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(57) Abstract:



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HUMANIZED ANTI-CD20 ANTIBODIES AND METHODS OF USE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application
5 Nos. 61/152,778 and 61/153,499, filed February 16, 2009 and February 18, 2009,
respectively; the entire contents of these applications are hereby incorporated by
reference herein.

REFERENCE TO SEQUENCE LISTING SUBMITTED VIA COMPUTER

10 READABLE CD

[0002] This application is being filed via Express Mail and includes a sequence
listing in .txt format on a computer readable CD. The .txt file contains a sequence
listing entitled "13790-90.txt " created on February 15, 2010 and is 92,065 bytes in
size. The sequence listing contained in this .txt file is part of the specification and is
15 hereby incorporated by reference herein in its entirety.

FIELD OF THE INVENTION

[0003] The invention relates to humanized anti-CD20 antibodies and their use in
the treatment of various diseases and conditions. The invention also relates to nucleic
20 acids encoding such antibodies, methods of making such antibodies, and compositions
comprising such antibodies.

BACKGROUND

[0004] Human CD20 (also called human B-lymphocyte-restricted differentiation
25 antigen as well as Bp35) is a hydrophobic transmembrane protein with a molecular
weight of approximately 35 kilodaltons located on pre-B and mature B lymphocytes.
CD20 is not found on hematopoietic stem cells, pro-B cells, normal plasma cells or
other normal tissues.

[0005] Human CD20 is a good target for immunotherapy of B cell neoplasms
30 because it is expressed on the surface of over 90% of malignant B cells but not
expressed on hemopoietic stem cells, normal plasma cells, myeloid, T lineage,
endothelial, or other nonlymphoid cells. Human CD20 is also a good target for
immunotherapy of autoimmune diseases, as B-cell depletion has been shown to be an

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effective strategy for treating autoimmune diseases. For example, rituximab, the chimeric anti-CD20 antibody C2B8 (available commercially as RITUXAN® and marketed by Biogen Idec Inc. and Genentech, Inc.), is approved for various indications, including non-Hodgkin's lymphoma and rheumatoid arthritis.

- 5 [0006] Upon binding antibody, CD20 does not significantly modulate nor is it shed. A plethora of antibody effector functions have been shown to be recruited by anti-CD20 antibodies, including antibody-dependent cell-mediated cytotoxicity (ADCC) by mononuclear effector cells, complement-dependent lysis, initiation of intracellular signals such as calcium fluxes, inhibition of cell growth, and induction of
10 cell differentiation. Anti-CD20 antibodies have also been shown to induce apoptosis of malignant B cell lines, especially after intensive cross-linking, for example, by cells expressing receptors for the Fc domain of IgG (FcγR).

[0007] The anti-CD20 monoclonal antibodies rituximab, anti-B1, and 1F5 have similar apoptotic effects on B cell lines.

- 15 [0008] Limitations of current murine and chimeric antibodies include the human anti-mouse antibody (HAMA) response and the human anti-chimeric antibody (HACA) response.

BRIEF SUMMARY

- 20 [0009] In one aspect, a humanized antibody is provided that comprises:
(a) a light chain comprising a light chain variable region comprising an amino acid sequence selected from the group consisting of:

- (1) Glu Ile Val Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Ser Ser Leu Ser Phe Met His Trp Tyr Gln
25 Gln Lys Pro Gly Lys Ala Pro Lys Xaa₄₅ Xaa₄₆ Ile Tyr Ala Thr Ser Asn Leu Ala Ser
Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Xaa₇₀ Thr Leu Thr Ile Ser
Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Xaa₈₆ Cys His Gln Trp Ser Ser Asn Pro
Leu Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys (SEQ ID NO: 1)

wherein

- 30 Xaa₄₅ is Pro or Leu;
Xaa₄₆ is Trp or Leu;
Xaa₇₀ is Tyr or Phe; and
Xaa₈₆ is Tyr or Phe; and

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(2) Glu Ile Val Leu Thr Gln Ser Pro Gly Thr Leu Ser Leu Ser Pro
 Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Ser Ser Leu Ser Phe
 Met His Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Xaa₄₅
 Xaa₄₆ Ile Tyr Ala Thr Ser Asn Leu Ala Ser Gly Ile Pro Ala Arg
 5 Phe Ser Gly Ser Gly Ser Gly Thr Asp Xaa₇₀ Thr Leu Thr Ile Ser
 Arg Leu Glu Pro Glu Asp Phe Ala Val Tyr Phe Cys His Gln Trp
 Ser Ser Asn Pro Leu Thr Phe Gly Gln Gly Thr Lys Val Glu Leu
 Lys (SEQ ID NO: 2)

wherein

10 Xaa₄₅ is Pro or Leu;
 Xaa₄₆ is Trp or Leu; and
 Xaa₇₀ is Tyr or Phe; and

(b) a heavy chain comprising a heavy chain variable region comprising the
 following amino acid sequence:

15 Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala
 Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Tyr Asn
 Met His Trp Val Arg Gln Ala Pro Gly Gln Arg Leu Glu Trp Ile Gly
 Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr Asn Gln Lys Phe Lys
 Gly Arg Xaa₆₈ Thr Xaa₇₀ Thr Xaa₇₂ Asp Xaa₇₄ Ser Ala Asn Thr Ala
 20 Tyr Met Glu Leu Ser Ser Leu Lys Ser Glu Asp Thr Ala Val Tyr Tyr
 Cys Ala Arg Ser His Tyr Gly Ser Asn Tyr Val Asp Tyr Phe Asp Tyr
 Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser (SEQ ID NO: 3)

wherein

Xaa₆₈ is Val or Ala;
 25 Xaa₇₀ is Val or Leu;
 Xaa₇₂ is Arg or Ala; and
 Xaa₇₄ is Thr or Lys; and

wherein the humanized antibody binds to human CD-20.

[0010] In another aspect, a humanized antibody is provided that comprises a light
 30 chain comprising a light chain variable region comprising the following amino acid
 sequence:

Glu Ile Val Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly Asp
 Arg Val Thr Ile Thr Cys Arg Ala Ser Ser Ser Leu Ser Phe Met His Trp

5 Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Xaa₄₅ Xaa₄₆ Ile Tyr Ala Thr
Ser Asn Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
Thr Asp Xaa₇₀ Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala
Thr Tyr Xaa₈₆ Cys His Gln Trp Ser Ser Asn Pro Leu Thr Phe Gly Gly
Gly Thr Lys Val Glu Ile Lys (SEQ ID NO: 1)

wherein

Xaa₄₅ is Pro or Leu;

Xaa₄₆ is Trp or Leu;

Xaa₇₀ is Tyr or Phe; and

10 Xaa₈₆ is Tyr or Phe;

wherein the humanized antibody binds to human CD-20.

[0011] In yet another aspect, a humanized antibody is provided that comprises a light chain comprising a light chain variable region comprising the following amino acid sequence:

15 Glu Ile Val Leu Thr Gln Ser Pro Gly Thr Leu Ser Leu Ser Pro Gly Glu
Arg Ala Thr Leu Ser Cys Arg Ala Ser Ser Ser Leu Ser Phe Met His
Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Xaa₄₅ Xaa₄₆ Ile Tyr Ala
Thr Ser Asn Leu Ala Ser Gly Ile Pro Ala Arg Phe Ser Gly Ser Gly Ser
Gly Thr Asp Xaa₇₀ Thr Leu Thr Ile Ser Arg Leu Glu Pro Glu Asp Phe
20 Ala Val Tyr Phe Cys His Gln Trp Ser Ser Asn Pro Leu Thr Phe Gly
Gln Gly Thr Lys Val Glu Leu Lys (SEQ ID NO: 2)

wherein

Xaa₄₅ is Pro or Leu;

Xaa₄₆ is Trp or Leu; and

25 Xaa₇₀ is Tyr or Phe;

wherein the humanized antibody binds to human CD-20.

[0012] In a further aspect, a humanized antibody is provided that comprises a heavy chain comprising a heavy chain variable region comprising the following amino acid sequence:

30 Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala
Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Tyr Asn
Met His Trp Val Arg Gln Ala Pro Gly Gln Arg Leu Glu Trp Ile Gly
Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr Asn Gln Lys Phe Lys

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Gly Arg Xaa₆₈ Thr Xaa₇₀ Thr Xaa₇₂ Asp Xaa₇₄ Ser Ala Asn Thr Ala
Tyr Met Glu Leu Ser Ser Leu Lys Ser Glu Asp Thr Ala Val Tyr Tyr
Cys Ala Arg Ser His Tyr Gly Ser Asn Tyr Val Asp Tyr Phe Asp Tyr
Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser (SEQ ID NO: 3)

5

wherein

Xaa₆₈ is Val or Ala;Xaa₇₀ is Val or Leu;Xaa₇₂ is Arg or Ala; andXaa₇₄ is Thr or Lys;

10 wherein the humanized antibody is capable of binding to human CD-20.

[0013] In yet a further aspect, a method of treating a B-cell disorder in a subject is provided. The method comprises administering to a subject in need thereof a therapeutically effective amount of any of the humanized antibodies disclosed herein.

[0014] In another aspect, a method of preventing a B-cell disorder in a subject is provided comprising administering to the subject a prophylactically effective amount of any of the humanized antibodies disclosed herein.

[0015] In a further aspect, a humanized anti-CD20 antibody composition comprising any of the humanized antibodies disclosed herein, wherein at least 90% of the N-glycans present in the composition are GlcNAc₂Man₃GlcNAc₂ (G0).

20 [0016] In another aspect, a pharmaceutical composition is provided comprising any of the humanized antibodies disclosed herein and a pharmaceutically acceptable excipient.

[0017] In yet a further aspect, an isolated nucleic acid is provided comprising a nucleic acid sequence encoding any of the humanized antibodies disclosed herein.

25 [0018] Also provided are host cells comprising any of the aforementioned isolated nucleic acids.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019] Figure 1A provides one embodiment of the nucleic acid sequences of the various nucleic acid molecules (including the X62109 signal peptide, U00570 Framework (FR) sequences, and 1F5VH complementarity determining regions (CDRs)) used to generate the 1F5RHAss nucleic acid molecule. Figure 1B illustrates the CDRs 1, 2, and 3 from 1F5VH (SEQ ID NOS: 77-79, respectively, from left to

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right) that were grafted into the acceptor FR of U00570 (SEQ ID NOS: 80-83, respectively, from left to right) to generate 1F5RHA.

[0020] Figure 2 provides the nucleic acid (SEQ ID NO: 15) and amino acid sequence (SEQ ID NO: 16) of the optimized 1F5RHA nucleic acid construct, containing no mutations in the framework region (FR).

[0021] Figure 3 provides the nucleic acid (SEQ ID NO: 17) and amino acid sequence (SEQ ID NO: 18) of the optimized 1F5RHB nucleic acid construct containing back-mutations at the 4 non-conserved vernier residues as follows: V67A; V69L; R71A; and T73K (Kabat numbering).

[0022] Figure 4A provides sequences of framework regions (FRs) and complementarity determining regions (CDRs) used to generate one example of a humanized anti-CD20 antibody, 1F5RKA11ss. Figure 4B illustrates the amino acid sequences of the FR (SEQ ID NOS: 87-90, respectively, from left to right) and CDRs (SEQ ID NOS: 84-86, respectively, from left to right) used to generate 1F5RKA11.

