplets may be adjusted to any length of repeats and their position within the oligonucleotide may be chosen so as to only substitute amino acids in a given CDR or framework region. By using (NNK), all twenty amino acids are possible at each position in the encoded variants. The overlapping sequence of 5-10 amino acids (15-30 nucleotides) will not be substituted, but this may be chosen to fall within the stacking regions of the framework, or may substituted by a separate or subsequent round of synthesis. Methods for synthesizing oligonucleotides are well known in the art and are also commercially available. Methods for generating the antibody variants from these oligonucleotides are also well known in the art, e.g., PCR.

[0073] The library of heavy and light chain variants, differing at random positions in their sequence, can be constructed in a any expression vector, such as a bacteriophage, specifically the vector of Fig.1, each of which contains DNA encoding a particular heavy and light chain variant

[0074] Following production of the antibody variants, the biological activity of variant relative to the parent antibody is determined. As noted above, this involves determining the binding affinity of the variant for the target. Numerous high-throughput methods exist for rapidly screen antibody variants for their ability to bind the target of interest.

[0075] One or more of the antibody variants selected from this initial screen may then be screened for enhanced binding affinity relative to the parent antibody. One common method for determining binding affinity is by assessing the association and dissociation rate constants using a BIACore™ surface plasmon resonance system (BIACore, Inc.). A biosensor chip is activated for covalent coupling of the target according to the manufacturer's (BIACore) instructions. The target is then diluted and injected over the chip to obtain a signal in response units (RU) of immobilized material. Since the signal in RU is proportional to the mass of immobilized material, this represents a range of immobilized target densities on the matrix. Dissociation data are fit to a one-site model to obtain k_{off} +/- s.d. (standard deviation of measurements). Pseudo-first order rate constant (k_s) are calculated for each association curve, and plotted as a function of protein concentration to obtain k_{on} +/- s.e. (standard error of fit). Equilibrium dissociation constants for binding, K_d 's, are calculated from SPR measurements as k_{off}/k_{on} . Since the equilibrium dissociation constant, K_d , is inversely proportional to k_{off} , an estimate of affinity improvement can be made assuming the association rate (k_{on}) is a constant for all variants.

[0076] The resulting candidate(s) with high affinity may optionally be subjected to one or more further biological activity assays to confirm that the antibody variant(s) with enhanced binding affinity still retain the desired therapeutic attributes. For example, in the case of an anti-IgE antibody, one may screen for those that block binding of IgE to its receptor and inhibit the release of histamine. The optimal antibody variant retains the ability to bind the target with a binding affinity significantly higher than the parent antibody.

[0077] The antibody variant(s) so selected may be subjected to further modifications oftentimes depending upon the intended use of the antibody. Such modifications may involve further alteration of the amino acid sequence, fusion to heterologous polypeptide(s) and/or covalent modifications such as those elaborated below. For example, any cysteines residues not involved in

maintaining the proper conformation of the antibody variant may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant cross linking. Conversely, (a) cysteine bond(s) may be added to the antibody to improve its stability (particularly where the antibody is an antibody fragment such as an Fv fragment).

VECTORS

[0078] The invention also provides isolated nucleic acid encoding an antibody variant as disclosed herein, vectors and host cells comprising the nucleic acid, and recombinant techniques for the production of the antibody variant. For recombinant production of the antibody variant, the nucleic acid encoding it is isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. DNA encoding the antibody variant is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody variant).

[0079] Many vectors are available. The vector components generally include one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

[0080] The phage expression vector depicted in Figure 1 is comprised of a commonly used M13 vector and M13's own gene III viral secretion signal for rapid secretion and screening variant Fabs for proper binding specificity and minimal affinity criteria. This vector does not use the entire gene III sequence, so there is no display on the surface of the bacterial cell, but rather the Fabs are secreted into the periplasmic space. Alternatively, the Fabs could be expressed in the cytoplasm and isolated. The heavy and light chains each have their own viral secretion signal, but are dependently expressed from a single strong inducible promoter.

[0081] The vector in Figure 1 also provides a His tag and a myc tag for easy purification, as well as detection. A skilled artisan would recognize that the Fabs could be independently expressed from separate promoters or that the secretion signal need not be the viral sequence chosen, but could be a prokaryotic or eukaryotic signal sequence suitable for the secretion of the antibody fragments from the chosen host cell. It should also be recognized that the heavy and light chains may reside on different vectors.

A. Signal Sequence Component

[0082] The antibody variant of this invention may be produced recombinantly. The variant may also be expressed as a fusion polypeptide fused with a heterologous polypeptide, which is preferably a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. The heterologous signal sequence selected preferably is one that is recognized and processed (i.e., cleaved by signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process the native antibody signal sequence, the signal sequence may be substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. Or in the case of the vector of Figure 1, the signal sequence chosen was a viral signal sequence from gene III. For yeast secretion the native signal sequence may be substituted by, e.g., the yeast invertase leader, α -factor leader (including *Saccharomyces* and *Kluyveromyces* α -factor leaders), or acid phosphatase leader, the *C. albicans* glucoamylase leader, or a signal described in e.g., WO 90/13646. In mammalian cell expression, mammalian signal sequences as well as viral secretory leaders, for example, the herpes simplex gD signal, are available. The DNA for such precursor region is ligated in reading frame to DNA encoding the antibody variant.

B. Origin of Replication Component

[0083] Vectors usually contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

C. Selection Gene Component

[0084] Vectors may contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for Bacilli.

[0085] One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin, mycophenolic acid and hygromycin.

[0086] Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the antibody nucleic acid, such as DHFR, thymidine kinase, metallothionein-I and -II preferably primate metallothionein genes, adenosine deaminase, ornithine decarboxylase, etc.

[0087] For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contain methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity.

[0088] Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding antibody, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3'-phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. (U.S. Pat. No. 4,965,199).

[0089] A suitable selection gene for use in yeast is the trp1 gene present in the yeast plasmid Yrp7 (Stinchcomb et al., *Nature* 282: 39 (1979)). The trp1 gene provides a selection marker for a variant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1. Jones, *Genetics* 85: 12 (1977). The presence of the trp1 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, Leu2-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the Leu2 gene.

D. Promoter Component

[0090] Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the antibody nucleic acid. Promoters suitable for use with prokaryotic hosts include the phoA promoter, β -lactamase and lactose promoter systems, alkaline phosphatase, a tryptophan (trp) promoter system, and hybrid promoters such as the tac promoter. However, other known bacterial promoters are suitable. Promoters for use in bacterial systems may also contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding the antibody.

[0091] Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CCAAT region where N may be any nucleotides. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

[0092] Examples of suitable promotor sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase or other glycolytic enzymes, such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

[0093] Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytchrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657. Yeast enhancers also are advantageously used with yeast promoters.

[0094] Antibody transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the

genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian virus 40 (SV40), from heterologous mammalian promoters, e.g., the actin promoter or an Immunoglobulin promoter, from heat-shock promoters-provided such promoters are compatible with the host cell systems.

[0095] The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Pat No. 4,419,446. A modification of this system is described in U.S. Pat. No. 4,601,978. Alternatively, human β -interferon cDNA has been expressed in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus. Alternatively, the rous sarcoma virus long terminal repeat can be used as the promoter.

E. Enhancer Element Component

[0096] Transcription of a DNA encoding the antibody of this invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α -fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, *Nature* 297: 17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the antibody-encoding sequence, but is preferably located at a site 5' from the promoter.

F. Transcription Termination Component

[0097] Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) may also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding the antibody. One useful transcription termination component is the bovine growth hormone polyadenylation region. See e.g., WO94/11026.

SELECTION AND TRANSFORMATION OF HOST CELLS

[0098] Suitable host cells for cloning or expressing the DNA in the vectors herein are prokaryotic, yeast, or higher eukaryotic cells. Suitable prokaryotes for this purpose include both Gram-negative and Gram-positive organisms, for example, Enterobacteria such as *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, *Serratia*, and *Shigella*, as well as *Bacilli*, *Pseudomonas*, and *Streptomyces*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli*-X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting.

[0099] In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for antibody-encoding vectors. *Saccharomyces cerevisiae* is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe*; *Kluyveromyces*; *Candida*; *Trichoderma*; *Neurospora crassa*; and filamentous fungi such as e.g., *Neurospora*, *Penicillium*, *Tolypocladium*, and *Aspergillus* hosts, such as *A. nidulans* and *A. niger*.

[0100] Suitable host cells for the expression of glycosylated antibodies are derived from multicellular organisms. In principal, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells, Luckow et al., *Bio/Technology* 6, 47-55 (1988); Miller et al., *Genetic Engineering*, Setlow et al. eds. Vol. 8, pp. 277-279 (Plenum publishing 1986); Mseda et al., *Nature* 315, 592-594 (1985). Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells. Moreover, plant cells

cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco and also be utilized as hosts.

[0101] Vertebrate cells, and propagation of vertebrate cells, in culture (tissue culture) has become a routine procedure. See *Tissue Culture*, Academic Press, Kruse and Patterson, eds. (1973). Examples of useful mammalian host cell lines are monkey kidney; human embryonic kidney line; baby hamster kidney cells; Chinese hamster ovary cells/-DHFR (CHO, Urlaub et al., *Proc. Natl. Acad. Sci. USA* 77: 4216 (1980)); mouse sertoli cells; human cervical carcinoma cells (HELA); canine kidney cells; human lung cells; human liver cells; mouse mammary tumor, and NS0 cells.

[0102] Host cells are transformed with the above-described vectors for antibody production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

[0103] The host cells used to produce the antibody variant of this invention may be cultured in a variety of media. Commercially available media such as Harn's F10 (Sigma), Minimal Essential Medium (MEM, Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium (DMEM, Sigma) are suitable for culturing host cells. In addition, any of the media described in Ham et al., *Meth. Enzymol.* 58: 44 (1979), Barnes et al., *Anal. Biochem.* 102: 255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,560,655; 5,122,469; 5,712,163; or 6,048,728 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as X-chlorides, where X is sodium, calcium, magnesium; and phosphates), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

ANTIBODY PURIFICATION

[0104] When using recombinant techniques, the antibody variant can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody variant is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, may be removed, for example, by centrifugation or ultrafiltration. Carter et al., *Bio/Technology* 10: 163-167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli*. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 minutes. Cell debris can be removed by centrifugation. Where the antibody variant is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

[0105] The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody variant. Protein A can be used to purify antibodies that are based on human IgG1, IgG2 or IgG4 heavy chains (Lindmark et al., *J. Immunol Meth.* 62: 1-13 (1983)). Protein G is recommended for all mouse isotypes and for human IgG3 (Guss et al., *EMBO J.* 5: 1567-1575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrene divinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody variant comprises a CH3 domain, the Bakerbond ABX™ resin (J. T. Baker, Phillipsburg, N.J.) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation. Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody variant to be recovered.

[0106] Following any preliminary purification step(s), the mixture comprising the antibody variant of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (e.g., from about 0-0.25M salt).

PHARMACEUTICAL FORMULATIONS

[0107] Therapeutic formulations of the polypeptide or antibody may be prepared for storage as lyophilized formulations or aqueous solutions by mixing the polypeptide having the desired degree of purity with optional "pharmaceutically-acceptable" carriers, excipients or stabilizers typically employed in the art (all of which are termed "excipients"). For example, buffering agents, stabilizing agents, preservatives, isotonicifiers, non-ionic detergents, antioxidants and other miscellaneous additives. (See Remington's Pharmaceutical Sciences, 16th edition, A. Osol, Ed. (1980)). Such additives must be nontoxic to the recipients at the dosages and concentrations employed.

[0108] Buffering agents help to maintain the pH in the range which approximates physiological conditions. They are preferably present at concentration ranging from about 2 mM to about 50 mM. Suitable buffering agents for use with the present invention include both organic and inorganic acids and salts thereof such as citrate buffers (e.g., monosodium citrate-disodium citrate mixture, citric acid-trisodium citrate mixture, citric acid-monosodium citrate mixture, etc.), succinate buffers (e.g., succinic acid-monosodium succinate mixture, succinic acid-sodium hydroxide mixture, succinic acid-disodium succinate mixture, etc.), tartrate buffers (e.g., tartaric acid-sodium tartrate mixture, tartaric acid-potassium tartrate mixture, tartaric acid-sodium hydroxide mixture, etc.), fumarate buffers (e.g., fumaric acid-monosodium fumarate mixture, etc.), fumarate buffers (e.g., fumaric acid-monosodium fumarate mixture, fumaric acid-disodium fumarate mixture, monosodium fumarate-disodium fumarate mixture, etc.), gluconate buffers (e.g., gluconic acid-sodium glycinate mixture, gluconic acid-sodium hydroxide mixture, gluconic acid-potassium glycinate mixture, etc.), oxalate buffer (e.g., oxalic acid-sodium oxalate mixture, oxalic acid-sodium hydroxide mixture, oxalic acid-potassium oxalate mixture, etc.), lactate buffers (e.g., lactic acid-sodium lactate mixture, lactic acid-sodium hydroxide mixture, lactic acid-potassium lactate mixture, etc.) and acetate buffers (e.g., acetic acid-sodium acetate mixture, acetic acid-sodium hydroxide mixture, etc.). Additionally, there may be mentioned phosphate buffers, histidine buffers and trimethylamine salts such as Tris.

[0109] Preservatives may be added to retard microbial growth, and may be added in amounts ranging from 0.2%-1% (w/v). Suitable preservatives for use with the present invention include phenol, benzyl alcohol, meta-cresol, methyl paraben, propyl paraben, octadecyldimethylbenzyl ammonium chloride, benzalconium halides (e.g., chloride, bromide, iodide), hexamethonium chloride, alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol, and 3-pentanol.

[0110] Isotonicifiers sometimes known as "stabilizers" may be added to ensure isotonicity of liquid compositions of the present invention and include polyhydric sugar alcohols, preferably trihydric or higher sugar alcohols, such as glycerin, erythritol, arabitol, xylitol, sorbitol and mannitol.

[0111] Stabilizers refer to a broad category of excipients which can range in function from a bulking agent to an additive which solubilizes the therapeutic agent or helps to prevent denaturation or adherence to the container wall. Typical stabilizers can be polyhydric sugar alcohols (enumerated above); amino acids such as arginine, lysine, glycine, glutamine, asparagine, histidine, alanine, ornithine, L-leucine, 2-phenylalanine, glutamic acid, threonine, etc., organic sugars or sugar alcohols, such as lactose, trehalose, stachyose, mannitol, sorbitol, xylitol, ribitol, myoinositol, galactitol, glycerol and the like, including cyclitols such as inositol; polyethylene glycol; amino acid polymers; sulfur containing reducing agents, such as urea, glutathione, thioctic acid, sodium thioglycolate, thioglycerol, .alpha.-monothioglycerol and sodium thio sulfate; low molecular weight polypeptides (i.e. <10 residues); proteins such as human serum albumin, bovine serum albumin, gelatin or immunoglobulins; hydrophylic polymers, such as polyvinylpyrrolidone monosaccharides, such as xylose, mannose, fructose, glucose; disaccharides such as lactose, maltose, sucrose and trisaccacharides such as raffinose; polysaccharides such as dextran. Stabilizers may be present in the range from 0.1 to 10,000 weights per part of weight active protein.

[0112] Non-ionic surfactants or detergents (also known as "wetting agents") may be added to help solubilize the therapeutic agent as well as to protect the therapeutic protein against agitation-induced aggregation, which also permits the formulation to be exposed to shear surface stressed without causing denaturation of the protein. Suitable non-ionic surfactants include polysorbates (20,80, etc.), polyoxamers (184,188 etc.), Pluronic.RTM. polyols, polyoxyethylene sorbitan monoethers (Tween.RTM.-20, Tween.RTM.-80, etc.). Non-ionic surfactants may be present in a range of about 0.05 mg/ml to about 1.0 mg/ml, preferably about 0.07 mg/ml to about 0.2 mg/ml.