[0023] Figure 5 provides the nucleic acid sequence (SEQ ID NO: 30) and amino acid sequence (SEQ ID NO: 31) of an example of an optimized antibody based on 1F5RKA11ss to generate the 1F5RKA11 construct. This construct contains no FR mutations.

[0024] Figure 6 provides the nucleic acid (SEQ ID NO: 32) and amino acid sequence (SEQ ID NO: 33) of an example of a humanized antibody, 1F5RKB11, based on 1F5RKA11, containing back-mutations at the 3 non-conserved vernier residues as follows: L46P; L47W; and F71Y (Kabat numbering); and at VH/VK interface residue Y87F (Kabat numbering).

[0025] Figure 7A provides sequences of framework regions (FRs) and complementarity determining regions (CDRs) used to generate one example of a humanized anti-CD20 antibody, 1F5RKA12ss. Figure 7B illustrates the CDRs 1, 2, and 3 from 1F5VK (SEQ ID NOS: 91-93, respectively, from left to right) that were grafted into the acceptor FR of human AY263415 (SEQ ID NOS: 94-97, respectively, from left to right) to generate 1F5RKA12.

[0026] Figure 8 provides the nucleic acid sequence (SEQ ID NO: 44) and amino acid sequence (SEQ ID NO: 45) of an example of an optimized antibody based on 1F5RKA12ss to generate the 1F5RKA12 construct. This construct contains no FR mutations.

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[0027] Figure 9 provides the nucleic acid sequence (SEQ ID NO: 46) and amino acid sequence (SEQ ID NO: 47) of an example of a humanized anti-CD20 antibody, 1F5RKB12, based on 1F5RKA12, containing back-mutations at the 3 non-conserved variable residues as follows: L46P; L47W; and F71Y (Kabat numbering).

5 [0028] Figure 10A provides an amino acid sequence alignment of examples of variants of 1F5RHA. Figure 10B provides an amino acid sequence alignment of examples of variants of 1F5RKA11. Figure 10C provides an amino acid sequence alignment of examples of variants of 1F5RKA12. The CDR amino acid residues are indicated by boxes labeled "CDR1", "CDR2", and "CDR3"; the underlined amino
10 acids indicate mouse FW residues that were introduced into the humanized sequences.

[0029] Figures 11A-E provide amino acid sequences of the mature variable and constant regions from 1F5RKG11, 1F5RKB11, 1F5RKF12, 1F5RKB12, and 1F5RHA proteins. Figure 11A provides the amino acid sequence for the mature variable and constant (underlined) regions of 1F5RKG11 (SEQ ID NO: 60). Figure
15 11B provides the amino acid sequence for the mature variable and constant (underlined) regions of 1F5RKB11 (SEQ ID NO: 61). Figure 11C provides the amino acid sequence for the mature variable and constant (underlined) regions of 1F5RKF12 (SEQ ID NO: 62). Figure 11D provides the amino acid sequence for the mature variable and constant (underlined) regions of 1F5RKB12 (SEQ ID NO: 63). Figure
20 11E provides the amino acid sequence for the mature variable and constant (underlined) regions of 1F5RHA (SEQ ID NO: 64). The constant regions of the amino acid sequences are underlined.

[0030] Figure 12A provides the results of Raji binding assays for chimeric VK x 1F5RHA (RHA) and chimeric VH x (1F5RKA11, 1F5RKA12, or 1F5RKB11)
25 compared with chimeric c1F5 antibody (Chimeric). Figure 12B provides the results of Raji binding assays for humanized 1F5RHA (RHA) or 1F5RHB (RHB) in association with chimeric VK, compared with Rituxan. Figures 12C and 12D provide results of Raji binding assays for humanized 1F5VK versions (KB11, etc.) in association with chimeric VH, compared to the chimeric antibody c1F5.

30 [0031] Figures 13A and 13B provide examples of Raji cell binding of antibodies encoded by humanized 1F5RHA in association with the humanized 1F5 kappa chains indicated, compared with the chimeric version of 1F5. Measurements are averages of duplicate wells.

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[0032] Figure 14 provides an example Raji cell binding of antibodies encoded by humanized 1F5RHA in association with the humanized 1F5 kappa chains indicated, compared with the chimeric version of 1F5. Measurements are averages of quadruplicate wells. Error bars indicate the standard deviation between the quadruplicates.

[0033] Figure 15 provides an example of thermostability results for some embodiments of humanized anti-CD20 antibodies. Each antibody was diluted to 1µg/ml in medium/ PBS, heated for 10 minutes at the indicated temperature, then cooled to 4°C before Raji cell binding ELISA at room temperature. The humanized antibodies (apart from Rituxan) are encoded by 1F5RHA together with the indicated light chain construct.

[0034] Figure 16 provides an example of the mean fluorescence intensity in a fluorescent antibody binding assay on Raji cells for several embodiments of humanized anti-CD20 antibodies.

[0035] Figure 17 provides an example of a pre-optimized chimeric c1F5VK nucleic acid (SEQ ID NO: 65) and corresponding amino acid sequence (SEQ ID NO: 66).

[0036] Figure 18 provides an example of an optimized chimeric c1F5VK nucleic acid (SEQ ID NO: 67) and corresponding amino acid sequence (SEQ ID NO: 68).

[0037] Figure 19 provides an example of a pre-optimized chimeric c1F5VH nucleic acid (SEQ ID NO: 69) and corresponding amino acid sequence (SEQ ID NO: 70).

[0038] Figure 20 provides an example of an optimized chimeric c1F5VH nucleic acid (SEQ ID NO: 71) and corresponding amino acid sequence (SEQ ID NO: 72).

[0039] Figure 21 shows the G0 glycan structure.

[0040] Figure 22 provides an example of a chimeric c1F5VH protein sequence (mature variable and constant region) (SEQ ID NO: 73). The constant region of the amino acid sequence is underlined.

[0041] Figure 23 provides an example of a chimeric c1F5VK protein sequence (mature variable and constant region) (SEQ ID NO: 74). The constant region of the amino acid sequence is underlined.

[0042] Figure 24 shows the results of the CDC assay of various concentrations of the tested anti-CD20 antibodies as explained in Example 5.

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[0043] Figure 25 shows the results of an assay measuring the dissociation of anti-CD20 antibodies (i.e., off-rate) from Raji cells as explained in Example 5.

[0044] Figure 26 shows a bar graph of the half-lives obtained from one of the off-rate studies on anti-CD20 antibodies explained in Example 5.

5 [0045] Figure 27 shows the results of a B-cell depletion assay using anti-CD20 antibodies at various concentrations.

[0046] Figure 28 shows the results of a B-cell depletion assay using anti-CD20 antibodies at various concentrations in the presence of anti-CD16 antibody.

10 DETAILED DESCRIPTION

[0047] The present invention relates to humanized 1F5 antibodies that bind to, or are capable of binding to, CD20, as well as to compositions comprising those antibodies. The present invention also relates to nucleic acid molecules encoding humanized 1F5 antibodies. That is, humanized anti-CD20 antibodies and nucleic acid
15 molecules encoding such anti-CD20 antibodies are provided.

[0048] The antibodies and compositions thereof provided herein are useful in methods for treating diseases and conditions associated with cells expressing CD20 (such as B cells), including lymphomas, autoimmune diseases, and transplant rejections. The compositions include the humanized anti-CD20 antibodies, antigen-
20 binding fragments of the humanized anti-CD20 antibodies, and pharmaceutical compositions of such antibodies. The compositions also include isolated nucleic acid molecules encoding the humanized anti-CD20 antibodies discussed herein, vectors comprising the nucleic acid molecules that encode the humanized anti-CD20 antibodies, host cells (including transfectomas and hybridomas) transformed with the
25 vectors or incorporating the nucleic acid molecules that express the humanized antibodies, pharmaceutical formulations of the anti-CD20 humanized antibodies, and methods of making and using the same. Methods for treating or preventing diseases or disorders associated with cells expressing CD20 (such as B-cell disorders) are also provided.

30 [0049] Prior to describing the invention in further detail, the following terms will first be defined. Unless otherwise noted, all nucleic acids sequences are written from left to right in 5' to 3' orientation, and all amino acid sequences are written from left to right in amino-terminal to carboxy-terminal orientation.

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[0050] Definitions:

[0051] The terms “antibody” and “immunoglobulin” are used interchangeably and are used in their broadest sense herein. Specifically, the term includes monoclonal antibodies, multispecific antibodies, antibody fragments, and other antibodies and immunoglobulins so long as they exhibit the desired biological activity and function.

[0052] “Antibody fragments” and “antibody fragment” comprise a portion of a full length antibody, and generally include the variable region thereof.

[0053] As used herein, a “CD20” or “human CD20” (also called human B-lymphocyte-restricted differentiation antigen, Bp35) refers to a transmembrane phosphoprotein with a molecular weight of approximately 35 kilodaltons that is expressed on normal and malignant B cells.

[0054] An “anti-CD20 antibody” as used herein means an antibody that specifically binds human CD20.

[0055] As used herein, a “chimeric antibody” refers to any antibody in which the immunoreactive or binding region or site is obtained or derived from a first species and the constant region (which may be intact, partial or modified) is obtained from a second species. For example, the target binding region or site is derived from a non-human source (e.g., mouse or primate) and the constant region is derived from a human antibody.

[0056] A “humanized antibody” refers to a polypeptide comprising at least a portion of a modified variable region of a human antibody wherein a portion of the variable region, preferably a portion substantially less than the intact human variable domain, has been substituted by the corresponding sequence from a non-human species and wherein the modified variable region is linked to at least another part of another protein, preferably the constant region of a human antibody. The expression “humanized antibodies” includes human antibodies in which one or more complementarity determining region (“CDR”) amino acid residues and/or one or more framework region (“FW” or “FR”) amino acid residues are substituted by amino acid residues from analogous sites in rodent or other non-human antibodies that are capable of binding to CD20. The expression “humanized antibody” also includes an immunoglobulin amino acid sequence variant or fragment thereof that is capable of binding to CD20 and that comprises an FR having substantially the amino acid sequence of a human immunoglobulin and a CDR having substantially the amino acid

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sequence of a non-human immunoglobulin.

[0057] Immunoglobulin heavy chains can be classified as gamma (γ), mu (μ), alpha (α), delta (δ), or epsilon (ϵ), with some subclasses among them (e.g., $\gamma 1 - \gamma 4$). The classification (or "class") of the antibody as IgG, IgM, IgA, IgD, or IgE, respectively, is determined by the nature of the heavy chain which confers functional specialization to the immunoglobulin. Immunoglobulin can be classified into subclasses (or "isotypes") for example, IgG₁, IgG₂, IgG₃, IgG₄, IgA₁, etc. Such isotypes are well-characterized and confers additional functional specialization to the immunoglobulin. With regard to IgG, a standard immunoglobulin molecule comprises two identical light chain polypeptides and two identical heavy chain polypeptides. The four chains are typically joined by disulfide bonds in a "Y" configuration where the light chains bracket the heavy chains starting at the mouth of the "Y" and continuing through the variable region.

[0058] There are two types of light chains: kappa (κ) or lambda (λ). Each heavy chain class may be bound with either a kappa or lambda light chain. In general, the light and heavy chains are covalently bonded to each other, and the "tail" portions of the two heavy chains are bonded to each other by covalent disulfide linkages and non-covalent linkages when the immunoglobulins are generated by either B cell hybridomas, B cells, or genetically engineered host cells. In the heavy chain, the amino acid sequences run from the amino-terminus at the forked ends of the Y configuration to the carboxyl-terminus at the bottom of each chain.