[0113] Additional miscellaneous excipients include bulking agents, (e.g. starch), chelating agents (e.g. EDTA), antioxidants (e.g., ascorbic acid, methionine, vitamin E), and cosolvents. The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desireable to further provide an immunosuppressive agent. Such molecules are suitably present in combination in amounts that are effective for the purpose intended. The active ingredients may also be entrapped in microcapsule prepared, for example, by coacervation techniques or by interfacial polymerization, for example,

hydroxymethylsilulose or gelatin-microcapsule and poly-(methylmethacrylate) microcapsule, respectively, in colloidal drug delivery systems (for example, liposomes, albumin micropheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, 16th edition, A. Osal, Ed. (1980).

[0114] The formulations to be used for in vivo administration must be sterile. This is readily accomplished, for example, by filtration through sterile filtration membranes. Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semi-permeable matrices of solid hydrophobic polymers containing the antibody variant, which matrices are in the form of shaped articles, e.g., films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37° C resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulphydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

[0115] The amount of therapeutic polypeptides, antibody or fragment thereof which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. Where possible, it is desireable to determine the dose-response curve and the pharmaceutical compositions of the Invention first in vitro, and then in useful animal model systems prior to testing in humans.

[0116] Preferably, an aqueous solution of therapeutic polypeptide, antibody or fragment thereof is administered by subcutaneous injection. Each dose may range from about 0.5 µg to about 50 µg per kilogram of body weight, or more preferably, from about 3 µg to about 30 µg per kilogram body weight.

[0117] The dosing schedule for subcutaneous administration may vary from once a month to daily depending on a number of clinical factors, including the type of disease, severity of disease, and the subject's sensitivity to the therapeutic agent.

USES FOR THE ANTIBODY VARIANT

[0118] The antibody variants of the Invention may be used as affinity purification agents. In this process, the antibodies are immobilized on a solid phase such as SEPHADEX™ resin or filter paper, using methods well known in the art. The immobilized antibody variant is contacted with a sample containing the target to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the target to be purified, which is bound to the immobilized antibody variant. Finally, the support is washed with another suitable solvent, such as glycine buffer, that will release the target from the antibody variant.

[0119] The variant antibodies may also be useful in diagnostic assays, e.g., for detecting expression of a target of interest in specific cells, tissues, or serum. For diagnostic applications, the antibody variant typically will be labeled with a detectable moiety. Numerous labels are available. Techniques for quantifying a change in fluorescence are described above. The chemiluminescent substrate becomes electronically excited by a chemical reaction and may then emit light which can be measured (using a chemiluminometer, for example) or donates energy to a fluorescent acceptor. Examples of enzymatic labels include luciferases (e.g., firefly luciferase and bacterial luciferase; U.S. Pat. No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, malate dehydrogenase, urease, peroxidase such as horseradish peroxidase (HRPO), alkaline phosphatase, beta-galactosidase, glucoamylase, lysozyme, saccharide oxidases (e.g., glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase), heterocyclic oxidases (such as uricase and xanthine oxidase), lactoperoxidase, microperoxidase, and the like. Techniques for conjugating enzymes to antibodies are described in O'Sullivan et al., Methods for the Preparation of Enzyme-Antibody Conjugates for Use in Enzyme Immunoassay, in Methods in Enzym. (Ed. J. Langone & H. Van Yunakis), Academic press, New York, 73: 147-166 (1981).

[0120] Sometimes, the label is indirectly conjugated with the antibody variant. The skilled artisan will be aware of various techniques for achieving this. For example, the antibody variant can be conjugated with biotin and any of the three broad

categories of labels mentioned above can be conjugated with avidin, or vice versa. Biotin binds selectively to avidin and thus, the label can be conjugated with the antibody variant in this Indirect manner. Alternatively, to achieve Indirect conjugation of the label with the antibody variant, the antibody variant is conjugated with a small hapten (e.g. digoxin) and one of the different types of labels mentioned above is conjugated with an anti-hapten antibody variant (e.g. anti-digoxin antibody). Thus, indirect conjugation of the label with the antibody variant can be achieved.

[0121] Specifically, the antibody variant need not be labeled, and the presence thereof can be detected using a labeled antibody which binds to the antibody variant.

[0122] The antibodies of the present invention may be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays. Zola, *Monoclonal Antibodies: A Manual of Techniques*, pp. 147-158 (CRC Press, Inc. 1987).

[0123] Competitive binding assays rely on the ability of a labeled standard to compete with the test sample for binding with a limited amount of antibody variant. The amount of target in the test sample is inversely proportional to the amount of standard that becomes bound to the antibodies. To facilitate determining the amount of standard that becomes bound, the antibodies generally are insolubilized before or after the competition. As a result, the standard and test sample that are bound to the antibodies may conveniently be separated from the standard and test sample which remain unbound.

[0124] Sandwich assays involve the use of two antibodies, each capable of binding to a different immunogenic portion, or epitope, or the protein to be detected. In a sandwich assay, the test sample to be analyzed is bound by a first antibody which is immobilized on a solid support, and thereafter a second antibody binds to the test sample, thus forming an insoluble three-part complex. See e.g., U.S. Pat. No. 4,376,110. The second antibody may itself be labeled with a detectable moiety (direct sandwich assays) or may be measured using an anti-immunoglobulin antibody that is labeled with a detectable moiety (indirect sandwich assay). For example, one type of sandwich assay is an ELISA assay, in which case the detectable moiety is an enzyme.

[0125] For immunohistochemistry, the tumor sample may be fresh or frozen or may be embedded in paraffin and fixed with a preservative such as formalin, for example.

[0126] The antibodies may also be used for in vivo diagnostic assays. Generally, the antibody variant is labeled with a radionuclide (such as ¹¹¹In, ⁹⁹Tc, ¹⁴C, ¹³¹I, ³H, ³²P or ³⁵S) so that the tumor can be localized using immunoscintigraphy. For example, a high affinity anti-IgE antibody of the present invention may be used to detect the amount of IgE present in, e.g., the lungs of an asthmatic patient.

[0127] The antibody of the present invention is provided in a kit, i.e., packaged combination of reagents in predetermined amounts with instructions for performing the diagnostic assay. Where the antibody variant is labeled with an enzyme, the kit may include substrates and cofactors required by the enzyme (e.g., a substrate precursor which provides the detectable chromophore or fluorophore). In addition, other additives may be included such as stabilizers, buffers (e.g., a block buffer or lysis buffer) and the like. The relative amounts of the various reagents may be varied widely to provide for concentrations in solution of the reagents which substantially optimize the sensitivity of the assay. Particularly, the reagents may be provided as dry powders, usually lyophilized, including excipients which on dissolution will provide a reagent solution having the appropriate concentration.

IN VIVO USES FOR THE ANTIBODY

[0128] It is contemplated that the antibodies of the present Invention may be used to treat a mammal. In one embodiment, the antibody is prepared to be administered to a nonhuman mammal for the purposes of obtaining preclinical data, for example. Exemplary nonhuman mammals to be treated include nonhuman primates, dogs, cats, rodents and other mammals in which preclinical studies are performed. Such mammals may be established animal models for a disease to be treated with the antibody or may be used to study toxicity of the antibody of interest. In each of these embodiments, dose escalation studies may be performed on the mammal.

[0129] The antibody or polypeptide is administered by any suitable means, including parenteral, subcutaneous, intraperitoneal, intrapulmonary, and intranasal, and, if desired for local immunosuppressive treatment, intralesional administration. Parenteral infusions include intramuscular, intravenous, intraarterial, intraperitoneal, or subcutaneous administration. In addition, the antibody variant is suitably administered by pulse infusion, particularly with declining doses of the antibody variant. Preferably the dosing is given by injections, most preferably intravenous or subcutaneous injections, depending in part on whether the

administration is brief or chronic.

[0130] For the prevention or treatment of disease, the appropriate dosage of the antibody or polypeptide will depend on the type of disease to be treated, the severity and course of the disease, whether the antibody variant is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody variant, and the discretion of the attending physician. The very high affinity anti-human IgE antibodies of the invention may be suitably administered to the patient at one time or over a series of treatments.

[0131] Depending on the type and severity of the disease, about 0.1 mg/kg to 150 mg/kg (e.g., 0.1-20 mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 mg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays. An exemplary dosing regimen for an anti-LFA-1. or anti-ICAM-1 antibody is disclosed in WO 94/04188.

[0132] The antibody variant composition will be formulated, dosed and administered in a manner consistent with good medical practice. Factors for consideration in this context include the particular disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners. The "therapeutically effective amount" of the antibody variant to be administered will be governed by such considerations, and is the minimum amount necessary to prevent, ameliorate, or treat a disease or disorder. The antibody variant need not be, but is optionally formulated with one or more agents currently used to prevent or treat the disorder in question. The effective amount of such other agents depends on the amount of antibody present in the formulation, the type of disorder or treatment, and other factors discussed above. These are generally used in the same dosages and with administration routes as used hereinbefore or about from 1 to 99% of the heretofore employed dosages.

[0133] The antibodies of the present invention which recognize IgE as their target may be used to treat "IgE-mediated disorders". These include diseases such as asthma, allergic rhinitis & conjunctivitis (hay fever), eczema, urticaria, atopic dermatitis., and food allergies. The serious physiological condition of anaphylactic shock caused by, e.g., bee stings, snake bites, food or medication, is also encompassed under the scope of this invention.

EXAMPLES

Example 1 Humanization of Anti-IgE Murine MAb TES-C21

[0134] The sequences of the heavy chain variable region (V_H) and the light chain variable region (V_L) of murine mAb TES-C21 were compared with human antibody germline sequences available in the public databases. Several criteria were used when deciding on a template as described in step 1 above, including overall length, similar CDR position within the framework, overall homology, size of the CDR, etc. All of these criteria taken together provided a result for choosing the optimal human template as shown in the sequence alignment between TES-C21 MAb heavy and light chain sequences and the respective human template sequences depicted in Figure 3A and 3B.

[0135] In this case, more than one human framework template was used to design this antibody. The human template chosen for the V_H chain was a combination of DP88 (aa residues 1-95) and JH4b (aa residues 103-113) (See Figure 3B). The human template chosen for the V_L chain was a combination of L16 (VK subgroup III, aa residues 1-87) combined with JK4 (aa residues 98-107) (See Figure 3A). The framework homology between the murine sequence and the human template was about 70% for V_H and about 74% for V_L .

[0136] Once the template was chosen, a Fab library was constructed by DNA synthesis and overlapping PCR as described above and depicted in Fig.2. The library was composed of synthesized TES-C21 CDRs synthesized with the respective chosen human templates. DP88/JH4b and L16/JK4. The complexity of the library was 4096 ($= 2^{12}$). The overlapping nucleotides encoding partial V_H and V_L sequences were synthesized in the range of about 63 to about 76 nucleotides with 18 to 21 nucleotide overlaps.

[0137] PCR amplification of V_L and V_H gene was performed using a biotinylated forward primer containing the specific sequence to the framework region FR1 and an overhanging sequence annealed to the end of leader sequence (Genell) and a reverse primer from the conserved constant region (C_K or $CH1$) under standard PCR conditions. The PCR product was purified by agarose gel electrophoresis, or by commercial PCR purification kit to remove unincorporated biotinylated primers and non-specific PCR.

[0138] 5'-Phosphorylation of PCR product was performed using 2 μ g PCR product, 1 μ L of T4 polynucleotide kinase (10 units/ μ L), 2 μ L of 10x PNK buffer, 1 μ L of 10mM ATP in a total volume of 20 μ L adjusted by ddH₂O. After incubating at 37°C for 45 minutes, and heat denaturation at 65°C for 10 min, the reaction volume was adjusted to 200 μ L by adding ddH₂O for the next step.

[0139] The 100 μ L of streptavidin-coated magnetic beads were washed twice with 200 μ L 2x B&W buffer and resuspended in 200 μ L 2x B&W buffer. The phosphorylated PCR product was mixed with beads, and incubated at room temperature (RT) for 16 min with mild shaking.

[0140] The beads were sedimented and washed twice with 200 μ L 2x B&W buffer. The non-biotinylated ssDNA (minus strand) was eluted with 300 μ L freshly prepared 0.15M NaOH at RT for 10 min with mild shaking. A second NaOH elution can increase the yield slightly (optional). The eluant was centrifuged to remove any trace beads.

[0141] The ssDNA was precipitated from the supernatant by adding 1 μ L glycogen (10mg/mL), 1/10 volume of 3M NaOAc (pH 5.2), and 2.5 volumes of EtOH. The precipitated ssDNA was then washed with 70% EtOH followed by lyophilizing for 3 min and dissolving in 20 μ L ddH₂O. The ssDNA was quantitated by spotting on an ethidium bromide (EtBr) agarose plate with DNA standards, or by measuring OD₂₆₀.

Example 2 Cloning of V_H and V_L into Phage-Expression Vector

[0142] V_H and V_L were cloned into a phage-expression vector by hybridization mutagenesis. Uridinylated templates were prepared by infecting CJ236 *E. coli* strain (dut⁻ ung⁻) with M13-based phage (phage-expression vector TN003).

[0143] The following components [200 ng of uridinylated phage vector (8.49 kb); 92 ng phosphorylated single-stranded H chain (489 bases); 100 ng phosphorylated single-stranded L chain (525 bases); 1 μ L 10X annealing buffer; adjust volume with ddH₂O to 1.0 μ L] were annealed (at about 8-fold molar ratio of insert to vector) by PCR holding the temperature at 85°C for 5 min (denaturation) and then ramping to 55°C over 1 hour. The samples were chilled on ice.

[0144] To the annealed product the following components were added: 1.4 μ L 10 X synthesis buffer; 0.5 μ L T4 DNA ligase (1 unit/ μ L); 1 μ L T4 DNA polymerase (1 unit/ μ L) followed by incubating on ice for 5 min, and 37°C for 1.5 hours. The product was then ethanol precipitated, and dissolved in 10 μ L of ddH₂O or TE.

[0145] DNA was digested with 1 μ L *Xba*I (10unit/ μ L) for 2 h, and heat inactivated at 65°C for 20 min. Digested DNA was transfected into 50 μ L of electro-competent DH10B cells by electroporation. The resulting phage were titered by growing on XL-1Blue bacterial lawn at 37°C overnight. Clones were sequenced to confirm composition.

Example 3 Deep Well Culture for Library Screening

A. Plating Phage Library

[0146] The phage library was diluted in LB media to achieve the desired number of plaques per plate. High titer phage were mixed with 200 μ L XL-1 B cell culture. 3mL LB top agar was mixed, poured onto an LB plate, and allowed to sit at room temperature for 10 minutes. The plate was incubated overnight at 37°C.

B. Phage Elution

[0147] 100 μ L of phage elution buffer (10mM Tris-Cl, pH 7.5, 10mM EDTA, 100mM NaCl) was added to each well of a sterile U-bottom 96 well plate. A single phage plaque from the overnight library plate was transferred with a filtered pipette tip to a well. The phage elution plate was incubated at 37°C for 1 hour. The plate may be stored at 4°C following incubation.

C. Culture for Deep Well Plates

[0148] XL1B cells from 50mL culture were added to 2xYT media at a 1:100 dilution. The cells were grown at 37°C in a shaker until the A_{600} was between 0.9 to 1.2 .

D. Infection with Phage in Deep Well Plates

[0149] When the cells reached the appropriate OD, 1M IPTG (1:2000) was added to the XL1B culture. The final concentration of IPTG was 0.5mM. 750 μ L of cell culture was transferred to each well of a 96 well - deep well plate (Fisher Scientific). Each well was inoculated with 25 μ L of eluted phage. The deep well plate was placed in the shaker (250rpm) and incubated overnight at 37°C.

E. Preparing Supernatant for ELISA Screening

[0150] Following incubation, the deep well plates were centrifuged at 3,250 rpm for 20 minutes using the Beckman JA-5.3 plate rotor. 50 μ L of supernatant was withdrawn from each well for ELISA.

F. Inoculation of 15mL Liquid Cultures of XL-1 cells

[0151] XL-1s were grown at 37°C in the shaker (250rpm) in 2xYT containing 10 μ g/mL of tetracycline until $A_{600} = 0.9$ to 1.2. IPTG was added at a final concentration of 0.5mM and 15mL of the culture was tranferred to a 50mL conical tube for each clone to be characterized. The cells were inoculated with 10 μ L of phage from the high titer stock (titer = $\sim 10^{11}$ pfu/mL) and incubated for 1 hour at 37°C. The cells were grown overnight at room temperature with shaking.