[0059] Both the light and heavy chains are divided into regions of structural and functional homology referred to as "constant regions" and "variable regions." The terms "constant" and "variable" are used functionally. The variable domains of both the light (V_L or VL) and heavy (V_H or VH) chain portions determine antigen recognition and specificity. Conversely, the constant domains of the light chain (C_L) and the heavy chain (C_{H1}, C_{H2}, or C_{H3}) confer important biological properties such as secretion, transplacental mobility, Fc receptor binding, complement binding, and the like. By convention the numbering of the constant region domains increases as they become more distal from the antigen binding site or amino-terminus of the antibody. The variable region is at the amino-terminus and the constant region is at the carboxyl-terminus; the C_{H3} and C_L domains actually comprise the carboxy-terminus of the heavy and light chain, respectively.

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[0060] The expressions “complementarity determining region,” “hypervariable region,” and “CDR” refer to one or more of the hyper-variable or complementarity determining regions (CDRs) found in the variable regions of light or heavy chains of an antibody (*See*, Kabat, E. A. et al., Sequences of Proteins of Immunological Interest, National Institutes of Health, Bethesda, Md., (1987)). These expressions include the hypervariable regions as defined by Kabat et al. (“Sequences of Proteins of Immunological Interest,” Kabat E., et al., US Dept. of Health and Human Services, 1983) or the hypervariable loops in 3-dimensional structures of antibodies (Chothia and Lesk, J Mol. Biol. 196 901-917 (1987)). The CDRs in each chain are held in close proximity by framework regions and, together with the CDRs from the other chain, contribute to the formation of the antigen binding site.

[0061] The expressions “framework region” and “FR” refer to one or more of the framework regions within the variable regions of the light and heavy chains of an antibody (*See*, Kabat, E. A. et al., Sequences of Proteins of Immunological Interest, National Institutes of Health, Bethesda, Md., (1987)). These expressions include those amino acid sequences of both light and heavy chains of an antibody, situated between the amino terminus and the first CDR, those interposed between the CDRs, and those between the third CDR and the start of the constant region.

[0062] CDR and FR residues can be determined according to a standard sequence definition (Kabat et al., Sequences of Proteins of Immunological Interest, National Institutes of Health, Bethesda Md. (1987)), and a structural definition (as in Chothia and Lesk, J. Mol. Biol. 196:901-217 (1987)).

[0063] Where indicated herein, reference is made to the numbering scheme from Kabat, E. A., et al., Sequences of Proteins of Immunological Interest (National Institutes of Health, Bethesda, Md. (1987) and (1991)). Kabat uses a method for assigning a residue number to each amino acid in a listed sequence, and this method for assigning residue numbers has become standard in the field. Where indicated, the Kabat numbering scheme is followed in this description. Where Kabat numbering is not indicated, sequential amino acid sequence numbering is used (i.e., the amino acids in a sequence are numbered using sequential integers (1, 2, 3, etc.) from left to right in amino-terminal to carboxy-terminal orientation).

[0064] An “antigen-binding fragment” of an antibody refers to biologically active fragments of the antibodies disclosed herein that function essentially the same as a

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full-length 1F5 antibody to bind to CD20. Such fragments comprise a portion of a full length antibody, generally the antigen binding or variable region thereof.

Examples of antibody fragments include Fab, Fab', F(ab')₂, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific

5 antibodies formed from antibody fragments.

[0065] "Fv" is the minimum antibody fragment which contains a complete antigen-recognition and antigen-binding site. This fragment consists of a dimer of one heavy- and one light-chain variable region domain in tight, non-covalent association. From the folding of these two domains emanate six hypervariable loops (3 loops each
10 from the H and L chain) that contribute the amino acid residues for antigen binding and confer antigen binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

15 [0066] The terms "rituximab" or "RITUXAN®" refer to a genetically engineered chimeric murine/human monoclonal antibody directed against CD20 that has the amino acid-sequence of the antibody designated "C2B8" in U.S. Pat. No. 5,736,137.

[0067] "Antibody-dependent cell-mediated cytotoxicity" and "ADCC" refer to a cell-mediated reaction in which nonspecific cytotoxic cells that express Fc receptors
20 (FcRs) (e.g. Natural Killer (NK) cells, neutrophils, and macrophages) recognize bound antibody on a target cell and subsequently cause lysis of the target cell. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII.

[0068] "Affinity" of an antibody for an antigen or epitope is a term well
25 understood in the art and means the extent, or strength, of binding of an antibody to an epitope. Affinity may be measured and/or expressed in a number of ways known in the art, including, but not limited to, equilibrium dissociation constant (K_D or K_d , which can be defined as the ratio of the off-rate and on-rate of the antibody, i.e., K_{off}/K_{on}), apparent equilibrium dissociation constant (K_D or K_d), and IC50 (amount
30 needed to effect 50% inhibition in a competition assay); relative affinity of humanized antibodies may also be determined as compared to, for example, related murine or chimeric antibodies. It is understood that, for purposes of this invention, an affinity is an average affinity for a given population of antibodies which bind to an antigen or

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epitope. Affinity (or relative affinity) for the humanized anti-CD20 antibodies described herein may be measured using an enzyme-linked immunosorbent assay (ELISA) or a fluorescent-activated cell sorting (FACS) assay as described in the Examples herein.

5 [0069] “Off-rate” means the dissociation rate constant (K_{off}) of an antibody from an antibody/antigen complex. Thus, antibodies with lower off-rates remain bound to the antibody longer than antibodies with higher off-rates.

[0070] “On-rate” means the association rate constant (K_{on}) of an antibody to an antigen to form an antibody/antigen complex.

10 [0071] An “effective amount” refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic or prophylactic result.

[0072] A “therapeutically effective amount” of an antibody may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the antibody to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the antibody are outweighed by the therapeutically beneficial effects. A “prophylactically effective amount” refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically but not necessarily, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount may be less than the therapeutically effective amount.

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20

[0073] “B-cell disorders” includes a variety of disorders, including, but not limited to, B-cell malignancies, autoimmune disorders, B-cell lymphomas, B-cell leukemias, and other disorders.

25

[0074] An “autoimmune disorder” or “autoimmune disease” as used herein refers to a non-malignant disease or disorder arising from and directed against an individual’s own tissues. Examples of autoimmune diseases or disorders include, but are not limited to, inflammatory responses such as inflammatory skin diseases including psoriasis and dermatitis (for example, atopic dermatitis); systemic scleroderma and sclerosis; responses associated with inflammatory bowel disease (such as Crohn’s disease and ulcerative colitis); respiratory distress syndrome (including adult respiratory distress syndrome; ARDS); dermatitis; meningitis;

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encephalitis; uveitis; colitis; glomerulonephritis; allergic conditions such as eczema and asthma and other conditions involving infiltration of T cells and chronic inflammatory responses; atherosclerosis; leukocyte adhesion deficiency; rheumatoid arthritis; systemic lupus erythematosus (SLE); diabetes mellitus (e.g. Type I diabetes mellitus or insulin dependent diabetes mellitus); multiple sclerosis; Raynaud's syndrome; autoimmune thyroiditis; allergic encephalomyelitis; Sjögren's syndrome; juvenile onset diabetes; and immune responses associated with acute and delayed hypersensitivity mediated by cytokines and T-lymphocytes typically found in tuberculosis, sarcoidosis, polymyositis, granulomatosis and vasculitis; pernicious anemia (Addison's disease); diseases involving leukocyte diapedesis; central nervous system (CNS) inflammatory disorder; multiple organ injury syndrome; hemolytic anemia (including, but not limited to cryoglobulinemia or Coombs positive anemia); myasthenia gravis; antigen-antibody complex mediated diseases; anti-glomerular basement membrane disease; antiphospholipid syndrome; allergic neuritis; Graves' disease; Lambert-Eaton myasthenic syndrome; bullous pemphigoid; pemphigus; autoimmune polyendocrinopathies; Reiter's disease; stiff-man syndrome; Behcet's disease; giant cell arteritis; immune complex nephritis; IgA nephropathy; IgM polyneuropathies; immune thrombocytopenic purpura (ITP) or autoimmune thrombocytopenia etc.

20

Humanized 1F5 Antibodies:

[0075] The invention provides humanized 1F5 antibodies and antigen-binding fragments thereof that are capable of binding to human CD20 (i.e., humanized anti-CD20 antibodies).

25 [0076] In one embodiment, a humanized antibody (including antigen-binding fragments thereof) is provided, comprising a light chain comprising a light chain variable region comprising an amino acid sequence selected from the group consisting of:

(1) Glu Ile Val Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg Val

30 Thr

Ile Thr Cys Arg Ala Ser Ser Ser Leu Ser Phe Met His Trp Tyr Gln Gln Lys Pro Gly
Lys Ala Pro Lys Xaa₄₅ Xaa₄₆ Ile Tyr Ala Thr Ser Asn Leu Ala Ser Gly Val Pro Ser
Arg

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Phe Ser Gly Ser Gly Ser Gly Thr Asp Xaa₇₀ Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu
Asp Phe Ala Thr Tyr Xaa₈₆ Cys His Gln Trp Ser Ser Asn Pro Leu Thr Phe Gly Gly
Gly

Thr Lys Val Glu Ile Lys (SEQ ID NO: 1)

5

wherein:

Xaa₄₅ is Pro or Leu;

Xaa₄₆ is Trp or Leu;

Xaa₇₀ is Tyr or Phe; and

Xaa₈₆ is Tyr or Phe; and

10

(2) Glu Ile Val Leu Thr Gln Ser Pro Gly Thr Leu Ser Leu Ser Pro Gly Glu Arg Ala
Thr

Leu Ser Cys Arg Ala Ser Ser Ser Leu Ser Phe Met His Trp Tyr Gln Gln Lys Pro Gly
Gln Ala Pro Arg Xaa₄₅ Xaa₄₆ Ile Tyr Ala Thr Ser Asn Leu Ala Ser Gly Ile Pro Ala
Arg

15

Phe Ser Gly Ser Gly Ser Gly Thr Asp Xaa₇₀ Thr Leu Thr Ile Ser Arg Leu Glu Pro Glu
Asp Phe Ala Val Tyr Phe Cys His Gln Trp Ser Ser Asn Pro Leu Thr Phe Gly Gln Gly
Thr Lys Val Glu Leu Lys (SEQ ID NO: 2)

wherein:

Xaa₄₅ is Pro or Leu;

20

Xaa₄₆ is Trp or Leu; and

Xaa₇₀ is Tyr or Phe.

[0077] In another embodiment, a humanized antibody (including antigen-binding
fragments thereof) is provided comprising a heavy chain comprising a heavy chain
variable region comprising the following amino acid sequence:

25

Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala Ser Val Lys Val
Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Tyr Asn Met His Trp Val Arg Gln Ala
Pro Gly Gln Arg Leu Glu Trp Ile Gly Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr
Asn Gln Lys Phe Lys Gly Arg Xaa₆₈ Thr Xaa₇₀ Thr Xaa₇₂ Asp Xaa₇₄ Ser Ala Asn Thr
Ala Tyr Met Glu Leu Ser Ser Leu Lys Ser Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg

30

Ser His Tyr Gly Ser Asn Tyr Val Asp Tyr Phe Asp Tyr Trp Gly Gln Gly Thr Leu Val
Thr Val Ser Ser (SEQ ID NO: 3)

wherein:

Xaa₆₈ is Val or Ala;

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Xaa₇₀ is Val or Leu;

Xaa₇₂ is Arg or Ala; and

Xaa₇₄ is Thr or Lys.

[0078] Thus, the light chain variable regions of the anti-CD20 antibodies may
 5 have various amino acid sequences, including the following amino acid sequences set forth in Figures 10B and 10C: SEQ ID NOS: 28, 49, 50, 51, 52, 53, 54, 42, 55, 56, 57, 58, and 59. In one example, the light chain variable region of the anti-CD20 antibody comprises the amino acid sequence of SEQ ID NO: 49. In another example, the light chain variable region of the anti-CD20 antibody comprises the amino acid
 10 sequence of SEQ ID NO: 54. In still another example, the light chain variable region of the anti-CD20 antibody comprises the amino acid sequence of SEQ ID NO: 59. In yet another example, the light chain variable region of the anti-CD20 antibody comprises the amino acid sequence of SEQ ID NO: 55.

[0079] The heavy chain variable regions of the anti-CD20 antibodies may have
 15 various amino acid sequences, including the following amino acid sequences set forth in Figure 10A: SEQ ID NO: 13 and SEQ ID NO: 48.

[0080] In one embodiment, a humanized antibody (including antigen-binding fragments thereof) is provided comprising
 (a) a light chain comprising a light chain variable region comprising an amino acid
 20 sequence selected from the group consisting of:

(1) Glu Ile Val Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
 Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Ser Ser Leu Ser Phe Met His Trp Tyr Gln
 Gln Lys Pro Gly

Lys Ala Pro Lys Xaa₄₅ Xaa₄₆ Ile Tyr Ala Thr Ser Asn Leu Ala Ser Gly Val Pro Ser
 25 Arg
 Phe Ser Gly Ser Gly Ser Gly Thr Asp Xaa₇₀ Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu
 Asp Phe Ala Thr Tyr Xaa₈₆ Cys His Gln Trp Ser Ser Asn Pro Leu Thr Phe Gly Gly
 Gly

Thr Lys Val Glu Ile Lys (SEQ ID NO: 1)

30 wherein:

Xaa₄₅ is Pro or Leu;

Xaa₄₆ is Trp or Leu;

Xaa₇₀ is Tyr or Phe; and

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Xaa₈₆ is Tyr or Phe; and

(2) Glu Ile Val Leu Thr Gln Ser Pro Gly Thr Leu Ser Leu Ser Pro Gly
Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Ser Ser Leu Ser Phe Met His Trp Tyr Gln
Gln Lys Pro Gly

5 Gln Ala Pro Arg Xaa₄₅ Xaa₄₆ Ile Tyr Ala Thr Ser Asn Leu Ala Ser Gly Ile Pro Ala
Arg
Phe Ser Gly Ser Gly Ser Gly Thr Asp Xaa₇₀ Thr Leu Thr Ile Ser Arg Leu Glu Pro Glu
Asp Phe Ala Val Tyr Phe Cys His Gln Trp Ser Ser Asn Pro Leu Thr Phe Gly Gln Gly
Thr Lys Val Glu Leu Lys (SEQ ID NO: 2)

10 wherein:

Xaa₄₅ is Pro or Leu;

Xaa₄₆ is Trp or Leu; and

Xaa₇₀ is Tyr or Phe; and

(b) a heavy chain comprising a heavy chain variable region comprising the following
15 amino acid sequence:

Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala
Ser Val Lys Val

Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Tyr Asn Met His Trp Val Arg Gln Ala
Pro Gly Gln Arg Leu Glu Trp Ile Gly Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr
20 Asn Gln Lys Phe Lys Gly Arg Xaa₆₈ Thr Xaa₇₀ Thr Xaa₇₂ Asp Xaa₇₄ Ser Ala Asn Thr
Ala Tyr Met Glu Leu Ser Ser Leu Lys Ser Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg
Ser His Tyr Gly Ser Asn Tyr Val Asp Tyr Phe Asp Tyr Trp Gly Gln Gly Thr Leu Val
Thr Val Ser Ser (SEQ ID NO: 3)

wherein:

25 Xaa₆₈ is Val or Ala;

Xaa₇₀ is Val or Leu;

Xaa₇₂ is Arg or Ala; and

Xaa₇₄ is Thr or Lys.

[0081] In one embodiment, the anti-CD20 antibody may comprise (1) a heavy
30 chain comprising a heavy chain variable region having the amino acid sequence set
forth in SEQ ID NO: 13 and (2) a light chain comprising a light chain variable region
having the amino acid sequence set forth in SEQ ID NO: 49.

[0082] In another embodiment, the anti-CD20 antibody may comprise (1) a heavy

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chain comprising a heavy chain variable region having the amino acid sequence set forth in SEQ ID NO: 13 and (2) a light chain comprising a light chain variable region having the amino acid sequence set forth in SEQ ID NO: 54.

[0083] In yet another embodiment, the anti-CD20 antibody may comprise (1) a heavy chain comprising a heavy chain variable region having the amino acid sequence set forth in SEQ ID NO: 13 and (2) a light chain comprising a light chain variable region having the amino acid sequence set forth in SEQ ID NO: 55.

[0084] In a further embodiment, the humanized anti-CD20 antibody may comprise (1) a heavy chain comprising a heavy chain variable region having the amino acid sequence set forth in SEQ ID NO: 13 and (2) a light chain comprising a light chain variable region having the amino acid sequence set forth in SEQ ID NO: 59.

[0085] The humanized anti-CD20 antibodies herein are capable of binding to human CD20. In some embodiments the humanized anti-CD20 antibodies bind to CD20 with an affinity similar to that of antibody 1F5 (i.e., with an affinity similar to that of the affinity that antibody 1F5 has for human CD20). In other embodiments, the humanized anti-CD20 antibodies bind to CD20 with an affinity at least as great as that of antibody 1F5. In yet another embodiment, the humanized anti-CD20 antibodies bind to CD20 with an affinity greater than that of antibody 1F5. As used herein an "affinity similar to that of antibody 1F5" means an affinity of sufficiently high degree of similarity to the affinity of antibody 1F5 such that one of skill in the art would consider the difference between the affinities to be of little or no biological significance.

[0086] In some embodiments the humanized anti-CD20 antibodies bind to CD20 with an affinity similar to that of the chimeric antibody c1F5 described in the Examples herein (i.e., with an affinity similar to that of the affinity that chimeric antibody c1F5 has for human CD20). In other embodiments, the humanized anti-CD20 antibodies bind to CD20 with an affinity at least as great as that of the chimeric antibody c1F5. In yet another embodiment, the humanized anti-CD20 antibodies bind to CD20 with an affinity greater than that of the chimeric antibody c1F5. As used herein an "affinity similar to that of chimeric antibody c1F5" means an affinity of sufficiently high degree of similarity to the affinity of chimeric antibody c1F5 such that one of skill in the art would consider the difference between the affinities to be of

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little or no biological significance.

[0087] In some embodiments the humanized anti-CD20 antibodies bind to CD20 with an off-rate lower than that of chimeric antibody c1F5. In other embodiments, the humanized anti-CD20 antibodies not lower than that of the chimeric antibody c1F5.

5 [0088] In further embodiments, the humanized anti-CD20 antibodies bind to CD20 with an affinity greater than that of rituximab. In other embodiments, the humanized anti-CD20 antibodies bind to CD20 with an off-rate lower than that of rituximab.

[0089] The anti-CD20 antibodies may comprise heavy chain constant regions (or
10 portions thereof) and/or light chain constant regions (or portions thereof) of any isotype, allotype, and idiotype. Such heavy and light constant regions may be naturally occurring or may contain deletion, substitution, or addition mutations. Human constant region DNA sequences are known and can be isolated from a variety of human cells. When present on the heavy and/or light chains, the constant regions
15 may be attached to the carboxyl-terminal end of the variable regions of the heavy and/or light chains.

Antibody Fragments

[0090] In some embodiments fragments of the humanized anti-CD20 antibodies described herein are provided, rather than whole antibodies.

20 [0091] Any method available can be used for the production of such antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies. However, these fragments can now be produced directly by recombinant host cells. Fab, Fv and ScFv antibody fragments can all be expressed in and secreted from E. coli, thus allowing the facile production of large amounts of
25 these fragments. Antibody fragments can be isolated from antibody phage libraries. Alternatively, Fab'-SH fragments can be directly recovered from E. coli and chemically coupled to form F(ab')₂ fragments. According to another approach, F(ab')₂ fragments can be isolated directly from recombinant host cell culture. In other embodiments, the antibody fragment is a single chain Fv fragment (scFv).

30 [0092] The antibody fragment may be antigen-binding fragments of the humanized anti-CD20 antibodies described herein. Any fragment of a whole antibody may be used so long as the fragment binds to CD20.

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Bispecific antibodies

[0093] The humanized anti-CD20 antibodies may also be used in the production of bispecific antibodies. Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may
5 bind to two different epitopes of the CD20 protein. Other such antibodies may combine a CD20 binding site with a binding site for another protein. Alternatively, an anti-CD20 binding site may be combined with a domain or arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (e.g. CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16), or NKG2D or other NK cell activating ligand, so as to focus and localize
10 cellular defense mechanisms to the CD20-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express CD20. These antibodies possess a CD20-binding arm and an arm which binds the cytotoxic agent (e.g. saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or
15 radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. F(ab')₂ bispecific antibodies).

Multivalent antibodies

[0094] The humanized anti-CD20 antibodies may also be used in the production of multivalent antibodies. A multivalent antibody may be internalized (and/or
20 catabolized) faster than a bivalent antibody by a cell expressing an antigen to which the antibodies bind. The humanized anti-CD20 antibodies may be multivalent antibodies (which are other than of the IgM class) with three or more antigen binding sites (e.g. tetravalent antibodies), which can be readily produced by recombinant expression of nucleic acid encoding the polypeptide chains of the antibody. The
25 multivalent antibody can comprise a dimerization domain and three or more antigen binding sites. The preferred dimerization domain comprises (or consists of) an Fc region or a hinge region. In this scenario, the antibody will comprise an Fc region and three or more antigen binding sites amino-terminal to the Fc region. The preferred multivalent antibody herein comprises (or consists of) three to about eight, but
30 preferably four, antigen binding sites. The multivalent antibody comprises at least one polypeptide chain (and may comprise two polypeptide chains), wherein the polypeptide chain(s) comprise two or more variable domains.

Recombinant Production Methods

[0095] The humanized anti-CD20 antibodies may be produced by any method available, such as, for example, recombinant expression techniques. Nucleic acids encoding the light and heavy chain variable regions, optionally linked to constant regions, can be inserted into expression vectors. The light and heavy chains can be cloned in the same or different expression vectors. The DNA segments encoding immunoglobulin chains can be operably linked to control sequences in the expression vector(s) that ensure the expression of immunoglobulin polypeptides. Expression control sequences include, but are not limited to, promoters (e.g., naturally-associated or heterologous promoters), signal sequences, enhancer elements, and transcription termination sequences. In one embodiment, the expression control sequences are eukaryotic promoter systems in vectors capable of transforming or transfecting eukaryotic host cells. Once the vector has been incorporated into the appropriate host, the host is maintained under conditions suitable for high level expression of the nucleotide sequences, and the collection and purification of the antibodies.

[0096] The expression vectors may be replicable in any host organism, either as episomes or as an integral part of the host chromosomal DNA. In one embodiment, expression vectors contain selection markers (e.g., ampicillin-resistance, hygromycin-resistance, tetracycline resistance or neomycin resistance) to permit detection of those cells transformed with the desired DNA sequences.