G. Isolation of Soluble Fab from Periplasm

[0152] The cells were pelleted in an IEC centrifuge at 4,500 rpm for 20 minutes. Culture medium was removed the pellet was resuspend in 650 μ L of resuspension buffer (50mM Tris, pH 8.0 containing 1mM EDTA and 500mM sucrose), vortexed, and placed on ice for 1 hour with gentle shaking. Cellular debris was removed by centrifugation at 9,000 rpm for 10 minutes at 4°C. The supernatent containing the soluble Fabs was collected and stored at 4°C.

Example 4 Framework Modification

[0153] There were twelve murine/human wobble residues within the framework at the potential key positions described above. Position 73 in V_H was kept as the murine residue threonine in the humanization library because this position was determined to affect binding. It was noted, however, that threonine at VH 73 is a common human residue in the human germline V_H subgroup 1 and 2.

[0154] The framework residues that differed between the TES-C21 sequence and the human template were randomly substituted as described above and then assessed for their potential affect on target binding, and antibody folding. Potential framework residues that may have affected the binding were identified. In this case, they were residues 12, 27, 43, 48, 67, 69 in V_H , and 1, 3, 4, 49, 60, 85 in V_L (Kabat number system). (See Figure 4) It was later demonstrated that only positions 27 and 69 significantly affected binding in the V_H region (clone number 1136-2C).

[0155] The primary screen used was a single point ELISA (SPE) using culture media (See description below). The primary screen selected clones that bind to the antibody's target molecule. Clones that gave equal or better signal than the parent molecule were selected for the next round of screening.

[0156] In the second round of screening, individual phage were grown in a 15 ml bacterial culture and periplasmic preparations were used for SPE and ELISA titration assays. The clones that retained higher binding in this assay were further characterized. Once all the selected primary clones were processed, the top 10-15% clones were sequenced and the clones arranged according to sequence. Representatives from each sequence group were compared against each other and the best clones selected. Sequences from these chosen clones were combined and the effects of various combinations were evaluated.

[0157] The constructed library was subjected to an ELISA screen for improved binding to the recombinant human IgE, SE44. Clones with binding affinity greater than murine TES-C21 Fab were isolated and sequenced. Clone ID #4, 49, 72, 76, and 136 were further characterized. ELISA titration curves for clone 4, 49, 72, 78, and 136 are shown in Figure 5A and 5B indicating that their affinity is similar to the parent, TES-C21. These clones compete with murine TES-C21 for binding to human IgE indicating that the binding epitope was not changed during the humanization process. The humanized Fabs did not bind to Fc ϵ RI-bound IgE suggesting that it is less likely that the humanized antibodies will crosslink the receptor to cause histamine release when they were constructed into divalent IgG.

[0158] Humanized clone 136 retained 5 murine heavy chain framework residues (= 94.3 % human V H framework homology), with a 100% human light chain framework selected for by affinity maturation. The inhibition of IgE binding to Fc ϵ RI by the humanized Fab was demonstrated (Figure 6).

Example 5 Single Point ELISA Protocol for Screening anti IgE

[0159] Plates were coated with 2ug/mL sheep anti-human Fd in carbonate coating buffer overnight at 4°C. The coating solution was removed and the plates were blocked with 200uL/well 3% BSA/PBS for 1 hour at 37°C. After washing the plates 4x with PBS/0.1% TWEEN® (PBST), 50uL/well Fab sample (i.e., supernatant containing high titer phage and secreted Fab or periplasmic prep from DMB block, or 15mL prep) was added. Plates were incubated for 1 hour at room temperature followed by washing 4x with PBST. 50uL/well of biotinylated SE44 at 0.015ug/mL diluted in 0.5% BSA/PBS and 0.05% TWEEN® was then added. Plates were then incubated for 2 hours at room temperature and washed 4X PBST. 50uL/well StreptAvidin HRP 1:2000 dilution in 0.5% BSA/PBS and 0.05% TWEEN® was added and the plates incubated 1 hour at room temperature. Plates were washed 6x with PBST. 50uL/well TMB substrate (sigma) was added to develop and then stopped by adding 50uL/well 0.2M H₂SO₄.

Example 6 ELISA Titration: anti IgE

[0160] Plates were coated with 0.25ug/mL (for purified Fab 0.1 ug/ml) SE44 in carbonate coating buffer overnight at 4°C. Coating solution was removed and the plates were blocked with 200uL/well 3 % BSA/PBS for 1 hour at 37°C.

[0161] The plates were washed 4x with PBS/0.1 % TWEEN® (PBST). 50uL/well Fab (from 15mL periplasmic prep) was added starting with a dilution of 1:2 and diluting 3 fold serially in 0.5% BSA/PBS and .05% TWEEN®20. Plates were incubated for 2 hours at room temperature.

[0162] The plates were washed 4x with PBST and 50uL/well 1:1000 (0.8ug/ml) dilution of biotin-sheep anti human Fd in 0.5% BSA/PBS and 0.05% TWEEN®20 was added. The plates were incubated again for 2 hours at room temperature.

[0163] Following a wash 4x with PBST, 50uL/well Neutra-avidin-AP 1: 2000(0.9 ug/ml) in 0.5% BSA/PBS and 0.05% TWEEN® 20 was added and the plates were incubated 1 hour at room temperature:

[0164] The plates were washed 4x with PBST. And developed by adding 50uL/well pNPP substrate. Development was stopped by adding 50uL/well 3M NaOH. The absorbance of each well was read at 405nm or 410nm.

Example 7 Protocol for Affinity Purification of M13 phage Expressed Soluble Fab

DAY 1

[0165] Two 500 mL cultures (2xYT) containing 10 mg/mL tetracycline were inoculated with 5 mL overnight stock XL1B and grown at 37°C to A600 = 0.9 to 1.2. IPTG was added to a concentration of 0.5mM. The cell culture was then infected with 200 μ L phage per culture and incubated for 1 hour at 37° C with shaking. Following infection, the cells were grown at 25°C overnight with shaking.

DAY 2

[0166] Cells were pelleted at 3500 x g for 30 minutes at 4°C in 250mL centrifuge tubes. Culture medium was aspirated and the pellets were resuspended in a total of 12-15 mL lysis buffer (Buffer A + protease inhibitor cocktail).

Buffer A: (1 liter)	
50mM NaH ₂ PO ₄	6.9 g NaH ₂ PO ₄ H ₂ O (or 6 g NaH ₂ PO ₄)
300mM NaCl	17.54 g NaCl
10mM imidazole adjust pH to 8.0 using NaOH	0.68 g imidazole (MW 68.08)
Lysis buffer: Mix 25 mL of Buffer A with one tablet of Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland).	

[0167] Resuspended cells were transferred into a 50mL conical tube and lysed with 100 μ L 100 mg/mL lysozyme by inverting the tube several times until the mixture moves together as a blob (due to the lysis). Cells were sonicated on ice followed by the addition of 10 μ L DNase I (about 1000 units) and gently rocked at 4°C for 30 minutes. Debris was pelleted by centrifugation at 12000 x g for 30 minutes at 4 °C, using 50 mL centrifuge tubes. Supernatants were transferred to a new conical tube and stored at 4 °C.

[0168] Ni-NT agarose (Qiagen, Valencia, CA) was used to purify the soluble Fabs according to the manufacturer's protocol. The lysate was mixed with Ni-NTA and loaded into a column. The flow through was collected for SDS-PAGE analysis. The column was washed with 20 mL buffer (50mM NaH₂PO₄, 300mM NaCl, 15mM imidazole, adjust pH to 8.0 with NaOH) followed by a 20 mL wash with 50mRNA NaH₂PO₄, 300mM NaCl, 20mM imidazole. Fabs were eluted with 6 x 500 μ L elution buffer (50mM NaH₂PO₄, 300mM NaCl, 450mM imidazole, adjust pH to 8.0 with NaOH) and analyzed by SDS PAGE. Column fractions were stored at 4°C. Column fractions were analyzed by SDS-PAGE and the fraction with the greatest amount of Fab was selected and dialyzed in PBS at 4 °C.

Example 8 Soluble Receptor Assay

[0169] A 96 well assay plate suitable for ELISA was coated with 0.05 mL 0.5 μ g/mL Fc ϵ RI alpha-chain receptor coating buffer (50 mM carbonate/bicarbonate, pH 9.6) for 12 hours at 4-8°C. The wells were aspirated and 250 μ L blocking buffer (PBS, 1% BSA, pH 7.2) was added and incubated for 1 hour at 37°C. In a separate assay plate the samples and reference TES-C21 MAbs were titrated from 200 to 0.001 μ g/mL by 1:4 dilutions with assay buffer (0.5% BSA and 0.05% Tween 20, PBS, pH 7.2) and an equal volume of 100ng/mL biotinylated IgE was added and the plate incubated for 2-3 hours at 25°C. The Fc ϵ RI - coated wells were washed three times with PBS and 0.05% TWEEN 20 and 50 μ L from the sample wells were transferred and incubated with agitation for 30 minutes at 25°C. Fifty μ L/well of 1 mg/mL Streptavidin-HRP, diluted 1:2000 in assay buffer, was incubated for 30 minutes with agitation and then the plate was washed as before. Fifty μ L/well of TMB substrate was added and color was developed. The reaction was stopped by adding an equal volume of 0.2 M H₂SO₄ and the absorbance measured at 450nm.

Example 9 Binding of Antibodies to IgE-loaded Fc ϵ RI

[0170] Antibody binding to human IgE associated with the alpha-subunit of Fc ϵ RI was determined by preincubating with 10 μ g/mL human IgE for 30 min at 4°C. Plates were washed three times followed by a one hour incubation with varying concentrations of

either murine anti-human IgE mAbs E-10-10 or the humanized Fab variant. Binding of Fabs was detected with a biotin labeled anti human Fd antibody followed by SA-HRP. Murine ab E10-10 was detected by Goat anti murine Ig Fc HRP -conjugated Ab.

Example 10 Clone Characterization

[0171] Each candidate was assayed for binding affinity and positive clones were sequenced. Antibody variants having beneficial mutations in CDR regions that increase binding affinity were further characterized. Assays included Biacore analysis; inhibition of IgE binding to its receptor; and cross linking of receptor bound IgE.

[0172] A library of variants was created. The amino acid sequences for the various CDRs which demonstrated improved affinity are depicted in Table 1. Figure 7 presents high affinity candidates having combinations of substitutions.

TABLE 1.

CDRL1:		CDRH1:	
P RASQSIGTNIH	SEQ ID NO 5	P MYWLE	SEQ ID NO 15
#1 RASRSIGTNIH	SEQ ID NO 6	#1 WYWLE	SEQ ID NO 16
#2 RASQRIGTNIH	SEQ ID NO 7	#2 YYWLE	SEQ ID NO 17
CDRL2:		CDRH2:	
P YASESIS	SEQ ID NO 8	P EISPGTFTTNYNEKFKA	SEQ ID NO 18
#1 YAYESIS	SEQ ID NO 9	#1 EIEPGTFTTNYNEKFKA	SEQ ID NO 19
#2 YASESIY	SEQ ID NO 10	#2 EIDPGTFTTNYNEKFKA	SEQ ID NO 20
#3 YASESDS	SEQ ID NO 11	#3 EISPDTFTTNYNEKFKA	SEQ ID NO 21
#4 YASESES	SEQ ID NO 12	#4 EISPETFTTNYNEKFKA	SEQ ID NO 22
CDRL3:		#6 EISPGTFETNYNEKFKA	SEQ ID NO 23
P QQSDSWPTT	SEQ ID NO 13	#6 EIEPGTFETNYNEKFKA	SEQ ID NO 24
#1 QQSWSWPTT	SEQ ID NO 14	#7 EIDPGTFETNYNEKFKA	SEQ ID NO 25
CDRH3:			
		P FSHFSGSNYDYFDY	SEQ ID NO 26
		#1 FSHFSGMNYDYFDY	SEQ ID NO 27
		#2 FSHFSGQNYDYFDY	SEQ ID NO 28
		#3 FSHFTGSNYDYFDY	SEQ ID NO 29

P = Parent

[0173] Nineteen heavy chain variants are presented in Figure 9 and 35 light chain variants are presented in Figure 8. Three candidates were further characterized for binding affinity and these are presented in Table 2.

TABLE 2 Binding Affinity

MAb	Kd	Fold Increase In Binding Affinity
TES-C21	614 ± 200 pM	
MAb 1 (CL-5A)	0.158 pM	3886
MAb 2 (CL-2C)	1.47 ± 0.5 pM	417
MAb 3 (CL-5I)	3.2 ± 2.2 pM	191

Example 11 Expression and purification of anti-IgE antibodies and HRP-conjugation

[0174] High affinity MAbs candidates were generated. For the generation of intact anti-IgE MAbs, the heavy and light chains variable regions were PCR amplified from phage vectors templates and subcloned separately into H-and L-chain expression vectors under the expression of a CMV promoter. Six full antibody clones were constructed and are represented in Figure 10 A-

F μ . Appropriate heavy and light chain plasmids were co-transfected into the mouse myeloma cell line NS0 using electroporation by techniques well known in the art. See, e.g., Liou et al. J Immunol. 143(12):3967-75 (1989). Antibodies were purified from the single stable cell line supernatants using protein A-sepharose (Pharmacia). The concentration of the antibody was determined using spectrophotometer at 280nm and FCA assay (IDEXX).

[0175] Purified antibodies were conjugated by horseradish peroxidase (HRP) using peroxidase conjugation kit (Zymed Labs, San Francisco, CA) according to the manufacturer's protocol. The titer of each conjugated anti-IgE MAb was determined using ELISA with plates coated with a monoclonal human IgE (SE44).

[0176] The following cultures have been deposited with the American Type Culture Collection, 10801 University Boulevard, Manassas Va. 20110-2209 USA (ATCC):

Hybridoma	ATCC NO.	Deposit Date
Anti-IgE CL-2C	PTA-5678	December 3, 2003
Anti-IgE CL-5A	PTA-5679	December 3, 2003
Anti-IgE CL-5I	PTA-5680	December 3, 2003

[0177] This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture for 30 years from the date of deposit. The organism will be made available by ATCC under the terms of the Budapest Treaty, which assures permanent and unrestricted availability of the progeny of the culture to the public upon issuance of the pertinent patent.

[0178] The assignee of the present application has agreed that if the culture on deposit should die or be lost or destroyed when cultivated under suitable conditions, it will be promptly replaced on notification with a viable specimen of the same culture. Availability of the deposited strain is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

[0179] Preferred items disclosed herein are described below and are referred to as embodiment E1 to embodiment E55.

E1. A variable light chain region comprising an amino acid sequence having the formula: FRL1-CORL1-FRL2-CDRL2-FRL3-CDRL3-FRL4, wherein FRL1 consists of any one of SEQ ID NOs: 30-37; CDRL1 consists of any one of SEQ ID NOs: 5-7; FRL2 consists of any one of SEQ ID NOs: 38-39; CDRL2 consists of any one of SEQ ID NOs: 8-12; FRL3 consists of any one of SEQ ID NOs: 40-43; CDRL3 consists of any of SEQ ID NOs: 13-14; and FRL4 consists of SEQ ID NO: 44.

E2. The variable light chain region of E1 comprising any one of SEQ ID NOs: 57, 61, 63, 65, 67 and 69.

E3. The variable light chain region of E1, further comprising a constant region.

E4. The variable light chain region of E3, wherein the constant domain has SEQ ID NO: 58.

E5. The variable light chain region of E2, further comprising a constant region.

E6. The variable light chain region of E5, wherein the constant region has SEQ ID NO: 58.

E7. The variable light chain region of E1, further comprising a signal peptide.

E8. The variable light chain region of E7, wherein the signal peptide has SEQ ID NO: 56.

E9. A variable heavy chain region comprising an amino acid sequence having the formula: FRH1-CDRH1-FRH2-CDRH2-FRH3-CDRH3-FRH4, wherein FRH1 consists of any one of SEQ ID NOs: 45-46; CDRH1 consists of any one of SEQ ID NOs: 15-17; FRH2 consists of any one of SEQ ID NOs: 47-50; CDRH2 consists of any one of SEQ ID NOs: 18-25; FRH3 consists of any one of SEQ ID NOs: 51-54; CDR3 consists of any of SEQ ID NOs: 26-29; and FRH4 consists of SEQ ID NO: 55.

E10. The variable heavy chain region of E9 comprising any one of SEQ ID NOs: 59, 62, 64, 66, 68 and 70.

E11. The variable heavy chain region of E7, further comprising at least the CH1 domain of a constant region.

E12. The variable heavy chain region of E11, comprising the CH1, CH2 and CH3 domains of a constant region.

E13. The variable heavy chain region of E11, wherein the constant region is from an IgG antibody.

E14. The variable heavy chain region of E13, wherein the IgG antibody is an IgG1 antibody, an IgG2 antibody, an IgG3 antibody, or an IgG4 antibody.

E15. The variable heavy chain region of E13, wherein the constant domain has SEQ ID NO: 60.

E16. The variable heavy chain region of E10, further comprising a constant region.

E17. The variable heavy chain region of E16, wherein the constant domain has SEQ ID NO: 60.

E18. The variable heavy chain region of E9, further comprising a signal peptide.

E19. The variable heavy chain region of E19, wherein the signal peptide has SEQ ID NO: 56.

E20. An antibody or antibody fragment comprising the variable light chain region of E1, wherein the antibody binds specifically to IgE.

E21. An antibody or antibody fragment comprising the variable heavy chain region of E9, wherein the antibody binds specifically to IgE.

E22. The antibody of E20, comprising a heavy chain region of E9.

E23. The antibody of E22, comprising a variable light chain region having the amino acid sequence set forth in SEQ ID NO: 57 and a variable heavy chain region having the amino acid sequence set forth in SEQ ID NO: 59.

E24. The antibody of E23, further comprising a constant light chain region and a constant heavy chain region.

E25. The antibody of E24, wherein the constant light chain region has the amino acid sequence set forth in SEQ ID NO: 58 and the constant heavy chain region has the amino acid sequence set forth in SEQ ID NO: 60.

E26. The antibody of E22, comprising a variable light chain region having the amino acid sequence set forth in SEQ ID NO: 61 and a variable heavy chain region having the amino acid sequence set forth in SEQ ID NO: 62.

E27. The antibody of E26, further comprising a constant light chain region and a constant heavy chain region.

E28. The antibody of E27, wherein the constant light chain region has the amino acid sequence set forth in SEQ ID NO: 58 and the constant heavy chain region has the amino acid sequence set forth in SEQ ID NO: 60.

E29. The antibody of E26, which is produced by a cell having ATCC Deposit number PTA-5678.

E30. The antibody of E22, comprising a variable light chain region having the amino acid sequence set forth in SEQ ID NO: 63 and a variable heavy chain region having the amino acid sequence set forth in SEQ ID NO: 64.

E31. The antibody of E30, further comprising a constant light chain region and a constant heavy chain region.

E32. The antibody of E31, wherein the constant light chain region has the amino acid sequence set forth in SEQ ID NO: 58 and the constant heavy chain region has the amino acid sequence set forth in SEQ ID NO: 60.

E33. The antibody of E30, which is produced by a cell having ATCC Deposit number PTA-5680.

E34. The antibody of E22, comprising a variable light chain region having the amino acid sequence set forth in SEQ ID NO: 65 and a variable heavy chain region having the amino acid sequence set forth in SEQ ID NO: 66.

E35. The antibody of E34, further comprising a constant light chain region and a constant heavy chain region.

E36. The antibody of E35, wherein the constant light chain region has the amino acid sequence set forth in SEQ ID NO: 58 and the constant heavy chain region has the amino acid sequence set forth in SEQ ID NO: 60.

E37. The antibody of E34, which is produced by a cell having ATCC Deposit number PTA-5679.

E38. The antibody of E22, comprising a variable light chain region having the amino acid sequence set forth in SEQ ID NO: 67 and a variable heavy chain region having the amino acid sequence set forth in SEQ ID NO: 68.

E39. The antibody of E38, further comprising a constant light chain region and a constant heavy chain region.

E40. The antibody of E39, wherein the constant light chain region has the amino acid sequence set forth in SEQ ID NO: 58 and the constant heavy chain region has the amino acid sequence set forth in SEQ ID NO: 60.

E41. The antibody of E22, comprising a variable light chain region having the amino acid sequence set forth in SEQ ID NO: 69 and a variable heavy chain region having the amino acid sequence set forth in SEQ ID NO: 70.

E42. The antibody of E41, further comprising a constant light chain region and a constant heavy chain region.

E43. The antibody of E42, wherein the constant light chain region has the amino acid sequence set forth in SEQ ID NO: 58 and the constant heavy chain region has the amino acid sequence set forth in SEQ ID NO: 60.

E44. A composition comprising a therapeutically effective amount of an antibody of E20 or 21 and a pharmaceutically acceptable carrier.

E45. The antibody of E20 or 21, further linked to a label.

E46. A diagnostic kit comprising an antibody of E20 or 21.

E47. A vector comprising a nucleic acid encoding the antibody of E22.

E48. A cell comprising the vector of E47.

E49. The cell of E48, wherein the cell is ATCC Deposit number PTA-5678, PTA-5679 or PTA-5680.

E50. A method for producing an antibody comprising culturing the cell of E48 under conditions appropriate for the production of an antibody, and isolating the antibody produced.

E51. A method for measuring the level of IgE in a subject, comprising contacting a sample of the subject, which sample comprises IgE molecules, with an antibody of E22; and determining the level of retention of the antibody by the sample relative to a control sample of a control subject, wherein a higher or lower level of retention of the antibody by the sample of the subject relative to the control sample indicates that the subject has a higher or lower level of IgE molecules relative to that in the control subject.

E52. A method for diagnosing a disorder associated with an abnormal level of IgE in a subject, comprising contacting a sample of the subject, which sample comprises IgE molecules, with an antibody of E22; and determining the level of retention of the antibody by the sample relative to a control sample of a control subject, wherein a higher or lower level of retention of the antibody by the sample of the subject relative to the control sample indicates that the subject has a disorder associated with an abnormal level of IgE.

E53. The method of E52, wherein the disorder associated with an abnormal level of IgE is asthma, allergic rhinitis, eczema, urticaria, or atopic dermatitis.

E54. A method for treating a disorder associated with an abnormally high IgE level in a subject, comprising administering to the subject a therapeutically effective amount of an antibody of E22, such that the disorder is treated in the subject.

E55. The method of E54, wherein the disorder is asthma, allergic rhinitis, eczema, urticaria, atopic dermatitis, or a food allergy.

SEQUENCE LISTING

[0180]

<110> TANOX, INC. SINGH, Sanjaya FOSTER, Catherine WU, Herren

<120> HIGH AFFINITY ANTI-HUMAN IgE ANTIBODIES

<130> TNX-1010 (2) PCT

<140> PCT/US2004/002894

<141> 2004-02-02

<150> 60/444,229

<151> 2003-02-01

<160> 70

<170> PatentIn version 3.4

<210> 1
 <211> 107
 <212> PRT
 <213> Mus musculus

<220>
 <221> misc_feature
 <223> TES-C21 LIGHT CHAIN

<400> 1
 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 Ser Ile Gly Thr Asn
 20 25 30
 Ile His Trp Tyr Gln Gln Arg Thr Asp Gly Ser Pro Arg Leu Leu Ile
 35 40 45
 Lys Tyr Ala Ser Glu Ser Ile Ser Gly Ile Pro Ser Arg Phe Ser Gly
 50 55 60
 Ser Gly Ser Gly Thr Glu Phe Thr Leu Asn Ile Asn Ser Val Glu Ser
 65 70 75 80
 Glu Asp Ile Ala Asp Tyr Tyr Cys Gln Gln Ser Asp Ser Trp Pro Thr
 85 90 95
 Thr Phe Gly Gly Thr Lys Leu Glu Ile Lys
 100 105

<210> 2
 <211> 107
 <212> PRT
 <213> Homo sapiens

<220>
 <221> misc_feature
 <223> L16/-JK4 human light chain consensus sequence template

<400> 2
 Glu Ile Val Met Thr Gln Ser Pro Ala Thr Leu Ser Val Ser Pro Gly
 1 5 10 15
 Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser Val Ser Ser Asn
 20 25 30
 Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu Ile
 35 40 45
 Tyr Gly Ala Ser Thr Arg Ala Thr Gly Ile Pro Ala Arg Phe Ser Gly
 50 55 60
 Ser Gly Ser Gly Thr Glu Phe Thr Leu Thr Ile Ser Ser Leu Gln Ser
 65 70 75 80
 Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Tyr Asn Asn Trp Pro Leu
 85 90 95
 Thr Phe Gly Gly Thr Lys Val Glu Ile Lys
 100 105

<210> 3
 <211> 123
 <212> PRT
 <213> Mus musculus

<220>
 <221> misc_feature
 <223> TES-C21 Heavy Chain

<400> 3

Gln Val Gln Leu Gln Gln Ser Gly Ala Glu Leu Met Lys Pro Gly Ala
1 5 10 15

Ser Val Lys Ile Ser Cys Lys Thr Thr Gly Tyr Thr Phe Ser Met Tyr
20 25 30

Trp Leu Glu Trp Val Lys Gln Arg Pro Gly His Gly Leu Glu Trp Val
35 40 45

Gly Glu Ile Ser Pro Gly Thr Phe Thr Thr Asn Tyr Asn Glu Lys Phe
50 55 60

Lys Ala Lys Ala Thr Phe Thr Ala Asp Thr Ser Ser Asn Thr Ala Tyr
65 70 75 80

Leu Gln Leu Ser Gly Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
85 90 95

Ala Arg Phe Ser His Phe Ser Gly Ser Asn Tyr Asp Tyr Phe Asp Tyr
100 105 110

Trp Gly Gln Gly Thr Ser Leu Thr Val Ser Ser
115 120

<210> 4

<211> 113

<212> PRT

<213> Homo sapiens

<220>

<221> misc_feature

<223> DP88/JH4b human heavy chain consensus sequence template

<400> 4

Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ser
1 5 10 15

Ser Val Lys Val Ser Cys Lys Ala Ser Gly Gly Thr Phe Ser Ser Tyr
20 25 30

Ala Ile Ser Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
35 40 45

Gly Gly Ile Ile Pro Ile Phe Gly Thr Ala Asn Tyr Ala Gln Lys Phe
50 55 60

Gln Gly Arg Val Thr Ile Thr Ala Asp Lys Ser Thr Ser Thr Ala Tyr
65 70 75 80

Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Tyr Phe Asp Tyr Leu Val Gln Gly Thr Ser Leu Thr Val Ser
100 105 110

Ser

<210> 5

<211> 11

<212> PRT

<213> ARTIFICIAL

<220>

<223> TES-C21 CDRL1 SEQUENCE (TABLE 1)

<400> 5

Arg Ala Ser Gln Ser Ile Gly Thr Asn Ile His
1 5 10

<210> 6

<211> 11

<212> PRT

<213> ARTIFICIAL

<220>

<223> CDRL1 VARIANT SEQUENCE #1 (TABLE 1)

<400> 6

Arg Ala Ser Arg Ser Ile Gly Thr Asn Ile His
1 5 10

<210> 7

<211> 11

<212> PRT

<213> ARTIFICIAL

<220>

<223> CDRL1 VARIANT SEQUENCE #2 (TABLE 1)

<400> 7

Arg Ala Ser Gln Arg Ile Gly Thr Asn Ile His
1 5 10

<210> 8

<211> 7

<212> PRT

<213> ARTIFICIAL

<220>

<223> TES-C21 CDRL2 SEQUENCE (TABLE 1)

<400> 8

Tyr Ala Ser Glu Ser Ile Ser
1 5

<210> 9

<211> 7

<212> PRT

<213> ARTIFICIAL

<220>

<223> CDRL2 VARIANT #1 (TABLE 1)

<400> 9

Tyr Ala Tyr Glu Ser Ile Ser
1 5

<210> 10

<211> 7

<212> PRT

<213> ARTIFICIAL

<220>

<223> CDRL2 VARIANT #2 (TABLE 1)

<400> 10

Tyr Ala Ser Glu Ser Ile Tyr
1 5

<210> 11

<211> 7

<212> PRT

<213> ARTIFICIAL

<220>

<223> CDRL2 VARIANT #3 (TABLE 1)

<400> 11

Tyr Ala Ser Glu Ser Asp Ser
1 5

<210> 12

<211> 7

<212> PRT
 <213> ARTIFICIAL

<220>
 <223> CDRL2 VARIANT #4 (TABLE 1)

<400> 12
 Tyr Ala Ser Glu Ser Glu Ser
 1 5

<210> 13
 <211> 9
 <212> PRT
 <213> ARTIFICIAL

<220>
 <223> TES-C21 CDRL3 (TABLE 1)

<400> 13
 Gln Gln Ser Asp Ser Trp Pro Thr Thr
 1 5

<210> 14
 <211> 9
 <212> PRT
 <213> ARTIFICIAL

<220>
 <223> CDRL3 VARIANT (TABLE)

<400> 14
 Gln Gln Ser Trp Ser Trp Pro Thr Thr
 1 5

<210> 15
 <211> 5
 <212> PRT
 <213> ARTIFICIAL

<220>
 <223> TES-C21 CDRH1

<400> 15
 Met Tyr Trp Leu Glu

1 5

<210> 16
 <211> 5
 <212> PRT
 <213> ARTIFICIAL

<220>
 <223> CDRH1 VARIANT #1 (TABLE 1)

<400> 16
 Trp Tyr Trp Leu Glu
 1 5

<210> 17
 <211> 5
 <212> PRT
 <213> ARTIFICIAL

<220>
 <223> CDRH1 #2 (TABLE 1)

<400> 17
 Tyr Tyr Trp Leu Glu
 1 5

<210> 18
 <211> 17
 <212> PRT
 <213> ARTIFICIAL

<220>
 <223> TES-C21 CDRH2 (TABLE 1)

<400> 18
 Glu Ile Ser Pro Gly Thr Phe Thr Thr Asn Tyr Asn Glu Lys Phe Lys
 1 5 10 15

Ala

<210> 19
 <211> 17
 <212> PRT
 <213> ARTIFICIAL

<220>
 <223> CDRH2 VARIANT #1 (TABLE 1)

<400> 19
 Glu Ile Glu Pro Gly Thr Phe Thr Thr Asn Tyr Asn Glu Lys Phe Lys
 1 5 10 15

Ala

<210> 20
 <211> 17
 <212> PRT
 <213> ARTIFICIAL

<220>
 <223> CDRH2 VARIANT #2 (TABLE 1)

<400> 20
 Glu Ile Asp Pro Gly Thr Phe Thr Thr Asn Tyr Asn Glu Lys Phe Lys
 1 5 10 15

Ala

<210> 21
 <211> 17
 <212> PRT
 <213> ARTIFICIAL

<220>
 <223> CDRH2 VARIANT #3 (TABLE 1)

<400> 21
 Glu Ile Ser Pro Asp Thr Phe Thr Thr Asn Tyr Asn Glu Lys Phe Lys
 1 5 10 15

Ala

<210> 22
 <211> 17
 <212> PRT
 <213> ARTIFICIAL

<220>
 <223> CDRH2 VARIANT #4 (TABLE 1)

<400> 22
 Glu Ile Ser Pro Glu Thr Phe Thr Thr Asn Tyr Asn Glu Lys Phe Lys
 1 5 10 15

Ala

<210> 23

<211> 17

<212> PRT

<213> ARTIFICIAL

<220>

<223> CDRH2 VARIANT #5 (TABLE 1)

<400> 23

Glu Ile Ser Pro Gly Thr Phe Glu Thr Asn Tyr Asn Glu Lys Phe Lys
 1 5 10 15

Ala

<210> 24

<211> 17

<212> PRT

<213> ARTIFICIAL

<220>

<223> CDRH2 VARIANT #6 (TABLE 1)

<400> 24

Glu Ile Glu Pro Gly Thr Phe Glu Thr Asn Tyr Asn Glu Lys Phe Lys
 1 5 10 15

Ala

<210> 25

<211> 17

<212> PRT

<213> ARTIFICIAL

<220>

<223> CDRH2 VARIANT #7 (TABLE 1)

<400> 25

Glu Ile Asp Pro Gly Thr Phe Glu Thr Asn Tyr Asn Glu Lys Phe Lys
 1 5 10 15

Ala

<210> 26

<211> 14

<212> PRT

<213> ARTIFICIAL

<220>

<223> TES-C21 CDRH3 (TABLE 1)