[0097] The expression vectors may be used to express the humanized anti-CD20 antibodies from any host cell, including prokaryotic host cells (e.g. *E. coli*), yeast host cells, mammalian host cells, plant host cells, and insect host cells.

[0098] In one embodiment, *E. coli* is used for production of the humanized antibodies. Other prokaryotic hosts suitable for such use include bacilli, such as *Bacillus subtilis*, and other enterobacteriaceae, such as *Salmonella*, *Serratia*, and various *Pseudomonas* species. In these prokaryotic hosts, one can also make expression vectors, which will typically contain expression control sequences compatible with the host cell (e.g., an origin of replication). In addition, any number of a variety of well-known promoters will be present, such as the lactose promoter system, a tryptophan (*trp*) promoter system, a beta-lactamase promoter system, or a promoter system from phage lambda. The promoters will typically control expression, optionally with an operator sequence, and have ribosome binding site

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sequences and the like, for initiating and completing transcription and translation.

[0099] Other microbes, such as yeast, are also useful for expression of the humanized antibodies. For example, *Saccharomyces* can be used as a yeast host, with suitable vectors having expression control sequences (e.g., promoters), an origin of replication, termination sequences and the like as desired. Promoters for use in yeast expression techniques include 3-phosphoglycerate kinase and other glycolytic enzymes. Inducible yeast promoters include, among others, promoters from alcohol dehydrogenase, isocytochrome C, and enzymes responsible for maltose and galactose utilization.

10 [00100] In another embodiment, mammalian tissue cell culture may be used to express and produce the humanized anti-CD20 antibodies (e.g., polynucleotides encoding immunoglobulins or fragments thereof). Any mammalian tissue cell may be used in such methods, and a number of suitable host cell lines capable of secreting heterologous proteins (e.g., intact immunoglobulins) have been developed in the art, and include CHO cell lines, various Cos cell lines, HeLa cells, preferably, myeloma cell lines, or transformed B-cells or hybridomas. In one embodiment the cells are nonhuman. Expression vectors for the mammalian cells can include expression control sequences, such as an origin of replication, a promoter, and an enhancer, and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. In one embodiment, the expression control sequences are promoters derived from immunoglobulin genes, SV40, adenovirus, bovine papilloma virus, cytomegalovirus and the like.

25 [00101] The vectors containing the polynucleotide sequences of interest (e.g., the heavy and light chain encoding sequences and expression control sequences) can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment, electroporation, lipofection, biolistics or viral-based transfection may be used for other cellular hosts.

30 Other methods used to transform mammalian cells include the use of polybrene, protoplast fusion, liposomes, electroporation, and microinjection. For production of transgenic animals, transgenes can be microinjected into fertilized oocytes, or can be incorporated into the genome of embryonic stem cells, and the nuclei of such cells

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transferred into enucleated oocytes.

[00102] When the nucleic acid molecules encoding the humanized heavy and light chains are cloned into separate expression vectors, the vectors can be co-transfected to obtain expression and assembly of intact immunoglobulins. Once expressed, the whole antibodies, their dimers, individual light and heavy chains, or other immunoglobulin forms of the antibodies may be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, HPLC purification, gel electrophoresis and the like. Substantially pure immunoglobulins of at least about 90 to 95% homogeneity can be prepared for pharmaceutical uses. In another embodiment, substantially pure humanized antibodies of at least about 98 to 99% or more homogeneity can be produced for use in the pharmaceutical formulations and methods.

[00103] Thus, also provided is a method of expressing the humanized anti-CD20 antibodies comprising: (a) transforming a host cell with a nucleic acid molecule encoding a humanized anti-CD20 antibody described herein, and (b) culturing the transformed host cells under conditions that allow for the expression of the humanized anti-CD20 antibodies. Known techniques may be used that include a selection marker on the vector so that host cells that express the humanized and chimeric antibodies and the marker can be easily selected.

[00104] In one preferred embodiment for producing the humanized anti-CD20 antibodies described herein, transformed duckweed plant or duckweed nodule cultures are used for the expression and the secretion of the antibodies. Genetic techniques for transforming duckweed and optimizing the nucleotide sequence of the expression cassette(s) encoding the antibody are described in U.S. Patent No. 6,815,184, the entire content of which is hereby incorporated herein by reference.

Glycosylation

[00105] The anti-CD20 antibodies described herein may be glycosylated or unglycosylated. When the anti-CD20 antibodies are glycosylated, the glycan structures that are present may vary as desired. For example, using different host cells for the recombinant production of the humanized anti-CD20 antibodies will vary the glycan structure(s) of the antibodies.

[00106] The glycan structures of the antibodies may be also be optimized using known techniques such as RNA interference, antisense, and knockout technologies,

etc. in the host cell line. For example, when plant cells and/or plants are used to produce the anti-CD20 antibodies, the native N-glycosylation pattern of the antibodies may be altered by using methods to inhibit the expression of α 1,3-fucosyltransferase and β 1,2-xylosyltransferase (e.g., using RNA interference constructs). Examples of such technology and plants are described in International Publication No. WO 2007/084926, the entirety of which is incorporated herein.

[00107] Thus, in one embodiment, the anti-CD20 antibodies may be prepared in plants such that the N-glycosylation pattern has reduced α 1,3-fucose and β 1,2-xylose as compared to such antibodies produced in plants without using methods to inhibit the expression of α 1,3-fucosyltransferase and β 1,2-xylosyltransferase.

[00108] In another embodiment, the anti-CD20 antibodies may have a glycosylation pattern that is devoid of fucose and xylose.

[00109] In yet another embodiment, the anti-CD20 antibodies may have an N-glycosylation pattern that is predominantly G0 glycan. "G0 glycan" means the complex N-linked glycan having the structure $\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2$ as shown in Figure 21, wherein GlcNAc is N-acetylglucosamine and Man is mannose, and wherein a GlcNAc is attached to both the 1,3 mannose arm and the 1,6 mannose arm of the core, which is attached to an amino acid residue of the antibody. Thus, for example, the anti-CD20 antibody composition may have a substantially homogeneous N-glycosylation profile wherein at least 80%, at least 85%, at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99% of the total amount of the N-glycans in the anti-CD20 antibody composition is represented by the G0 glycan species.

[00110] Some immunoglobulins have conserved N-linked glycosylation of the Fc regions of the heavy chains. For example, IgG type immunoglobulins have glycosylated $\text{C}_{\text{H}}2$ domains having N-linked oligosaccharides at asparagines 297. Antibodies with reduced fucose content in the oligosaccharides attached to the asparagines at position 297 of the Fc region may enhance the affinity of Fc for $\text{Fc}\gamma\text{RIII}$, which in turn may increase ADCC of the antibodies.

[00111] Thus, the antibodies having an N-glycosylation profile that is predominantly G0 may have increased ADCC activity relative to antibodies produced in plants that do not have inhibited expression or function of α 1,3-fucosyltransferase (and thus produce antibodies with more α 1,3-fucose).

Methods of Treatment

[00112] The humanized anti-CD20 antibodies of, including antigen-binding fragments thereof, may be used in the treatment of subjects having any disease or disorder associated with CD20-expressing cells, such as normal and malignant B cells
5 expressing CD20 antigen. Thus, the humanized anti-CD20 antibodies are useful for the treatment of B-cell disorders.

[00113] By "malignant" B cell is intended any neoplastic B cell, including but not limited to B cells derived from lymphomas including low, intermediate-, and high-grade B cell lymphomas, immunoblastic lymphomas, non-Hodgkin's lymphomas,
10 Hodgkin's disease, Epstein-Barr Virus (EBV) induced lymphomas, and AIDS-related lymphomas, as well as B cell acute lymphoblastic leukemias, myelomas, chronic lymphocytic leukemias, acute myeloblastic leukemias, and the like.

[00114] Thus, the humanized anti-CD20 antibodies are useful to treat a number of malignant and non-malignant diseases including autoimmune diseases and related
15 conditions, and CD20 positive cancers including B cell lymphomas and leukemias. Stem cells (B-cell progenitors) in bone marrow lack the CD20 antigen, allowing healthy B-cells to regenerate after treatment and return to normal levels within several months.

[00115] Autoimmune diseases or autoimmune related conditions that can be treated
20 using the anti-CD20 antibodies, include arthritis (rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, psoriatic arthritis), psoriasis, dermatitis including atopic dermatitis; chronic autoimmune urticaria, polymyositis/dermatomyositis, toxic epidermal necrolysis, systemic scleroderma and sclerosis, responses associated with inflammatory bowel disease (IBD) (Crohn's disease, ulcerative colitis), respiratory
25 distress syndrome, adult respiratory distress syndrome (ARDS), meningitis, allergic rhinitis, encephalitis, uveitis, colitis, glomerulonephritis, allergic conditions, eczema, asthma, conditions involving infiltration of T cells and chronic inflammatory responses, atherosclerosis, autoimmune myocarditis, leukocyte adhesion deficiency, systemic lupus erythematosus (SLE), lupus (including nephritis, non-renal, discoid,
30 alopecia), juvenile onset diabetes, multiple sclerosis, allergic encephalomyelitis, immune responses associated with acute and delayed hypersensitivity mediated by cytokines and T-lymphocytes, tuberculosis, sarcoidosis, granulomatosis including Wegener's granulomatosis, agranulocytosis, vasculitis (including ANCA), aplastic

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- anemia, Coombs positive anemia, Diamond Blackfan anemia, immune hemolytic anemia including autoimmune hemolytic anemia (AIHA), pernicious anemia, pure red cell aplasia (PRCA), Factor VIII deficiency, hemophilia A, autoimmune neutropenia, pancytopenia, leukopenia, diseases involving leukocyte diapedesis, CNS
- 5 inflammatory disorders, multiple organ injury syndrome, myasthenia gravis, antigen-antibody complex mediated diseases, anti-glomerular basement membrane disease, anti-phospholipid antibody syndrome, allergic neuritis, Bechet's disease, Castleman's syndrome, Goodpasture's Syndrome, Lambert-Eaton Myasthenic Syndrome, Raynaud's syndrome, Sjögren's syndrome, Stevens-Johnson syndrome, solid organ
- 10 transplant rejection (including pretreatment for high panel reactive antibody titers, IgA deposit in tissues, etc), graft versus host disease (GVHD), pemphigoid bullous, pemphigus (all including vulgaris, foliaceus), autoimmune polyendocrinopathies, Reiter's disease, stiff-man syndrome, giant cell arteritis, immune complex nephritis, IgA nephropathy, IgM polyneuropathies or IgM mediated neuropathy, idiopathic
- 15 thrombocytopenic purpura (ITP), thrombotic thrombocytopenic purpura (TTP), autoimmune thrombocytopenia, autoimmune disease of the testis and ovary including autoimmune orchitis and oophoritis, primary hypothyroidism; autoimmune endocrine diseases including autoimmune thyroiditis, chronic thyroiditis (Hashimoto's Thyroiditis), subacute thyroiditis, idiopathic hypothyroidism, Addison's disease,
- 20 Grave's disease, autoimmune polyglandular syndromes (or polyglandular endocrinopathy syndromes), Type I diabetes (also referred to as insulin-dependent diabetes mellitus (IDDM)), Sheehan's syndrome, autoimmune hepatitis, Lymphoid interstitial pneumonitis (HIV), bronchiolitis obliterans (non-transplant) vs NSIP, Guillain-Barré Syndrome, Large Vessel Vasculitis (including Polymyalgia
- 25 Rheumatica and Giant Cell (Takayasu's) Arteritis), Medium Vessel Vasculitis (including Kawasaki's Disease and Polyarteritis Nodosa), ankylosing spondylitis, Berger's Disease (IgA nephropathy), Rapidly Progressive Glomerulonephritis, Primary biliary cirrhosis, Celiac sprue (gluten enteropathy), Cryoglobulinemia, ALS, coronary artery disease.
- 30 **[00116]** CD20 positive cancers that may be treated using the humanized anti-CD20 antibodies described herein include those comprising abnormal proliferation of cells that express CD20 on the cell surface. The CD20 positive B cell neoplasms include CD20-positive Hodgkin's disease, including lymphocyte predominant Hodgkin's

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disease (LPHD); non-Hodgkin's lymphoma (NHL); follicular center cell (FCC) lymphomas; acute lymphocytic leukemia (ALL); chronic lymphocytic leukemia (CLL); and Hairy cell leukemia. The non-Hodgkins lymphomas include low grade/follicular non-Hodgkin's lymphoma (NHL), small lymphocytic lymphoma (SLL), intermediate grade/follicular NHL, intermediate grade diffuse NHL, high grade immunoblastic NHL, high grade lymphoblastic NHL, high grade small non-cleaved cell NHL, bulky disease NHL, plasmacytoid lymphocytic lymphoma, mantle cell lymphoma, AIDS-related lymphoma, and Waldenstrom's macroglobulinemia. Treatments of relapses of these cancers are also contemplated.