<400> 26

Phe Ser His Phe Ser Gly Ser Asn Tyr Asp Tyr Phe Asp Tyr
 1 5 10

<210> 27

<211> 14

<212> PRT

<213> ARTIFICIAL

<220>

<223> CDRH3 VARIANT #1 (TABLE 1)

<400> 27

Phe Ser His Phe Ser Gly Met Asn Tyr Asp Tyr Phe Asp Tyr
 1 5 10

<210> 28

<211> 14

<212> PRT

<213> ARTIFICIAL

<220>

<223> CDRH3 VARIANT #2 (TABLE 1)

<400> 28

Phe Ser His Phe Ser Gly Gln Asn Tyr Asp Tyr Phe Asp Tyr
 1 5 10

<210> 29

<211> 14

<212> PRT

<213> ARTIFICIAL

<220>

<223> CDRH3 VARIANT #3 (TABLE 1)

<400> 29

Phe Ser His Phe Thr Gly Ser Asn Tyr Asp Tyr Phe Asp Tyr
 1 5 10

<210> 30

<211> 23

<212> PRT

<213> ARTIFICIAL

<220>

<223> FRL1 VARIANT 136

<400> 30

Glu Ile Val Met Thr Gln Ser Pro Ala Thr Leu Ser Val Ser Pro Gly
 1 5 10 15

Glu Arg Ala Thr Leu Ser Cys
 20

<210> 31

<211> 23

<212> PRT

<213> ARTIFICIAL

<220>

<223> FRL1 VARIANT 1

<400> 31

Asp Ile Leu Met Thr Gln Ser Pro Ala Thr Leu Ser Val Ser Pro Gly
 1 5 10 15

Glu Arg Ala Thr Leu Ser Cys
 20

<210> 32

<211> 23

<212> PRT

<213> ARTIFICIAL

<220>

<223> FRL1 VARIANT 2

<400> 32

Asp Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser Val Ser Pro Gly
 1 5 10 15

Glu Arg Ala Thr Leu Ser Cys
 20

<210> 33

<211> 23

<212> PRT

<213> ARTIFICIAL

<220>

<223> FRL1 VARIANT 4

<400> 33

Asp Ile Leu Leu Thr Gln Ser Pro Ala Thr Leu Ser Val Ser Pro Gly
 1 5 10 15

Glu Arg Ala Thr Leu Ser Cys
 20

<210> 34

<211> 23

<212> PRT

<213> ARTIFICIAL

<220>

<223> FRL1 VARIANT 13

<400> 34

Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser Val Ser Pro Gly
 1 5 10 15

Glu Arg Ala Thr Leu Ser Cys
 20

<210> 35

<211> 23

<212> PRT

<213> ARTIFICIAL

<220>

<223> FRL1 VARIANT 18

<400> 35

Glu Ile Leu Leu Thr Gln Ser Pro Ala Thr Leu Ser Val Ser Pro Gly
 1 5 10 15

Glu Arg Ala Thr Leu Ser Cys
 20

<210> 36

<211> 23

<212> PRT

<213> ARTIFICIAL

<220>

<223> FRL1 VARIANT 25

<400> 36

Asp Ile Val Met Thr Gln Ser Pro Ala Thr Leu Ser Val Ser Pro Gly
 1 5 10 15

Glu Arg Ala Thr Leu Ser Cys
 20

<210> 37

<211> 23

<212> PRT

<213> ARTIFICIAL

<220>

<223> FRL1 VARIANT 27

<400> 37

Glu	Ile	Leu	Met	Thr	Gln	Ser	Pro	Ala	Thr	Leu	Ser	Val	Ser	Pro	Gly
1			5		10				15						

Glu	Arg	Ala	Thr	Leu	Ser	Cys
	20					

<210> 38

<211> 15

<212> PRT

<213> ARTIFICIAL

<220>

<223> FRL2 VARIANT 136

<400> 38

Trp	Tyr	Gln	Gln	Lys	Pro	Gly	Gln	Ala	Pro	Arg	Leu	Leu	Ile	Tyr
1				5		10			15					

<210> 39

<211> 15

<212> PRT

<213> ARTIFICIAL

<220>

<223> FRL2 VARIANT 1

<400> 39

Trp	Tyr	Gln	Gln	Lys	Pro	Gly	Gln	Ala	Pro	Arg	Leu	Leu	Ile	Lys
1				5		10			15					

<210> 40

<211> 32

<212> PRT

<213> ARTIFICIAL

<220>

<223> FRL3 VARIANT 136

<400> 40

Gly	Ile	Pro	Ala	Arg	Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr	Glu	Phe	Thr
1				5		10			15						

Leu	Thr	Ile	Ser	Ser	Leu	Gln	Ser	Glu	Asp	Phe	Ala	Val	Tyr	Tyr	Cys
		20			25			30							

<210> 41

<211> 32

<212> PRT

<213> ARTIFICIAL

<220>

<223> FRL3 VARIANT 1

<400> 41

Gly	Ile	Pro	Ser	Arg	Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr	Glu	Phe	Thr
1				5		10			15						

Leu	Thr	Ile	Ser	Ser	Leu	Gln	Ser	Glu	Asp	Phe	Ala	Val	Tyr	Tyr	Cys
		20			25			30							

<210> 42

<211> 32

<212> PRT

<213> ARTIFICIAL

<220>

<223> FRL3 VARIANT 13

<400> 42

Gly	Ile	Pro	Ala	Arg	Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr	Glu	Phe	Thr
1				5			10				15				

Leu	Thr	Ile	Ser	Ser	Leu	Gln	Ser	Glu	Asp	Phe	Ala	Asp	Tyr	Tyr	Cys
					20		25						30		

<210> 43

<211> 32

<212> PRT

<213> ARTIFICIAL

<220>

<223> FRL3 VARIANT 18

<400> 43

Gly	Ile	Pro	Ser	Arg	Phe	Ser	Gly	Ser	Gly	Thr	Glu	Phe	Thr
1				5			10			15			

Leu	Thr	Ile	Ser	Ser	Leu	Gln	Ser	Glu	Asp	Phe	Ala	Asp	Tyr	Tyr	Cys
					20		25						30		

<210> 44

<211> 10

<212> PRT

<213> ARTIFICIAL

<220>

<223> FRL4 VARIANT

<400> 44

Phe	Gly	Gly	Gly	Thr	Lys	Val	Glu	Ile	Lys
1				5			10		

<210> 45

<211> 30

<212> PRT

<213> ARTIFICIAL

<220>

<223> FRH1 VARIANT 136

<400> 45

Gln	Val	Gln	Leu	Val	Gln	Ser	Gly	Ala	Glu	Val	Met	Lys	Pro	Gly	Ser
1				5			10		15						

Ser	Val	Lys	Val	Ser	Cys	Lys	Ala	Ser	Gly	Tyr	Thr	Phe	Ser
				20				25			30		

<210> 46

<211> 30

<212> PRT

<213> ARTIFICIAL

<220>

<223> FRH1 VARIANT 2

<400> 46

Gln	Val	Gln	Leu	Val	Gln	Ser	Gly	Ala	Glu	Val	Lys	Lys	Pro	Gly	Ser
1				5			10		15						

Ser	Val	Lys	Val	Ser	Cys	Lys	Ala	Ser	Gly	Tyr	Thr	Phe	Ser
				20				25			30		

<210> 47

<211> 14

<212> PRT

<213> ARTIFICIAL

<220>

<223> FRH2 VARIANT 136

<400> 47

Trp Val Arg Gln Ala Pro Gly His Gly Leu Glu Trp Met Gly
1 5 10

<210> 48

<211> 14

<212> PRT

<213> ARTIFICIAL

<220>

<223> FRH2 VARIANT 2

<400> 48

Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met Gly
1 5 10

<210> 49

<211> 14

<212> PRT

<213> ARTIFICIAL

<220>

<223> FRH2 VARIANT 8

<400> 49

Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Val Gly
1 5 10

<210> 50

<211> 14

<212> PRT

<213> ARTIFICIAL

<220>

<223> FRH2 VARIANT 21

<400> 50

Trp Val Arg Gln Ala Pro Gly His Gly Leu Glu Trp Val Gly
1 5 10

<210> 51

<211> 32

<212> PRT

<213> ARTIFICIAL

<220>

<223> FRH3 VARIANT 136

<400> 51

Arg Val Thr Phe Thr Ala Asp Thr Ser Thr Ser Thr Ala Tyr Met Glu
1 5 10 15

Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg
20 25 30

<210> 52

<211> 32

<212> PRT

<213> ARTIFICIAL

<220>

<223> FRH3 VARIANT 1

<400> 52

Arg Ala Thr Phe Thr Ala Asp Thr Ser Thr Ser Thr Ala Tyr Met Glu
1 5 10 15Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg
20 25 30

<210> 53

<211> 32

<212> PRT

<213> ARTIFICIAL

<220>

<223> FRH3 VARIANT 43

<400> 53

Arg Val Thr Ile Thr Ala Asp Thr Ser Thr Ser Thr Ala Tyr Met Glu
1 5 10 15Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg
20 25 30

<210> 54

<211> 32

<212> PRT

<213> ARTIFICIAL

<220>

<223> FRH3 VARIANT 103

<400> 54

Arg Ala Thr Ile Thr Ala Asp Thr Ser Thr Ser Thr Ala Tyr Met Glu
1 5 10 15Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg
20 25 30

<210> 55

<211> 11

<212> PRT

<213> ARTIFICIAL

<220>

<223> FRH4 VARIANT 136

<400> 55

Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
1 5 10

<210> 56

<211> 19

<212> PRT

<213> Bacteriophage M13mp18

<220>

<221> misc_feature

<223> Gene III signal Sequence

<400> 56

Met Glu Trp Ser Gly Val Phe Met Phe Leu Leu Ser Val Thr Ala Gly

1 5 10 15

Val His Ser

<210> 57

<211> 107

<212> PRT

<213> ARTIFICIAL

<220>

<223> LIGHT CHAIN VARIABLE REGION OF CLONE 136

<400> 57

Glu	Ile	Val	Met	Thr	Gln	Ser	Pro	Ala	Thr	Leu	Ser	Val	Ser	Pro	Gly
1					5				10					15	

Glu	Arg	Ala	Thr	Leu	Ser	Cys	Arg	Ala	Ser	Gln	Ser	Ile	Gly	Thr	Asn
					20				25			30			

Ile	His	Trp	Tyr	Gln	Gln	Lys	Pro	Gly	Gln	Ala	Pro	Arg	Leu	Leu	Ile
					35			40			45				

Tyr	Tyr	Ala	Ser	Glu	Ser	Ile	Ser	Gly	Ile	Pro	Ala	Arg	Phe	Ser	Gly
					50			55			60				

Ser	Gly	Ser	Gly	Thr	Glu	Phe	Thr	Leu	Thr	Ile	Ser	Ser	Leu	Gln	Ser
65					70			75		80					

Glu	Asp	Phe	Ala	Val	Tyr	Tyr	Cys	Gln	Gln	Ser	Asp	Ser	Trp	Pro	Thr
					85			90			95				

Thr	Phe	Gly	Gly	Thr	Lys	Val	Glu	Ile	Lys						
					100			105							

<210> 58

<211> 106

<212> PRT

<213> ARTIFICIAL

<220>

<223> LIGHT CHAIN CONSTANT REGION OF CLONE 136

<400> 58

Thr	Val	Ala	Ala	Pro	Ser	Val	Phe	Ile	Phe	Pro	Pro	Ser	Asp	Glu	Gln
1						5		10			15				

Leu	Lys	Ser	Gly	Thr	Ala	Ser	Val	Val	Cys	Leu	Leu	Asn	Asn	Phe	Tyr
							20	25			30				

Pro	Arg	Glu	Ala	Lys	Val	Gln	Trp	Lys	Val	Asp	Asn	Ala	Leu	Gln	Ser
						35	40			45					

Gly	Asn	Ser	Gln	Glu	Ser	Val	Thr	Glu	Gln	Asp	Ser	Lys	Asp	Ser	Thr
							50			55			60		

Tyr	Ser	Leu	Ser	Ser	Thr	Leu	Thr	Leu	Ser	Lys	Ala	Asp	Tyr	Glu	Lys
65					70			75		80					

His	Lys	Val	Tyr	Ala	Cys	Glu	Val	Thr	His	Gln	Gly	Leu	Ser	Ser	Pro
					85			90		95					

Val	Thr	Lys	Ser	Phe	Asn	Arg	Gly	Glu	Cys						
					100			105							

<210> 59

<211> 123

<212> PRT

<213> ARTIFICIAL

<220>

<223> HEAVY CHAIN VARIABLE REGION OF CLONE 136

<400> 59

Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Met Lys Pro Gly Ser
1 5 10 15

Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Ser Met Tyr
20 25 30

Trp Leu Glu Trp Val Arg Gln Ala Pro Gly His Gly Leu Glu Trp Met
35 40 45

Gly Glu Ile Ser Pro Gly Thr Phe Thr Thr Asn Tyr Asn Glu Lys Phe
50 55 60

Lys Ala Arg Val Thr Phe Thr Ala Asp Thr Ser Thr Ser Thr Ala Tyr
65 70 75 80

Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Phe Ser His Phe Ser Gly Ser Asn Tyr Asp Tyr Phe Asp Tyr
100 105 110

Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
115 120

<210> 60

<211> 330

<212> PRT

<213> ARTIFICIAL

<220>

<223> CONSTANT REGION OF HUMAN IgG1

<400> 60

Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys

1 5 10 15

Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr
20 25 30

Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser
35 40 45

Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser
50 55 60

Leu Ser Ser Val Val Thr Val Pro Ser Ser Leu Gly Thr Gln Thr
65 70 75 80

Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn Thr Lys Val Asp Lys
85 90 95

Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys
100 105 110

Pro Ala Pro Glu Leu Leu Gly Pro Ser Val Phe Leu Phe Pro Pro
115 120 125

Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys
130 135 140

Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp
145 150 155 160

Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu
165 170 175

Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu
180 185 190

His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn
195 200 205

Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly
210 215 220

Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu
225 230 235 240

Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr
245 250 255

Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn
260 265 270

Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe
275 280 285

Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn
290 295 300

Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr
305 310 315 320

Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
325 330

<210> 61

<211> 107

<212> PRT

<213> ARTIFICIAL

<220>

<223> LIGHT CHAIN VARIABLE REGION OF CLONE CL-2C

<400> 61

Glu Ile Val Met Thr Gln Ser Pro Ala Thr Leu Ser Val Ser Pro Gly
 1 5 10 15

Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser Ile Gly Thr Asn
 20 25 30

Ile His Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu Ile
 35 40 45

Tyr Tyr Ala Ser Glu Ser Ile Ser Gly Ile Pro Ala Arg Phe Ser Gly
 50 55 60

Ser Gly Ser Gly Thr Glu Phe Thr Leu Thr Ile Ser Ser Leu Gln Ser
 65 70 75 80

Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Ser Trp Ser Trp Pro Thr
 85 90 95

Thr Phe Gly Gly Thr Lys Val Glu Ile Lys
 100

<210> 62

<211> 123

<212> PRT

<213> ARTIFICIAL

<220>

<223> HEAVY CHAIN VARIABLE REGION OF CLONE CL-2C

<400> 62

Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Met Lys Pro Gly Ser
 1 5 10 15

Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Ser Trp Tyr

20 25 30

Trp Leu Glu Trp Val Arg Gln Ala Pro Gly His Gly Leu Glu Trp Met

35 40 45

Gly Glu Ile Asp Pro Gly Thr Phe Thr Thr Asn Tyr Asn Glu Lys Phe

50 55 60

Lys Ala Arg Val Thr Phe Thr Ala Asp Thr Ser Thr Ser Thr Ala Tyr

65 70 75 80

Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys

85 90 95

Ala Arg Phe Ser His Phe Ser Gly Ser Asn Tyr Asp Tyr Phe Asp Tyr

100 105 110

Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser

115 120

<210> 63

<211> 107

<212> PRT

<213> ARTIFICIAL

<220>

<223> LIGHT CHAIN VARIABLE REGION OF CLONE CL-5I

<400> 63

Glu Ile Val Met Thr Gln Ser Pro Ala Thr Leu Ser Val Ser Pro Gly
 1 5 10 15

Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser Ile Gly Thr Asn
 20 25 30

Ile His Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu Ile
 35 40 45

Tyr Tyr Ala Ser Glu Ser Ile Ser Gly Ile Pro Ala Arg Phe Ser Gly
 50 55 60

Ser Gly Ser Gly Thr Glu Phe Thr Leu Thr Ile Ser Ser Leu Gln Ser
 65 70 75 80

Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Ser Trp Ser Trp Pro Thr
 85 90 95

Thr Phe Gly Gly Thr Lys Val Glu Ile Lys
 100 105

<210> 64

<211> 123

<212> PRT

<213> ARTIFICIAL

<220>

<223> HEAVY CHAIN VARIABLE REGION OF CLONE CL-5I

<400> 64

Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Met Lys Pro Gly Ser
 1 5 10 15

Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Ser Met Tyr
 20 25 30

Trp Leu Glu Trp Val Arg Gln Ala Pro Gly His Gly Leu Glu Trp Met
 35 40 45

Gly Glu Ile Asp Pro Gly Thr Phe Glu Thr Asn Tyr Asn Glu Lys Phe
 50 55 60

Lys Ala Arg Val Thr Phe Thr Ala Asp Thr Ser Thr Ser Thr Ala Tyr
 65 70 75 80

Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
 85 90 95

Ala Arg Phe Ser His Phe Ser Gly Ser Asn Tyr Asp Tyr Phe Asp Tyr
 100 105 110

Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
 115 120

<210> 65

<211> 107

<212> PRT

<213> ARTIFICIAL

<220>

<223> LIGHT CHAIN VARIABLE REGION OF CLONE CL-5A

<400> 65

Glu Ile Val Met Thr Gln Ser Pro Ala Thr Leu Ser Val Ser Pro Gly
 1 5 10 15

Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser Ile Gly Thr Asn
 20 25 30

Ile His Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu Ile
 35 40 45

Tyr Tyr Ala Ser Glu Ser Ile Ser Gly Ile Pro Ala Arg Phe Ser Gly
 50 55 60

Ser Gly Ser Gly Thr Glu Phe Thr Leu Thr Ile Ser Ser Leu Gln Ser
 65 70 75 80

Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Ser Trp Ser Trp Pro Thr
 85 90 95

Thr Phe Gly Gly Thr Lys Val Glu Ile Lys
 100 105

<210> 66

<211> 123

<212> PRT

<213> ARTIFICIAL

<220>

<223> HEAVY CHAIN VARIABLE REGION OF CLONE CL-5A

<400> 66

Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Met Lys Pro Gly Ser
 1 5 10 15

Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Ser Trp Tyr
 20 25 30

Trp Leu Glu Trp Val Arg Gln Ala Pro Gly His Gly Leu Glu Trp Met
 35 40 45

Gly Glu Ile Glu Pro Gly Thr Glu Thr Thr Asn Tyr Asn Glu Lys Phe
 50 55 60

Lys Ala Arg Val Thr Phe Thr Ala Asp Thr Ser Thr Ser Thr Ala Tyr
 65 70 75 80

Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
 85 90 95

Ala Arg Phe Ser His Phe Ser Gly Ser Asn Tyr Asp Tyr Phe Asp Tyr
 100 105 110

Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
 115 120

<210> 67

<211> 107

<212> PRT

<213> ARTIFICIAL

<220>

<223> LIGHT CHAIN VARIABLE REGION OF CLONE CL-2B

<400> 67

Glu Ile Val Met Thr Gln Ser Pro Ala Thr Leu Ser Val Ser Pro Gly
 1 5 10 15

Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser Ile Gly Thr Asn
 20 25 30

Ile His Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu Ile
 35 40 45

Tyr Tyr Ala Ser Glu Ser Ile Ser Gly Ile Pro Ala Arg Phe Ser Gly
 50 55 60

Ser Gly Ser Gly Thr Glu Phe Thr Leu Thr Ile Ser Ser Leu Gln Ser
 65 70 75 80

Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Ser Trp Ser Trp Pro Thr
 85 90 95

Thr Phe Gly Gly Thr Lys Val Glu Ile Lys
 100 105

<210> 68

<211> 123

<212> PRT

<213> ARTIFICIAL

<220>

<223> HEAVY CHAIN VARIABLE REGION OF CLONE CL-2B

<400> 68

Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Met Lys Pro Gly Ser
1 5 10 15Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Ser Tyr Tyr
20 25 30Trp Leu Glu Trp Val Arg Gln Ala Pro Gly His Gly Leu Glu Trp Met
35 40 45Gly Glu Ile Asp Pro Gly Thr Phe Thr Thr Asn Tyr Asn Glu Lys Phe
50 55 60Lys Ala Arg Val Thr Phe Thr Ala Asp Thr Ser Thr Ser Thr Ala Tyr
65 70 75 80Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95Ala Arg Phe Ser His Phe Ser Gly Ser Asn Tyr Asp Tyr Phe Asp Tyr
100 105 110Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
115 120

<210> 69

<211> 107

<212> PRT

<213> ARTIFICIAL

<220>

<223> LIGHT CHAIN VARIABLE REGION OF CLONE CL-1136-2C

<400> 69

Glu Ile Val Met Thr Gln Ser Pro Ala Thr Leu Ser Val Ser Pro Gly
1 5 10 15Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser Ile Gly Thr Asn
20 25 30Ile His Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu Ile
35 40 45Tyr Tyr Ala Ser Glu Ser Ile Ser Gly Ile Pro Ala Arg Phe Ser Gly
50 55 60Ser Gly Ser Gly Thr Glu Phe Thr Leu Thr Ile Ser Ser Leu Gln Ser
65 70 75 80Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Ser Trp Ser Trp Pro Thr
85 90 95Thr Phe Gly Gly Thr Lys Val Glu Ile Lys
100 105

<210> 70

<211> 123

<212> PRT

<213> ARTIFICIAL

<220>

<223> HEAVY CHAIN VARIABLE REGION OF CLONE CL-1136-2C

<400> 70

Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ser
 1 5 10 15

Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Ser Met Tyr
 20 25 30

Trp Leu Glu Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
 35 40 45

Gly Glu Ile Ser Pro Gly Thr Phe Thr Thr Asn Tyr Asn Glu Lys Phe
 50 55 60

Lys Ala Arg Val Thr Phe Thr Ala Asp Thr Ser Thr Ser Thr Ala Tyr
 65 70 75 80

Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
 85 90 95

Ala Arg Phe Ser His Phe Ser Gly Ser Asn Tyr Asp Tyr Phe Asp Tyr

100 105 110

Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
 115 120

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- [WO9962550A \[0007\]](#)
- [US5958706A \[0008\]](#)
- [EP0407392A \[0010\]](#)
- [US5449760A \[0010\]](#)
- [US6180379B \[0010\]](#)
- [US4816567A \[0065\] \[0066\]](#)
- [WO9316185A \[0068\]](#)
- [EP239400B1 \[0069\]](#)
- [WO9013646A \[0082\]](#)
- [US4965199A \[0088\]](#)
- [EP73657A \[0093\]](#)
- [US4419446A \[0095\]](#)
- [US4601978A \[0095\]](#)
- [WO9411026A \[0097\]](#)
- [US4767704A \[0103\]](#)
- [US4667866A \[0103\]](#)
- [US4560655A \[0103\]](#)
- [US5122469A \[0103\]](#)
- [US5712163A \[0103\]](#)
- [US6048728A \[0103\]](#)
- [US3773919A \[0114\]](#)
- [US4737456A \[0119\]](#)
- [US4376110A \[0124\]](#)
- [WO9404188A \[0131\]](#)
- [US2004002894W \[0180\]](#)
- [US60444239B \[0180\]](#)

Non-patent literature cited in the description

- **HASEGAWA, S.**Hematopoiesis, 1999, vol. 93, 2543-2551 [0003]
- **KLUBAL R. et al.**J. Invest. Dermatol., 1997, vol. 108, 3336-42 [0003]
- **DIERKS, A.E. et al.**J. Immunol., 1993, vol. 150, 2372-2382 [0004]
- **SANON, A. et al.**J. Allergy Clin. Immunol., 1990, vol. 86, 333-344 [0004]
- **BONNEFOY, J. et al.**Eur. Resp. J., 1996, vol. 9, 63s-66s [0004]
- **SUTTONGOULD**Nature, 1993, vol. 366, 421-428 [0004] [0005]
- **YANG, P.P.**J. Clin. Invest., 2000, vol. 106, 879-886 [0004]
- **PRESTA et al.**J. Biol. Chem., 1994, vol. 269, 26368-26373 [0005]
- **HENRY A.J. et al.**Biochemistry, 1997, vol. 36, 15568-15578 [0005]
- **SHI, J. et al.**Biochemistry, 1997, vol. 36, 2112-2122 [0005]
- **MALVEAUX et al.**J. Clin. Invest., 1978, vol. 62, 176- [0006]
- **MACGLASHAN et al.**J. Immunol., 1997, vol. 158, 1438-1445 [0006]
- **COME, J. et al.**J. Clin. Invest., 1997, vol. 99, 879-887 [0007]
- **RACINE-POON, A. et al.**Clin. Pharmacol. Ther., 1997, vol. 62, 675-690 [0007]
- **FAHY, J.V. et al.**Am. J. Resp. Crit. Care Med., 1997, vol. 155, 1824-1834 [0007]
- **BOULET, L. P.**Am. J. Resp. Crit. Care Med., 1997, vol. 155, 1835-1840 [0007]
- **MILGROM, E. et al.**N. Engl. J. Med., 1999, vol. 341, 1966-1973 [0007]
- **KOLBINGER et al.**Protein Engineering, 1993, vol. 6, 8971-980 [0008]
- **CHANG et al.**Biotechnology, 1990, vol. 8, 122-128 [0010]
- **WARD, E. S. et al.**Nature, 1989, vol. 341, 544-546 [0012]
- **CHOTHIA et al.**Nature, 1989, vol. 342, 887-883 [0013]
- **QUEEN et al.**Proc. Natl. Acad. Sci. USA, 1989, vol. 86, 10029-10033 [0015]
- **KABAT et al.**Sequences of Proteins of Immunological InterestNational Institute of Health19870000 [0034]
- **KOHLERMILSTEIN**Nature, 1975, vol. 256, 495- [0037]
- **FIELD et al.**Mol Cell. Biol., 1988, vol. 8, 2159-2165 [0044]
- **EVAN et al.**Mol Cell. Biol., 1985, vol. 5, 123610-3616 [0044]
- **PABORSKY et al.**Protein Engineering, 1990, vol. 3, 6547-553 [0044]
- **ED HARLOWDAVID LANE**Antibodies: A Laboratory ManualCold Spring Harbor Laboratory19880000 [0050]
- **ED HARLOWDAVID LANE**Antibodies, A Laboratory ManualCold Spring Harbor Laboratory19880000 [0060]
- **CHAMPEJ.**Biol. Chem., 1995, vol. 270, 1388-1394 [0060]
- **KOHLER et al.**Nature, 1975, vol. 256, 495- [0061]
- **GODING**Monoclonal Antibodies: Principles and PracticeAcademic Press19860000590-103 [0062]
- **KOZBAR**J. Immunol., 1984, vol. 133, 3001- [0063]
- **BRODEUR et al.**Monoclonal Antibody Production Techniques and ApplicationsMarcel Dekker, Inc.1987000051-63 [0063]
- **GODING**Monoclonal Antibodies: Principles and PracticeAcademic Press1986000059-103 [0064]
- **MORRISON et al.**Proc. Natl Acad. Sci. USA, 1984, vol. 81, 6851- [0065]
- **JONES**Nature, 1986, vol. 321, 522-525 [0066]
- **RIECHMAN et al.**Nature, 1988, vol. 332, 323-327 [0066]
- **VERHOEYENS et al.**Science, 1988, vol. 239, 1534-1536 [0066]
- **SIMS et al.**J. Immunol., 1993, vol. 151, 2296- [0067]
- **CHOTHIA et al.**J. Mol. Biol., 1987, vol. 196, 901- [0067]
- **CARTER et al.**Proc. Natl. Acad. Sci. USA, 1992, vol. 89, 4285- [0067]
- **PRESTA et al.**J. Immunol., 1993, vol. 151, 2623- [0067]
- **MORIMOTO et al.**Journal of Biochemical and Biophysical Methods, 1992, vol. 24, 107-117 [0068]
- **BRENNAN et al.**Science, 1985, vol. 229, 81- [0068]
- **CARTER et al.**Bio/Technology, 1992, vol. 10, 163-167 [0068] [0104]
- **AMIT et al.**Science, 1986, vol. 233, 747-753 [0069]
- **CHOTHIA**J. Mol. Biol., 1987, vol. 196, 901-917 [0069]
- **STINCHCOMB et al.**Nature, 1979, vol. 282, 39- [0069]
- **JONES**Genetics, 1977, vol. 85, 12- [0089]
- **YANIV**Nature, 1982, vol. 297, 17-18 [0096]
- **LUCKOW et al.**Bio/Technology, 1988, vol. 6, 47-55 [0100]

- **MILLER et al.** Genetic Engineering Plenum publishing 19860000 vol. 8, 277-279 [0100]
- **MSEDA et al.** Nature, 1985, vol. 315, 592-594 [0100]
- Tissue Culture Academic Press 19730000 [0101]
- **URLAUB et al.** Proc. Natl. Acad. Sci. USA, 1980, vol. 77, 4216- [0101]
- **HAM et al.** Meth. Enzymol., 1979, vol. 58, 44- [0103]
- **BARNES et al.** Anal. Biochem., 1980, vol. 102, 255- [0103]
- **LINDMARK et al.** J. Immunol Meth., 1983, vol. 62, 1-13 [0105]
- **GUSS et al.** EMBO J., 1986, vol. 5, 1567-1575 [0105]
- Remington's Pharmaceutical Sciences 19800000 [0107] [0113]
- Methods for the Preparation of Enzyme-Antibody Conjugates for Use in Enzyme Immunoassay **O'SULLIVAN et al.** Methods in Enzym. Academic press 19810000 vol. 73, 147-166 [0119]
- **ZOLA** Monoclonal Antibodies: A Manual of Techniques CRC Press, Inc. 19870000147-158 [0122]
- **LIOU et al.** J Immunol., 1989, vol. 143, 123967-75 [0174]

Patentkrav

1. Isoleret antistof eller antigenbindende fragment deraf, som omfatter en variabel region med letkæde, omfattende CDRL1, CDRL2 og CDRL3, og en variabel region med tungkæde, omfattende CDRH1, CDRH2 og CDRH3, hvor CDRL1 består af aminosyresekvensen angivet i SEQ ID NO:5, CDRL2 består af aminosyresekvensen angivet i SEQ ID NO:8, CDRL3 består af aminosyresekvensen QQSWSWPPTT, CDRH1 består af aminosyresekvensen angivet i SEQ ID NO:15, CDRH2 består af aminosyresekvensen angivet i SEQ ID NO:25, og CDRH3 består af aminosyresekvensen angivet i SEQ ID NO:26, hvor antistoffet eller det antigenbindende fragment deraf binder specifikt til IgE.
5
2. Antistof eller antigenbindende fragment deraf ifølge krav 1, hvor den variable region med letkæde omfatter aminosyresekvensen angivet i SEQ ID NO:63, eller hvor den variable region med tungkæde omfatter aminosyresekvensen angivet i SEQ ID NO:64.
10
3. Antistof eller antigenbindende fragment deraf ifølge krav 1 eller 2, hvor den variable region med letkæde har aminosyresekvensen angivet i SEQ ID NO:63, og den variable region med tungkæde har aminosyresekvensen angivet i SEQ ID NO:64.
15
4. Antistof eller antigenbindende fragment deraf ifølge et af kravene 1 til 3, hvor antistoffet eller det antigenbindende fragment deraf endvidere omfatter en konstant region med letkæde og en konstant region med tungkæde.
20
5. Antistof eller antigenbindende fragment deraf ifølge krav 4, hvor den konstante region med letkæde har aminosyresekvensen, der er angivet i SEQ ID NO:58, og den konstante region med tungkæde har aminosyresekvensen, der er angivet i SEQ ID NO:60.
25
6. Antistof eller antigenbindende fragment deraf ifølge et af kravene 1 til 5, endvidere forbundet med en markering.
30

7. Sammensætning, som omfatter en terapeutisk virksom mængde af antistoffet eller det antigenbindende fragment deraf ifølge et af kravene 1 til 5 og et farmaceutisk acceptabelt bærestof.