10 [00117] In some embodiments, the humanized anti-CD20 antibodies may be used to treat non-Hodgkin's lymphoma (NHL), lymphocyte predominant Hodgkin's disease (LPHD), small lymphocytic lymphoma (SLL), chronic lymphocytic leukemia, rheumatoid arthritis and juvenile rheumatoid arthritis, systemic lupus erythematosus (SLE), Wegener's disease, inflammatory bowel disease, idiopathic thrombocytopenic purpura (ITP), thrombotic thrombocytopenic purpura (TTP), autoimmune
15 thrombocytopenia, multiple sclerosis, psoriasis, IgA nephropathy, IgM polyneuropathies, myasthenia gravis, vasculitis, diabetes mellitus, Raynaud's syndrome, Sjögren's syndrome, and glomerulonephritis.

[00118] The humanized anti-CD20 antibodies described herein are useful as a
20 single-agent treatment in, e.g., for relapsed or refractory low-grade or follicular, CD20-positive, B-cell NHL, or can be administered to patients in conjunction with other drugs in a multi drug regimen.

[00119] Depending on the indication to be treated and factors relevant to the dosing that a physician of skill in the field would be familiar with, the humanized anti-CD20
25 antibodies may be administered at a dosage that is efficacious for the treatment of that indication while minimizing toxicity and side effects. For the treatment of a CD20 positive cancer or an autoimmune disease, the therapeutically effective dosage may be in the range of about 100 mg/m² to about 600 mg/m², although higher and lower doses may also be used. In different embodiments, the dosage may be 100 mg/dose, 125
30 mg/dose, 150 mg/dose, 175 mg/dose, 200 mg/dose, 225 mg/dose, 250 mg/dose, 275 mg/dose, 300 mg, 325 mg/dose, 350 mg/dose, 375 mg/dose, 400 mg/dose, 425 mg/dose, 450 mg/dose, 475 mg/dose, 500 mg/dose, 525 mg/dose, 550 mg/dose, 575 mg/dose, 600 mg/dose. Other dosing amounts, regimens, and intervals may also be

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used.

[00120] In treating disease, the humanized anti-CD20 antibodies may be administered to the patient chronically or intermittently, as determined by the physician of skill in the disease.

- 5 [00121] In some embodiments, a patient may receive an initial conditioning dose(s) of the antibody followed by a therapeutic dose to alleviate or minimize any adverse events. The conditioning dose(s) will be lower than the therapeutic dose to condition the patient to tolerate higher dosages.

- [00122] The humanized anti-CD20 antibodies may be administered using any
10 available route of administration, including by intravenous administration (e.g., as a bolus or by continuous infusion over a period of time), or by subcutaneous, intramuscular, intraperitoneal, intracerebrospinal, intra-articular, intrasynovial, intrathecal, or inhalation routes.

- [00123] In one embodiment, the humanized anti-CD20 antibodies are administered
15 by intravenous infusion with 0.9% sodium chloride solution as an infusion vehicle.

- [00124] In treating the B cell neoplasms described above, the patient may also be treated with the humanized anti-CD20 antibodies described herein in conjunction with one or more therapeutic agents such as a chemotherapeutic agent in a multidrug regimen. The humanized anti-CD20 antibodies may be administered concurrently,
20 sequentially, or alternating with the chemotherapeutic agent, or after non-responsiveness with other therapy. Standard chemotherapy for lymphoma treatment may include cyclophosphamide, cytarabine, melphalan and mitoxantrone plus melphalan. In another embodiment, the humanized CD20 antibody may be used in conjunction with CHOP, which is one of the most common chemotherapy regimens
25 for treating Non-Hodgkin's lymphoma. The following are the drugs used in the CHOP regimen: cyclophosphamide (brand names cytoxan, neosar); adriamycin (doxorubicin/hydroxydoxorubicin); vincristine (Oncovin); and prednisolone (sometimes called Deltasone or Orasone). In particular embodiments, the humanized anti-CD20 antibodies are administered to a patient in need thereof in combination
30 with one or more of the following chemotherapeutic agents: doxorubicin, cyclophosphamide, vincristine, and prednisolone. In a specific embodiment, a patient suffering from a lymphoma (such as a non-Hodgkin's lymphoma) may be treated with the anti-CD20 antibodies described herein in conjunction with CHOP

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(cyclophosphamide, doxorubicin, vincristine and prednisone) therapy. In another embodiment, the cancer patient may be treated with the humanized anti-CD20 antibody in combination with CVP (cyclophosphamide, vincristine, and prednisone) chemotherapy.

5 [00125] In treating the autoimmune diseases or autoimmune related conditions described above, the patient may be treated with the humanized anti-CD20 antibodies in conjunction with a second therapeutic agent, such as an immunosuppressive agent (such as in a multi drug regimen). The humanized anti-CD20 antibody may be administered concurrently, sequentially, or alternating with the immunosuppressive
10 agent, or upon non-responsiveness with other therapy.

[00126] Immunosuppressive agents for use in adjunct therapy include any substances that act to suppress or mask the immune system of a patient. Such agents include, but are not limited to, substances that suppress cytokine production, down regulate or suppress self-antigen expression, or mask the MHC antigens. Examples of
15 such agents include steroids such as glucocorticosteroids (e.g., prednisone, methylprednisolone, and dexamethasone); 2-amino-6-aryl-5-substituted pyrimidines, azathioprine; bromocryptine; glutaraldehyde; anti-idiotypic antibodies for MHC antigens and MHC fragments; cyclosporin A; cytokine or cytokine receptor antagonists including anti-interferon- γ , - β , or - α antibodies; anti-tumor necrosis
20 factor- α antibodies; anti-tumor necrosis factor- β antibodies; anti-interleukin-2 antibodies and anti-IL-2 receptor antibodies; anti-L3T4 antibodies; heterologous anti-lymphocyte globulin; pan-T antibodies; soluble peptide containing a LFA-3 binding domain; streptokinase; TGF- β ; streptodornase; deoxyspergualin; rapamycin; T-cell receptor; T-cell receptor fragments; and T cell receptor antibodies.

25 [00127] For the treatment of rheumatoid arthritis, the patient may be treated with a humanized anti-CD20 antibody in conjunction with any one or more of the following drugs: DMARDS (disease-modifying anti-rheumatic drugs (e.g., methotrexate)), NSAI or NSAID (non-steroidal anti-inflammatory drugs), HUMIRA™ (adalimumab; Abbott Laboratories), ARAVA® (leflunomide), REMICADE® (infliximab; Centocor
30 Inc., of Malvern, Pa.), ENBREL (etanercept; Immunex, Wash.), and COX-2 inhibitors.

Formulations

[00128] Therapeutic formulations of the humanized anti-CD20 antibodies may be

prepared for storage or use by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients, or stabilizers in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g., Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

[00129] The formulations herein may also contain more than one active compound as necessary for the particular indication being treated. For example, it may be desirable to further provide a cytotoxic agent, chemotherapeutic agent, cytokine, or immunosuppressive agent. The effective amount of such other agents depends on the amount of antibody present in the formulation, the type of disease or disorder or treatment, and other factors discussed above.

[00130] The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions.

[00131] Sustained-release preparations may also be prepared comprising the humanized anti-CD20 antibodies. Suitable examples of sustained-release preparations include semi-permeable matrices of solid hydrophobic polymers containing the

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antagonist, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include, but are not limited to, polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides, copolymers of L-glutamic acid and ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers, and poly-D-(-)-3-hydroxybutyric acid.

Conjugated antibodies and other methods

[00132] The humanized anti-CD20 antibodies may be conjugated with one or more therapeutic or diagnostic agents.

10 [00133] The humanized antibodies described herein may be used in therapeutic methods and diagnostic methods. Accordingly, the humanized antibodies may be administered alone as a naked antibody or administered as a multimodal therapy, temporally according to a dosing regimen, but not necessarily conjugated to a therapeutic agent. In one embodiment, the efficacy of the naked humanized and

15 chimeric antibodies can be enhanced by supplementing naked antibodies with one or more other naked antibodies, i.e., monoclonal antibodies to specific antigens, such as CD4, CD5, CD8, CD14, CD15, CD19, CD21, CD22, CD23, CD25, CD33, CD37, CD38, CD40, CD40L, CD46, CD52, CD54, CD74, CD80, CD126, B7, MUC1, Ia, HM1.24, or HLA-DR, tenascin, VEGF, PlGF, an oncogene, an oncogene product, or a

20 combination thereof with one or more immunoconjugates of anti-CD20, or antibodies to these recited antigens, conjugated with therapeutic agents, including drugs, toxins, immunomodulators, hormones, therapeutic radionuclides, etc., with one or more therapeutic agents, including drugs, oligonucleotides, toxins, immunomodulators, hormones, therapeutic radionuclides, etc., administered concurrently or sequentially

25 or according to a prescribed dosing regimen, with the humanized antibodies.

[00134] Immunoconjugates may be administered for diagnostic and therapeutic uses in B cell lymphomas, autoimmune diseases, transplant rejections, and other disease or disorders. Thus, the humanized anti-CD20 antibodies may be conjugated to a cytotoxic agent such as a toxin or a radioactive isotope. In some embodiments,

30 the toxin is calicheamicin, a maytansinoid, a dolastatin, auristatin E and analogs or derivatives thereof.