5 8. Diagnosekit, som omfatter antistoffet eller det antigenbindende fragment deraf ifølge et af kravene 1 til 6.

10 9. Fremgangsmåde til måling af niveauet af IgE i et individ, hvilken fremgangsmåde omfatter at bringe en prøve fra individet, hvilken prøve omfatter IgE-molekyler, i kontakt med antistoffet eller det antigenbindende fragment deraf ifølge et af kravene 1 til 6; og at bestemme retentionsniveauet af antistoffet eller det antigenbindende fragment deraf via prøven i forhold til en kontrolprøve fra et kontrolindivid, hvor et højere eller lavere retentionsniveau af antistoffet eller det antigenbindende fragment deraf via prøven fra individet i forhold til kontrolprøven viser, at individet har et højere eller lavere niveau af IgE-molekyler i forhold til det i kontrolindividet.

15 10. Fremgangsmåde til diagnosticering af en sygdom associeret med et abnormt niveau af IgE i et individ, hvilken fremgangsmåde omfatter at bringe en prøve fra individet, hvilken prøve omfatter IgE-molekyler, i kontakt med antistoffet eller det antigenbindende fragment deraf ifølge et af kravene 1 til 6; og at bestemme retentionsniveauet af antistoffet eller det antigenbindende fragment deraf via prøven i forhold til en kontrolprøve af et kontrolindivid, hvor et højere eller lavere retentionsniveau af antistoffet eller det antigenbindende fragment deraf via prøven fra individet i forhold til kontrolprøven viser, at individet har en sygdom associeret med et abnormt niveau af IgE.

20 11. Antistof eller antigenbindende fragment deraf ifølge et af kravene 1 til 5 til anvendelse i en fremgangsmåde til behandling af en sygdom associeret med et abnormt højt niveau af IgE i et individ.

25 12. Fremgangsmåde ifølge krav 10 eller antistof eller antigenbindende fragment deraf til anvendelse ifølge krav 11, hvor sygdommen er astma, allergisk rhinitis, eksem, nældefeber, atopisk dermatitis eller en fødevareallergi.

13. Vektor, omfattende en nukleinsyre, der koder antistoffet eller det antigen-bindende fragment deraf ifølge et af kravene 1 til 6.

14. Celle, som omfatter vektoren ifølge krav 13.

5

15. Celle ifølge krav 14, hvor cellen er ATCC deponeringsnummer PTA-5680.

10

DRAWINGS

Figure 1 Phage Vector

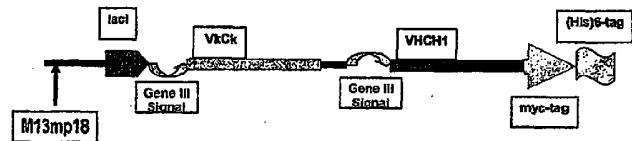


FIGURE 2

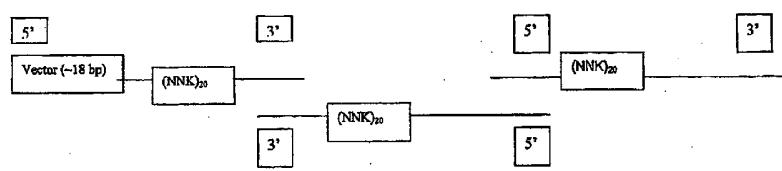


Figure 3 TES-C21 and Template Comparison

A. Light Chain (V_κ) (Underlined: Kabat CDR; Bold/italic: Chothia CDR) (TES-C21 LIGHT CHAIN – SEQ ID NO 1 L16/JK4 – SEQ ID NO2)

Mu TES-C21	D I L L T Q S P A I L S V S P G E R V S F S C R A S Q S I G T N I H W Y Q Q
L16 VH	B V N T A T L R A S Q S V S S N L R

40	50	60	70
TES-C21	R T D G S P R L L I K Y A S E S I S G I P S R F S G S G S G T B F T L N I N		
L16 VH	K P G Q A Y G A S T R A T A		

80	90	100	107
TES-C21	S V E S S D I A D Y Y C Q Q S D S W F T T F G G G T K L E I K		
L16 VX-JK4	L Q F V Q Q Y H N W P L T V		

B. Heavy Chain (V_λ)

TES-C21 HEAVY CHAIN – SEQ ID NO 3 DP88/JH4b – SEQ ID NO 4

1	10	20	30
Murine TES-C21	Q V Q L Q Q S G A E L M K P G A S V K I S C K T T G Y T F S M Y W L E W V		
DP88 VH	V V K S V A S G S Y A I S		

40	50	52a 53	60	65	70
TES-C21	K Q F P G H G L E W V G E I S P G T E T T N Y H E K F K A K A T F T A D T				
DP88 VH	R A Q M G I P Y F G T A N Y A Q K F O G R V I K				

80	82a b c 83	90	95	100 b c d e f 101
TES-C21	S S N T A Y L Q L S G L T S E D S A V Y F C A R F S H F S G S N Y D Y F D Y			
DP88 VH	T S M E S R T Y - - - - - X F D Y			

103	110	113
TES-C21	W Q G T S L T V S S	
DP88-JH4b	L V	

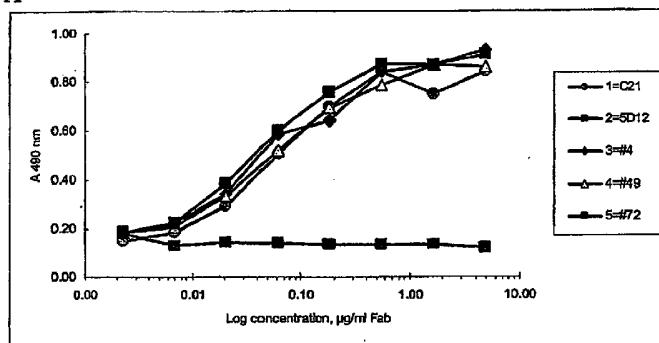
Figure 4 Framework Sequences of High Affinity Candidates*

VK Positions Mouse Human	1 3 4 49 60 85							VH Positions Mouse Human								
	D	L	L	K	S	D	M	Y	A	V	M	Y	H	M	A	F
1 (clone ID)	D	L	M	K	S	V	M	Y	H	M	A					
2	D	V	L	K	A	V	K	Y	Q	M	V					
4	D	D	L	L	Y	S	M	Y	Q	M	V					
8	D	V	M	Y	S	V	M	Y	Q	V	A					
13	D	D	L	M	Y	A	D	M	Y	Q	V	V				
15	D	D	L	L	K	S	D	M	Y	Q	M	V	V			
16	D	D	L	L	K	S	D	M	Y	Q	M	V	V			
18	D	D	V	L	K	S	D	M	Y	Q	V	V	V			
21	D	D	V	L	K	A	D	M	Y	Q	V	A	V			
23	D	D	V	L	M	Y	A	M	Y	Q	V	V	V			
25	D	D	V	L	M	Y	A	M	Y	Q	V	A	V			
27	D	D	V	L	M	Y	S	M	Y	Q	V	V	V			
30	D	D	V	L	M	Y	A	D	K	Y	Q	V	V			
31	D	D	V	L	M	Y	S	V	M	Y	H	M	V	V		
33	D	D	V	L	M	Y	A	D	M	Y	H	M	V	V		
35	D	D	V	L	M	Y	S	D	M	Y	H	M	V	V		
38	D	D	V	L	M	K	A	D	K	Y	H	M	V	V		
43	D	D	V	L	M	Y	S	D	M	Y	H	M	V	V		
44	D	D	V	L	M	Y	A	D	M	Y	H	M	V	V		
45	D	D	V	V	M	K	A	V	M	Y	Q	M	V	V		
46	D	D	V	V	M	Y	A	D	M	Y	H	M	V	V		
48	D	D	V	V	M	Y	A	D	M	Y	H	M	V	V		
49	D	D	V	V	M	Y	A	D	M	Y	H	M	V	V		
50	D	D	V	V	M	Y	A	D	M	Y	H	M	V	V		
52	D	D	V	V	L	Y	A	D	K	Y	Q	M	V	V		
53	D	D	V	V	L	Y	S	V	M	Y	H	M	A	V		
56	D	D	V	V	L	M	Y	S	M	Y	H	M	V	V		
58	D	D	V	V	L	M	Y	S	M	Y	H	M	V	V		
61	D	D	V	V	L	M	K	S	M	Y	H	M	V	V		
63	D	D	V	V	L	M	K	S	D	M	Y	Q	V	V		
64	D	D	V	V	L	M	K	S	M	Y	Q	V	V	V		
68	D	D	V	V	L	M	K	S	D	M	Y	Q	V	V		
70	D	D	V	V	L	M	K	S	D	M	Y	Q	M	A		
72	D	D	V	V	L	M	K	S	D	M	Y	Q	M	A		
75	D	D	V	V	L	M	K	S	D	M	Y	Q	M	A		
76	D	D	V	V	L	M	K	S	D	M	Y	Q	M	A		
81	D	D	V	V	L	M	K	S	D	M	Y	Q	M	A		
83	D	D	V	V	L	M	K	S	D	M	Y	Q	M	A		
86	D	D	V	V	L	M	K	S	D	M	Y	Q	M	A		
89	D	D	V	V	L	M	K	S	D	M	Y	Q	M	A		
90	D	D	V	V	L	M	K	S	D	M	Y	Q	M	A		
93	D	D	V	V	L	M	K	S	D	M	Y	Q	M	A		
103	D	D	V	V	L	M	K	S	D	M	Y	Q	M	A		
109	D	D	V	V	L	M	K	S	D	M	Y	Q	M	A		
114	D	D	V	V	L	M	K	S	D	M	Y	Q	M	A		
124	D	D	V	V	L	M	K	S	D	M	Y	Q	M	A		
128	D	D	V	V	L	M	K	S	D	M	Y	Q	M	A		
135	D	D	V	V	L	M	K	S	D	M	Y	Q	M	A		
136	D	D	V	V	L	M	K	S	D	M	Y	Q	M	A		
152	D	D	V	V	L	M	K	S	D	M	Y	Q	M	A		
153	D	D	V	V	L	M	K	S	D	M	Y	Q	M	A		
157	D	D	V	V	L	M	K	S	D	M	Y	Q	M	A		
1136-2C	E	V	M	M	Y	S	D	K	Y	Q	M	M	V	V		

*P=parent sequence; T=human template sequence; amino acids listed in bold are murine residues

Figure 5 ELISA TITRATION CURVE

A



B

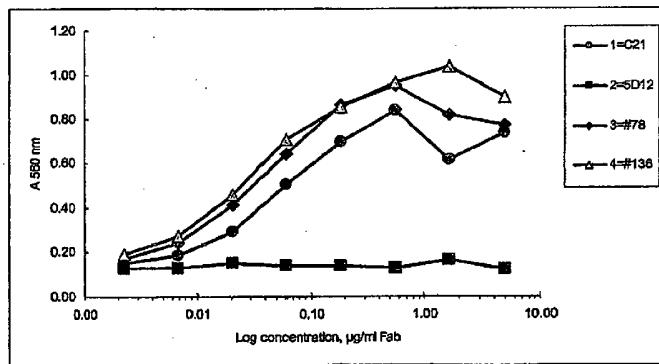


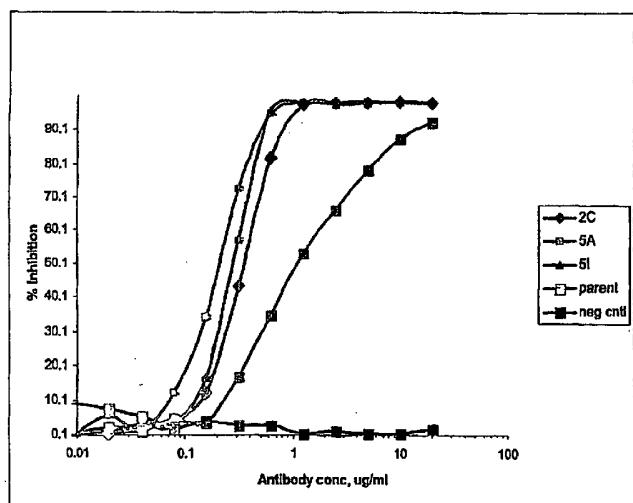
Figure 6 Inhibition Assay

Figure 7: List of High Affinity Candidates from Library

Clone	CDR-L1	CDR-L2	CDR-L3	CDR-H1	CDR-H2	CDR-H3
136	RASRSIGTNH	YASESIS	QQSWSWPITT	MYWLE	EIEPGTFTTINYNEKFKA	FSHFSGGSNYDVFDV
L3-9	wt	wt	QQSWSWPITT	wt	wt	wt
CL-2G	wt	wt	QQSWSWPITT	WWWLE	wt	wt
R5-E	wt	wt	QQSWSWPITT	YYWLE	wt	wt
R87-E	wt	wt	QQSWSWPITT	wt	EIEPGTFTTINYNEKFKA	wt
CL-2I	wt	wt	QQSWSWPITT	wt	EIDPGTFTTINYNEKFKA	wt
R2-C	wt	wt	QQSWSWPITT	WYWLE	EIEPGTFTTINYNEKFKA	wt
CL-2C	wt	wt	QQSWSWPITT	WWWLE	EIDPGTFTTINYNEKFKA	wt
CL-2H	wt	wt	QQSWSWPITT	YYWLE	EIEPGTFTTINYNEKFKA	wt
CL-2B	wt	wt	QQSWSWPITT	YYWLB	EIDPGTFTTINYNEKFKA	wt
CL-3A	wt	wt	QQSWSWPITT	wt	EISPETFTTINYNEKFKA	wt
R47-E	wt	wt	QQSWSWPITT	wt	EISPDFTFTINYNEKFKA	wt
CL-3G	wt	wt	QQSWSWPITT	YYWLE	EISPETFTTINYNEKFKA	wt
R3-A	wt	wt	QQSWSWPITT	YYWLE	EISPDFTFTINYNEKFKA	wt
CL-3C	wt	wt	QQSWSWPITT	WWWLE	EISPETFTTINYNEKFKA	wt
CL-3B	wt	wt	QQSWSWPITT	WWWLB	EISPDFTFTINYNEKFKA	wt
R5-K	wt	wt	QQSWSWPITT	wt	EISPGTFTTINYNEKFKA	wt
CL-4B	wt	wt	QQSWSWPITT	WWWLE	EISPGTFTTINYNEKFKA	wt
R5-D	wt	wt	QQSWSWPITT	YYWLE	EISPGTFTTINYNEKFKA	wt
CL-5G	wt	wt	QQSWSWPITT	wt	EIEPGTFTTINYNEKFKA	wt
CL-5I	wt	wt	QQSWSWPITT	wt	EIDPGTFTTINYNEKFKA	wt
CL-5A	wt	wt	QQSWSWPITT	WWWLE	EIEPGTFTTINYNEKFKA	wt
CL-5H	wt	wt	QQSWSWPITT	WWWLB	EIDPGTFTTINYNEKFKA	wt
R5-H	wt	wt	QQSWSWPITT	YYWLE	EIEPGTFTTINYNEKFKA	wt
RS-N	wt	wt	QQSWSWPITT	YYWLE	EIDPGTFTTINYNEKFKA	wt

Changes from parent amino acid sequence are listed in **BOLD**.