[00135] A wide variety of diagnostic and therapeutic agents can be conjugated to the humanized anti-CD20 antibodies. Such therapeutic agents can also be used for

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- administration separately with the naked antibody as described above. Therapeutic agents include, for example, chemotherapeutic drugs such as vinca alkaloids, anthracyclines, epidophyllotoxin, taxanes, antimetabolites, alkylating agents, antikinase agents, antibiotics, Cox-2 inhibitors, antimitotic, antiangiogenic and
- 5 apoptotic agents, particularly doxorubicin, methotrexate, taxol, CPT-11, camptothecins, and others from these and other classes of anticancer agents, and the like. Other useful cancer chemotherapeutic drugs for the preparation of immunoconjugates and antibody fusion proteins include nitrogen mustards, alkyl sulfonates, nitrosoureas, triazines, folic acid analogs, COX-2 inhibitors, pyrimidine
- 10 analogs, purine analogs, platinum coordination complexes, hormones, and the like.
- [00136] Additional toxins for use in conjugation with the humanized antibodies include any pharmaceutically acceptable toxins, and include, but are not limited to DNA damaging agents, inhibitors of microtubule polymerization or depolymerization and antimetabolites. Classes of cytotoxic agents include, for example, the enzyme
- 15 inhibitors such as dihydrofolate reductase inhibitors, and thymidylate synthase inhibitors, DNA intercalators, DNA cleavers, topoisomerase inhibitors, the anthracycline family of drugs, the vinca drugs, the mitomycins, the bleomycins, the cytotoxic nucleosides, the pteridine family of drugs, diynenes, the podophyllotoxins and differentiation inducers.
- 20 [00137] The humanized anti-CD20 antibodies may also be conjugated with a radioactive isotope. For example, for selective destruction of a tumor, the antibody may comprise a highly radioactive atom. A variety of radioactive isotopes are available for the production of radioconjugated anti-CD20 antibodies. When the conjugate is to be used for diagnosis, it may comprise a radioactive atom for
- 25 scintigraphic studies, or a spin label for nuclear magnetic resonance (NMR) imaging.
- [00138] The labels may be incorporated into the conjugate using any method. For example, the peptide may be biosynthesized or may be synthesized by chemical amino acid synthesis using suitable amino acid precursors involving, for example, fluorine-19 in place of hydrogen.
- 30 [00139] An oligonucleotide, such as an antisense molecule, may also be conjugated to or be administered with the humanized anti-CD20 antibodies.
- [00140] The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Example 1: Preparation of Humanized 1F5 Antibodies

[00141] Chimeric expression vectors were prepared using the heavy and light chain
5 sequences of the mouse antibody 1F5 (Press et al. *Blood*, 69(2):584-591 (1987)).
Genbank accession number AY058906 provides the sequence of 1F5 VK, the light
chain of mouse antibody 1F5. This VK uses the AJ231219 germline V gene to
whose DNA sequence it is 98% identical. GenBank AY058907 accession number is
the sequence of 1F5 VH, the heavy chain of mouse antibody 1F5. This VH uses the
10 AC090843 germline V gene to whose DNA sequence it is 95% identical.

[00142] The construction of the chimeric expression vectors included adding a
suitable leader sequence to the mouse VH and VK, preceded by a HindIII restriction
site and a Kozak sequence. The Kozak sequence ensures efficient translation of the
variable region sequence. It defines the correct AUG codon from which a ribosome
15 can commence translation. An important base in the Kozak sequence is the adenine at
position -3, upstream of the AUG start. The human 1210 VK leader sequence was
predicted to cut correctly by signal protease when contiguous with 1F5 VK. The
human 1210 VH leader sequence was predicted to cut correctly by signal protease
when contiguous with 1F5 VH. The appropriate 1210 leader sequences were
20 therefore cloned upstream (5') of the 1F5 VH and VK coding regions.

[00143] Construction of the chimeric expression vectors also included introducing
a 5' fragment of the human $\gamma 1$ constant region, up to a natural ApaI restriction site,
contiguous with the 3' end of the J region of 1F5. The human $\gamma 1$ constant region is
encoded in the expression vector, downstream of the inserted VH sequence but, unlike
25 the kappa construct, it lacks a V-C intron. For the kappa chain, the natural splice
donor site and a BamHI site is added downstream of the V region. The splice donor
sequence facilitates the splicing out of the kappa V-C intron, which is useful for in-
frame attachment of VK to the kappa constant region.

[00144] The mouse 1F5 VH and VK genes were analyzed to identify any extra
30 restriction sites which may interfere with the subcloning. One restriction site was
found in the pre-optimized 1F5VK sequence (Figure 17) but none were identified in
1F5VH sequence (Figure 19). These DNA sequences were optimized for expression,
by removing internal TATA-boxes, chi-sites and ribosomal entry sites, AT-rich or

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GC-rich sequence stretches; ARE, INS, CRS sequence elements; repeat sequences and RNA secondary structures; (cryptic) splice donor and acceptor sites, branch points; by adapting codon usage to the codon bias of *Cricetulus griseus*/ *homo sapiens*; and by increasing the GC content to improve mRNA stability. The optimized c1F5VK (Figure 18) and c1F5VH (Figure 20) DNA construct sequences were synthesized and cloned into cloning vector pGA4 (Geneart AG, Regensburg, Germany). The VK and VH inserts were excised with (BamHI + HindIII) or (HindIII + ApaI) respectively. The excised fragments were then gel purified, ligated into pKN100 or pG1D200, respectively, which had been cut similarly, and phosphatase digested. The ligated constructs were transformed into competent TOP10 bacteria. Expression clone colonies were screened by PCR for the presence of an insert of the correct size using primers g10545 (5'-TGTTCCCTTCCATGGGTCTT) (SEQ ID NO:75) and g23070 (5'-GTGTGCACGCCGCTGGTC) (SEQ ID NO:76). Minipreps were generated of the correct expression clones and used to transform HEK293T cells.

[00145] Figure 22 shows the amino acid sequence of the heavy chain of the mature chimeric 1F5 antibody with constant region underlined. Figure 23 shows the amino acid sequence of the light chain of the mature chimeric 1F5 antibody with constant region underlined.

[00146] The chimeric heavy and light chain sequences of the murine monoclonal antibody 1F5 were then humanized by identifying appropriate human FR sequences from sequence comparisons to human and mouse immunoglobulins. The protein sequences of human and mouse immunoglobulins from the International Immunogenetics Database 2006 (Lefranc, M.P., *Nucl. Acids Res.*, 31:307-310(2003)) and the Kabat Database (Kabat, E.A., et al., NIH National Technical Information Service, 1-3242(1991)) Release 5 of Sequences of Proteins of Immunological Interest (last update 17-Nov-1999) were used to compile a database of immunoglobulin protein sequences in Kabat alignment. The database contained 9322 human VH and 2689 human VK sequences. A sequence analysis program, Gibbs, was used to query the human VH and VK databases with 1F5 VH and VK protein sequences.

Generation of 1F5RHA and 1F5RHB

[00147] To generate a humanized VH sequence, human VH sequences in the

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database were identified that had the highest identity to 1F5VH at Vernier, Canonical and VH-VK Interface (VCI) residues, located within the V-region framework. Of the VH sequences identified, accession number U00570 was chosen as the human FR donor for 1F5RHA. The human germline V-gene most identical to U00570 is the

5 VHI family gene X62109 (VI-3B), from which the signal peptide was extracted. The SignalP algorithm predicted that it would cut appropriately with signal peptidase. Figure 1A shows the sequences used in the generation of the DNA sequence 1F5RHAss from the natural DNA sequences of the X62109 signal, 1F5VH CDRs and U00570 FR. CDRs 1, 2 and 3 from 1F5VH were grafted into the acceptor FR of

10 U00570 to generate 1F5RHA (Figure 1B). The DNA sequence was optimized by silent mutagenesis (GeneArt) to remove internal TATA-boxes, chi-sites and ribosomal entry sites, AT-rich or GC-rich sequence stretches, ARE, INS, CRS sequence elements, repeat sequences and RNA secondary structures (cryptic) splice donor and acceptor sites and branch points. The optimized 1F5RHA construct DNA

15 and protein sequence is shown in Figure 2. This construct contains no mutations in the framework region.

[00148] A second construct, 1F5RHB (Figure 3), was based on the 1F5RHA sequence, to contain 4 back-mutations at the non-conserved vernier residues as follows: V67A; V69L; R71A and T73K (Kabat numbering) (Figure 10A).

20

Generation of 1F5RKA11 and 1F5RKA12

[00149] Humanized VK sequences were generated based on sequence comparisons to human VK sequences with the highest identity to 1F5VK at VCI residues, located within the V-region framework. From sequence comparisons, there were no human

25 VK genes identified with the same length of CDR1 as 1F5VK (10 amino acids). Two groups of human FRs with the closest CDR1 lengths (11 and 12 amino acids) were then analyzed that had identical lengths of CDR 2 and 3 as 1F5VK.

[00150] For the 11 amino acid CDR1 sequences, Accession number AB064140 was chosen as the FW donor on which to base 1F5RKA11. Figure 4A shows the

30 generation of 1F5RKA11ss from the natural DNA sequences of 1F5 VK, the human VK sequence AB064140 and the human Z00013 signal sequence. This sequence also was optimized to generate the 1F5RKA11 construct (Figure 5), which contains no FR mutations. Figure 4B provides the amino acid sequences of the FR and CDRs used to

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generate 1F5RKA11.

[00151] CDRs 1, 2 and 3 from 1F5VK were grafted into the acceptor FR of human AB064140 to generate 1F5RKA11. The nearest germline V-gene to AB064140 is Z00013 (Vd/ L8), from which the signal peptide was obtained. The SignalP

5 algorithm predicted appropriate cutting of this signal peptide.

[00152] A second construct, 1F5RKB11 (Figure 6), was generated from 1F5RKA11 by introducing four back mutations at the three non-conserved vernier residues: L46P; L47W and F71Y (Kabat numbering); together with VH/VK interface residue Y87F (Kabat numbering) (Figure 10B). Further versions: 1F5RKB11;

10 1F5RKD11; 1F5RKE11 and 1F5RKF11 were based on 1F5RKB11, each containing a different reversal of one of these 4 mutations (Figure 10B).

[00153] A second set of humanized VK sequences were also prepared based on a human kappa sequence with CDR1 sequences containing 12 amino acids. Accession number AY263415 was selected as the FR donor on which to base 1F5RKA12.

15 [00154] Figure 7A shows the nucleic acid sequences used to generate 1F5RKA12ss from the natural DNA sequence of 1F5 VK, the human VK sequence AY263415 and the human X12686 signal sequence. CDRs 1, 2 and 3 from 1F5VK were grafted into the acceptor FR of human AY263415 to generate 1F5RKA12 (Figure 7B). The nearest germline V-gene to accession AY263415 is the VKIII family member,
20 X12686 (A27), from which the signal peptide was obtained. The SignalP algorithm predicted appropriate cutting of this signal peptide. The optimized 1F5RKA12 construct DNA and protein sequence is shown in Figure 8. This construct contains no FR mutations. A second construct 1F5RKB12 (Figure 9) was generated from 1F5RKA12 by introducing three back mutations at the three non-conserved vernier
25 residues: L46P; L47W and F71Y (Kabat numbering). Further versions: 1F5RKC12; 1F5RKD12 and 1F5RKE12 were based on 1F5RKB12, each containing a different reversal of one of these 3 mutations.

[00155] Expression vectors were prepared that included, for the heavy chains, a human γ 1 constant region attached to the variable region, and for the light chains, a
30 kappa constant region attached to the variable region.

[00156] Figures 11A-E provide the amino acid sequences of the mature variable and constant regions from 1F5RKG11, 1F5RKB11, 1F5RKF12, 1F5RKB12, and 1F5RHA proteins, respectively. Figures 22 and 23 provide the amino acid sequences

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for a chimeric protein sequence of mature variable and constant regions for c1F5VH and c1F5VK, respectively. The constant regions of the amino acid sequences are underlined.