FIGURE 8A

FIGURE 8B

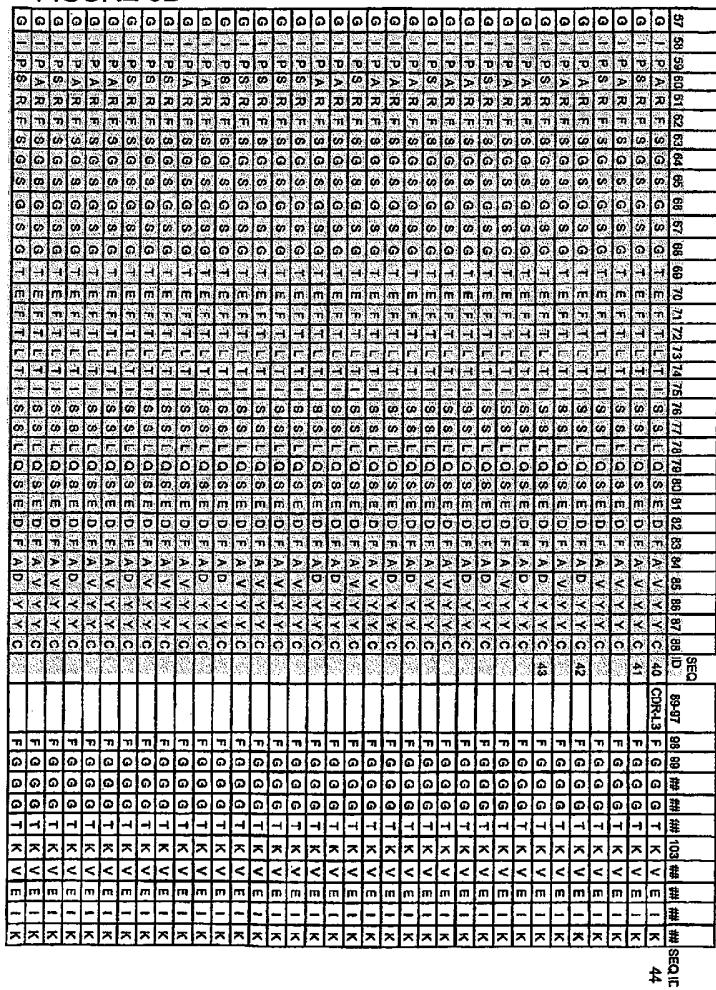


FIGURE 9A

VH alignments	
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 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55
18	Q V Q L V Q S G A E V M K P G S S V K V S C K A S G Y T F S 45 D R Q A P G H G L E W M G 47 C R R H Z
1	Q V Q L V Q S G A E V M K P G S S V K V S C K A S G Y T F S
2	Q V Q L V Q S G A E V K K P G S S V K V S C K A S G Y T F S 46 W V R Q A P G G L E W M G 48
4	Q V Q L V Q S G A E V M K P G S S V K V S C K A S G Y T F S
8	Q V Q L V Q S G A E V M K P G S S V K V S C K A S G Y T F S
13	Q V Q L V Q S G A E V M K P G S S V K V S C K A S G Y T F S
15	Q V Q L V Q S G A E V M K P G S S V K V S C K A S G Y T F S
21	Q V Q L V Q S G A E V M K P G S S V K V S C K A S G Y T F S
30	Q V Q L V Q S G A E V K K P G S S V K V S C K A S G Y T F S
31	Q V Q L V Q S G A E V M K P G S S V K V S C K A S G Y T F S
36	Q V Q L V Q S G A E V K K P G S S V K V S C K A S G Y T F S
43	Q V Q L V Q S G A E V M K P G S S V K V S C K A S G Y T F S
44	Q V Q L V Q S G A E V M K P G S S V K V S C K A S G Y T F S
53	Q V Q L V Q S G A E V M K P G S S V K V S C K A S G Y T F S
54	Q V Q L V Q S G A E V M K P G S S V K V S C K A S G Y T F S
55	Q V Q L V Q S G A E V M K P G S S V K V S C K A S G Y T F S

FIGURE 9B

Figure 10A Polypeptide sequence of Mab 136

LIGHT CHAIN

SIGNAL PEPTIDE

MEWSGVFMFLLSVTAGVHS (SEQ ID NO 56)

VARIABLE REGION V_L

EIVMTQSPAT LSVSPGERAT LSCRASQSIG TNIHWYQQKP GQAPRLLIYY
 ASESISGIPA RFSGSGSGTE FTIITISLQS EDFAVVYCCQQ SDSWPTTFGG
 GTKVEIK (SEQ ID NO 57)

CONSTANT REGION C_L

TVAAPSVFIF PPSDEQLKSG TASVVCLLNN FYPREAKVQW KVDNALQSGN
 SQESVTEQDS KDSTYSLSS LTLSKADYEK HKVYACEVTH QGLSSPVTKS
 FNRGEC (SEQ ID NO 58)

HEAVY CHAIN (IgG1)

SIGNAL PEPTIDE

MEWSGVPMFLLSVTAGVHS (SEQ ID NO 56)

VARIABLE REGION V_H

QVQLVQSGAR VMKPQSSVKV SCKASGYTFS MYWLEWVRQA PGHGLEWMGE
 ISPGTFTTNY NEKKKARVTF TADTSTSTAY MELSSLRSED TAVYYCARFS
 HFSGSNYDVF DYNGQQGTLVT VSS (SEQ ID NO 59)

CONSTANT REGION CH_1 -3(IgG1) (SEQ ID NO 60)

ASTKGPSVFP LAPSSKSTSG GTAALGCLVK DYFPEPVTVS WNSGALTSGV
 HTFPAVLQSS GLYSLSSVVT VPSSSLGTQY YICNVNHKPS NTKVDKKKVEP
 KSCDKTHTCP PCPAABELLGG PSVFLFPKPK KDTLMISRTP EYTCVVVDVS
 HEDPEVKFPN WYDGVVEVHNKA KTKPREEQYN STYRVVSVLT VLHQDWLNGK
 EYKCKVSNKA LPAPIEKTIIS KAKGQPREPQ VYTLPSSRDE LTKNQVSLTC
 LVKGKFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSSFFLY SKLTVDKSRW
 QQGNVPSCSV MHEALHNHYT QKSLSLSPGK

Figure 10B Polypeptide Sequence of MAb CL-2C

LIGHT CHAIN**SIGNAL PEPTIDE**

MEWSGVFMFLLSVTAGVHS (SEQ ID NO 56)

VARIABLE REGION VRBIVMTQSPAT LSVSPGERAT LSCRASQSIG TNIHWYQQKP GQAPRLLIYY
ASESISGIPA RFSGSGSGTE FTLTISSSLQS EDRAVYYCQQ SWSWPTTFGG
GTKVEIK (SEQ ID NO 61)**CONSTANT REGION CK**TVAAPSVFIF PPSDEQLKSG TASVVCLLNN FYPREAKVQW KVDNALQSGN
SQESVTEQDS KDSTYSLSSST LTL SKADYEK HKVYACEVTH QGLSSPVTKS
FNRGEC (SEQ ID NO 58)**HEAVY CHAIN (IgG1)****SIGNAL PEPTIDE**

MEWSGVFMFLLSVTAGVHS (SEQ ID NO 56)

VARIABLE REGION VH (2C)QVQLVQSGAE VMKPGSSVKV SCKASGYTFS WYWLEWVRQA PGHGLEWMGE
IDPGTFTTNY NEKFKARVTF TADTSTSTAY MELSSLRSED TAVYYCARFS
HPSGSNYDYP DYWGQQTLVT VSS (SEQ ID NO 62)**CONSTANT REGION CH1-3(IgG1) (SEQ ID NO 60)**ASTKGPSVFP LAPSSKSTSG GTAALGCLVK DYFPPEPVTVS WNSGALTSGV
HTFPAVLQSS GLYSLSVVIT VPSSSLGTQT YICNVNHNKPS NTKVDKKVEP
KSCDKTHTCR PCPAPELLGG PSVFLFPKPKD TLMISRTP EVTCVVVDVS
HEDPEVKFNW YVDGVVEVHNA KTKPREEQYN STYRVVSVLT VLIHQDWLNGK
EYKCKVSNKA LPAPIEKTIIS KAKGQPREPQ VYTLPSSRDE LTKNQVSLTC
LVKGFYPSDI AVEVESNGOP ENNYKTTPPV LDSDGSSFFLY SKLTVDKSRW
QQGNVFCSV MHEALHNHYT QKSLSLSPGK

Figure 10C Polypeptide Sequence of MAb CL-51

LIGHT CHAIN

SIGNAL PEPTIDE

MEWSGVFMFLLSVTAGVHS (SEQ ID NO 56)

VARIABLE REGION VK

EIVMTQSPAT LSVSPGERAT LSCRASQSIG TNIRHWYQQKP GQAPRLLIYY
ASESISGIPA RFSGSGSGTE FTLTISLQS EDFAVYYCQQ SWSWPTTFGG
GTKWEIK (SEQ ID NO 63)

CONSTANT REGION CK (SEQ ID NO 58)

TVAAPSVFIF PPSDEQLKSG TASVVCLLNN FYPREAKVQW KVDNALQSGN
SQESVTEQDS KDSTYSLSST LTLSKADYEK HKVYACEVTH QGLSSPVTKS
FNRGECHEAVY CHAIN (IgG1)

SIGNAL PEPTIDE

MEWSGVFMFLLSVTAGVHS (SEQ ID NO 56)

VARIABLE REGION VH (51)

QVQLVQSGAE VMKPGSSVKV SCKASGYTFS MYWLEWVRQA PGHGLEWMGE
IDPGTFRTNY NEKFKARVTF TADTSTSTAY MELSSLRSED TAVYYCARFS
HFSGSNYDVF DYWGCGTLVT VSS (SEQ ID NO 64)

CONSTANT REGION CH1-3(IgG1) (SEQ ID NO 60)

ASTKGPSVFP LAPSSKSTSG GTAALGCLVK DYFPEPVTVS WNSGALTSGV
HTPPAVLQSS GLYSLSSVVT VPSSSLGTQY YICNVNHKPS NTKVDKKVEP
KSCDKTHTCP PCPAPELLGG PSVFLPPKPK DDTLMISRTP EVICVVVVDVS
HEDEVKFENW YVDGVEVHNAA KTKPREEQYN STYRVSVLT VLIHQDWLNGK
EYKCKVSNKA LPAPIEKTIK KAKGQPREPQ VYTLPPSRDE LTKNQVSLTC
LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSSFFLY SKLTVDKSRW
QQGNVFSCSV MHEALHNHYT QKSLSLSPFGK

Figure 10D Polypeptide Sequence of MAb CL-5A**LIGHT CHAIN****SIGNAL PEPTIDE**

MEWSGVFMFLLSVTAGVHS (SEQ ID NO 56)

VARIABLE REGION V_kEIVMTQSPAT LSVPGERAT LSCRASQSIG TNIHWYQQKP GQAPRLLIYY
ASESISGIPA RFSGSGSCTE FTLTISSLQS EDPAVYYCQQ SWSWPITFGG
GTKVEIK (SEQ ID NO 65)**CONSTANT REGION C_k** (SEQ ID NO 58)TVAAPSVFIF PPSDEQLKSG TASVVCLLNN FYFREAKVQW KVDNALQSGN
SQESVTEQDS KDSTYSLSST LTLSKADYEK HKVYACEVTH QGLSSPVTKS
FNRGEC**HEAVY CHAIN (IgG1)****SIGNAL PEPTIDE**

MEWSGVFMFLLSVTAGVHS (SEQ ID NO 56)

VARIABLE REGION V_H (5A)QVQLVQSGAE VMKPGSSVKV SCKASGYTFS WYWLEWVRQA PGHGLEWMGE
IEPGTETTNY NEKFKARVTP TADTSTSTAY MELSSLESED TAVYYCARFS
HFSGSNYDYF DYWGQGTIVT VSS (SEQ ID NO 66)**CONSTANT REGION CH1-3(IgG1)** (SEQ ID NO 60)ASTKGPSVFP LAPESSKSTSC GAAALGCLVK DYPPEPVTVS WNSGALTSGV
HTFPAPVLQSS GLYSLSSVVT VPSSSLGQT YTCNVNHHKPS NTKVDKKVEP
KSCDKTHTCP PCFAPEELLGG PSVPLFPPPKP KDTLMISRTP EVTCVVVDVS
HEDPEVKFNW YVDGVEVHNA KTKPREEQYN STYRVVSVLT VLHQDWLNKG
EYKCKVSNKA LPAPIEKTIS KAKGQPREFQ VYTLPPSRD3 LTKNQVSLTC
LVKGFPYPSDI AVEWESNGQI ENNYKTTTPPV LDSDGSFFLY SKLTVDKSRW
QQGNVFSCSV MHEALHNHYT QKSLSLSPGK

Figure 10E Polypeptide Sequence of MAb CL-2B

LIGHT CHAIN**SIGNAL PEPTIDE**

MEWSGVFMFLLSVTAGVHS (SEQ ID NO 56)

VARIABLE REGION VKEIVMTQSPAT LSVSPGERAT LSCRASQSIG TNIHWYQQKP GQAPRLLIYY
ASSEISGIPA RFSGSGSGTE FTTLTISLQS EDFAVYYCQQ SWSWPTTFGG
GTKVEIK (SEQ ID NO 67)

CONSTANT REGION CK (SEQ ID NO 58)

TVAAPSVFIF PPSDEQLKSG TASVVCALLNN FYPREAKVQW KVDNALQSGN
SQESVTZQDS KDSTYSLSSST LTLISKADYEK HKVYACEVTH QGLSSPVTKS
FNRGEC**HEAVY CHAIN (IgG1)****SIGNAL PEPTIDE**

MEWSGVFMFLLSVTAGVHS (SEQ ID NO 56)

VARIABLE REGION VH (2B)QVOLVQSGAE VMXPGSSVKV SCKASGYTFS YYWLEWVRQA PGHGLEWMGE
IDPGTFTTNY NEKFKARVTF TADTSTSTAY MELSSLRSED TAVYYCARFS
HPSGSNYDYP DYWCGQTLVT VSS (SEQ ID NO 68)

CONSTANT REGION CH1-3(IgG1) (SEQ ID NO 60)

ASTKGPSVFP LAPSSKSTSG GTAALGCLVK DYPPEPVTVS WNSGALTSGV
HTPPAVLQSS GLYSLSSVVT VPSSSLGTQT YICNVNHKPS NTKVDKKKVEP
KSCDKTHTCP PCPAPELGG PSVFLPPKP KDTLMISRTP EVTCAVVDVS
HEDPEVKFNW YVDGVEVHNA KTKPREEQYN STYRVVSVLVT VLHQDWLNGK
EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ VYTLPPSRDE LTKNOVSLTC
LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SKLTVDKSRW
QQGNVFSCSV MHEALHNHYT QKSLSLSPGK

Figure 10F Polypeptide Sequence of MAb CL-1136-2C

LIGHT CHAIN**SIGNAL PEPTIDE**

MEWSGVFMFLLSVTAGVHS (SEQ ID NO 56)

VARIABLE REGION VKBIVMTQSPAT LSVSPGERAT LSCRASQSIG TNIHWYQQKP GQAPRLLIYY
ASESISGIPA RFSGSGSGTS FTIITSSLQS EDFAVYYCQQ SWSWPTTFGG
GTKVEIK (SEQ ID NO 69)**CONSTANT REGION CK** (SEQ ID NO 58)TVAAPSVFIF PPSDEQLKSG TASVVCCLNN FYPREAKVQW KVDNALQSGN
SQESVTEQDS KDSTYSLSST LTLSKADYEK HKVYACEVTH QGLSSPVTKS
FNRGEC**HEAVY CHAIN (IgG1)****SIGNAL PEPTIDE**

MEWSGVFMFLLSVTAGVHS (SEQ ID NO 56)

VARIABLE REGION (VH)QVQLVQSGAE VKKPGSSVKV SCKASGYTFS WYWLEWVRQA PGQGLEWMGE
ISPQTFTINY NEKKVKARVTF TADITSTSTAY MELSSLRSED TAVYYCARFS
HFSGSNYDVF DYWGQGTLVT VSS (SEQ ID NO 70)**CONSTANT REGION CH1-3(IgG1)** (SEQ ID NO 60)ASTKGPSVFP LAPSSKSTSG GTAALGCLVK DYFPEPVTVS WNSGALTSGV
HTPAVQLQSS GLYSLSSVVT VPSSSLGTQT YICNVNHHKPS NTKVDKKVEP
KSCDKTHTCP PCPAPELLGG PSVFLFPPKP KDTIMISRTP EVTCVVVDVS
HEDEPVKPNW YVDGVEVHNA KTKPREEQYN STYRVVSVLT VLIHQDWLNGK
SYKCKVSNKA LPAPIEKCTIS KARGQPREPQ VYTLPPSRDE LTKNQVSLTC
LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SKLTVDKSRW
QQGNVFSCSV MHEALHNHYT QKSLSLSPGK