[00157] The expression plasmid preparations encoding (humanized or chimeric) heavy and light chains were used to transfect HEK293t cells. For transfection, cells were initially grown in DMEM plus GlutaMax supplemented with 10% FCS, penicillin, and streptomycin in a flask and incubated in a CO₂-gassed cell culture chamber. The cell cultures were split 1:3 to every two days or 1:4 or 1:5 every 3-4 days. Light trypsinisation was used to detach cells from flasks during passaging. One day before transfection, 6-well plates were prepared by adding 2 ml of culture media to each well, followed by the addition of cells (2×10^5 cells/well). Cells were checked the following day to ensure at least 80% confluency and the media was replaced with 2 ml/well. For transfection, 96 μ l of OPTiPRO SFM was aliquoted into a sterile 1.5 ml tube and 6 μ l of Fugene 6 was added directly to the media, and incubated at room temperature for 5 minutes. DNA (1 μ g total; 0.5 μ g heavy chain and 0.5 μ g light chain vector) was added and incubated at room temperature for 15 minutes. A mixture of the Fugene 6, DNA, and OptiPro SFM mixture was added, dropwise, around the well, and the plates were then incubated for 4 days in a cell culture incubator. The conditioned medium was harvested after 4 days, IgG was quantified, and antigen-binding assays performed.

[00158] IgG in these conditioned media was measured by ELISA to measure Raji cell binding (Example 2). The concentrations of IgG1 κ antibody in all transfected 293 cell-conditioned media used are shown in Table 1. The humanized and chimeric antibodies were expressed at good to excellent levels, and were used for the further Examples 2-4 below.

Table 1

Constructs used for 293 transfection		IgG	
VH	VK	ng/ml	
chimeric	chimeric	3685	5580
chimeric	chimeric	4335	5084
chimeric	chimeric	8714	8479

chimeric	KB11	847	1576
chimeric	KC11	4603	6381
chimeric	KD11	2352	4940
chimeric	KE11	1537	1543
chimeric	KF11	1730	1730
chimeric	KB12	813	1062
chimeric	KC12	1861	1498
chimeric	KD12	2772	2684
chimeric	KE12	2964	2964
RHA	chimeric	3222	4215
RHB	chimeric	4876	3306
RHA	chimeric	6291	6895
RHA	RKB12	1960	2146
RHA	RKC12	447	494
RHA	RKD12	4789	4651
RHA	RKE12	2517	2375
RHA	RKF12	3773	3394
RHA	RKB11	6258	2267
RHA	RKC11	6258	6609
RHA	RKD11	8600	8366
RHA	RKE11	9182	7957
RHA	RKF11	8517	5529
RHA	RKG11	8516	8184

Example 2: Raji Binding By Humanized 1F5 Antibodies

- [00159] Assays were performed to measure binding of the chimeric and humanized
- 5 1F5 antibodies to Raji cells (a human B cell line expressing CD20). Raji cells (80 μ l; $1-2 \times 10^6$ cells/ml in 24 ml with fresh growth medium diluted with 0.11 vol of 10x PBS (MPBS) and 8ml 8% paraformaldehyde (in PBS)) were added to 96-well poly-D-lysine-coated plate and centrifuged at 2500 rpm (Beckman 6L) at 25° C for 60 minutes. The pellets were washed 4 times with 400 μ l PBS, Tween 20 (0.02% v/v).

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50 µl of MPBS was added to each well after the final wash, and incubated for 1 hour at room temperature, and the blocking buffer was then discarded. Serial dilutions of the 1F5 antibodies were prepared in MPBS in a separate 96-well plate, transferred to the Raji-cell coated plate and incubated at room temperature for 1 hour. The antibody solution was then discarded and the plates were washed 6 times with 400 µl PBS buffer. Goat anti-human kappa (Sigma A7164) was prepared by diluting the stock antibody solution 1:2000 in MPBS, added to the washed wells, and incubated again at room temperature for 1 hour. The plates were again washed 6 times in 400 µl PBS buffer. 100 µl of K-blue TMB solution was added to each well. The reactions were stopped by added 50 µl red stop after 30 minutes, and the absorbance was read at 650 nm.

[00160] As illustrated in Figure 12, the binding to Raji cells of antibodies encoded by chimeric c1F5VH, in association with humanized VKs: 1F5RKA11; 1F5RKA12; 1F5RKC11 or 1F5RKC12 was reduced compared with that of the chimeric c1F5 antibody (Figures 12A, 12C, and 12D). In contrast, the Raji cell binding by antibodies encoded by chimeric c1F5VK in combination with either 1F5RHA or 1F5RHB was indistinguishable, and similar to that of Rituxan (Figure 12B). Other VK versions: 1F5RKB11; 1F5RKD11; 1F5RKE11; 1F5RKF11 (Figure 12D); 1F5RKB12; 1F5RKD12; 1F5RKE12; (Figure 12C), in combination with chimeric c1F5VH, bound to Raji cells similarly to, or better than, the chimeric antibody.

[00161] In light of the low binding potency of the 1F5RKC11 and 1F5RKC12 versions, in which the vernier L46P backmutation (Kabat numbering) was reversed, 1F5RKG11 and 1F5RKF12 were designed (Figure 10 B and C, respectively) based on 1F5RKA11 or 1F5RKA12, respectively, with the sole introduction of the L46P back mutation (Kabat numbering).

Additional assays (Figures 13-15) investigated fully humanized antibodies encoded by 1F5RHA together with various humanized kappa chains. Figures 13A-B show that the Raji cell binding by fully humanized antibodies encoded by 1F5RHA, in association with all kappa chain constructs except the 1F5RKC versions, is similar to that of the chimeric antibody. The Raji cell binding by antibodies encoded by the kappa constructs 1F5RKC12 and 1F5RKC11 (not shown) was again poor. Figure 14 confirms that the Raji cell binding potency of antibody encoded by 1F5RHA in association with 1F5RKF12 appears to be slightly superior to that of the antibody

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encoded by 1F5RKG11, and that their potencies are similar to that of the chimeric antibody.

Example 3: FACS Binding Assay

5 [00162] Raji cell binding by the humanized 1F5 antibodies was also analyzed with a FACSort flow cytometer (Becton Dickinson, San Jose CA). Standard FACS (fluorescent-activated cell sorting) procedures were utilized for the assays. For measurement of cell binding, debris and cell clumps were gated out based on forward versus side scatter. Dead cells were excluded from analysis ("gated out") based on PI
10 uptake. 5,000 to 10,000 viable cells were analyzed per sample and the geometric mean of the fluorescence distribution (mean fluorescence intensity, or MFI) was determined using instrument software (CellQuest, Becton-Dicknson). MFI values are reported for all studies. Two studies were conducted for each of humanized antibodies RHA x RKF12; RHA x RKB11; and RHA x RKG11. The results of the
15 FACS binding assay are provided in Figure 16. As shown in the figure, the humanized antibodies that were tested (RHA x RKF12; RHA x RKB11; and RHA x RKG11) all had higher binding affinity than the chimeric 1F5.

Example 4: Thermostability

20 [00163] To investigate whether the two antibodies (1F5RKG11 x 1F5RHA and 1F5RKF12 x 1F5RHA) were of similar structural stability, 1 µg/ml dilutions of each antibody in culture medium, buffered by PBS, were heated for 10 minutes at seven temperatures between 50 and 80 °C (50°, 55°, 60°, 65°, 70°, 75°, and 80°C). After rapid cooling to 4°C, Raji binding analysis was performed as described above. The
25 results of the Raji binding assay (Figure 15) indicated that the 1F5RKG11 x 1F5RHA antibody retained binding potency at a temperature higher than did the 1F5RKF12 x 1F5RHA antibody. At 75°C the 1F5RKG11 antibody retained a greater residual potency than Rituxan, which was inactivated by 10 minutes at this temperature.

30 **Example 5: CDC Activity, Off-Rate, and B-cell depletion of humanized 1F5 antibodies**

[00164] Four of the humanized 1F5 antibodies described above (RHA x RKB11 (Variant D); RHA x RKG11 (Variant E); RHA x RKB12 (Variant F); and RHA x

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RKF12 (Variant G)) as well as chimeric antibody c1F5 were produced in transgenic *Lemna* plants providing for primarily G0 glycosylation. Analysis of the chimeric and humanized antibodies (e.g., by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis) confirmed that the antibodies

5 had predominantly G0 glycosylation (data not shown).

[00165] The purpose of the present example was to evaluate several aspects of the *in vitro* activity of these antibodies, including complement-dependent cytotoxic activity (CDC) using Raji cells as targets, dissociation of binding ("off rate") from B-cells, and *in vitro* B-cell depleting activity in whole blood. The values for rituximab

10 (RTX) were also measured and compared to the values determined for the humanized and chimeric 1F5 antibodies.

CDC Activity

[00166] Complement dependent cytotoxicity (CDC) of variant antibodies was measured in Raji Burkitt Lymphoma cells (ATCC Accession Number CCL-86) using

15 flow cytometry to enumerate dead cells. Briefly, cells were treated with varying concentrations of different antibodies in 90 microliters PBS followed by the addition of 10 microliters normal human serum to give a final concentration of 10% serum. Cells were incubated for 30 minutes at 37°C and then placed on ice. Cold (4°C) PBS containing propidium iodide (PI) was added to the cells and the frequency of PI-

20 positive cells determined by FACS.

[00167] Figure 24 shows the results of the CDC assay of various concentrations of the tested antibodies. As shown in the Figure, the G0-glycosylated humanized 1F5 antibodies had much less complement dependent cytotoxicity than rituximab, with the CDC of antibody E being approximately 10 times less than rituximab.

25 [00168] In terms of EC₅₀'s, the antibodies ranked as follows:
RTX < Variant G < Variant F < Variant D < chimeric < Variant E (Table 2).

Table 2

Variant	RTX	chimeric	Variant D	Variant E	Variant F	Variant G
LogEC ₅₀	-1.726	-0.7810	-1.063	-0.773	-1.133	-1.146
EC ₅₀	0.01879	0.1656	0.08642	0.1685	0.07368	0.07138

30 Antibody Off-Rates From Cells

[00169] The rate at which fluorescently-labeled antibodies dissociated from Raji

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cells was compared with RTX over a 4-hour time period. The assay format used followed that described in Goldenberg D.M et al. (2009. Blood, Vol 113(5):1062) and is briefly outlined below.

[00170] All antibodies were conjugated to a fluorescein-like fluorophore, DyLight 488, according to vendor instructions (Pierce Cat. No. 53025). Following conjugation, labeled antibody was separated from free dye using chromatography columns provided in the labeling kit. Protein concentration after labeling was measured spectrophotometrically and concentrations found to vary between 0.93 and 1.1 mg/ml. Purified labeled antibodies were stored at 4°C in PBS protected from light.

[00171] To measure antibody dissociation from cells, 10⁶ Raji cells were suspended in PBS containing 1% bovine serum albumin (PBS/BSA). Labeled antibody was added to a final concentration of 5ug/ml in a volume of 1.0 ml. Following a 30 minute incubation at 37°C, unlabeled ("cold") antibody was added to a final concentration of 1000 ug/ml. Labeled cells mixed with cold antibody were returned to 37°C for 4 hours. At 20-minute intervals, 100 ul aliquots of cells were withdrawn from each sample, 400 uL cold PBS/BSA containing propidium iodide was added and mean fluorescence intensity (MFI) of the cell population excluding dead cells (PI⁺) determined by FACS. It was presumed with this protocol that labeling does not influence antibody affinity.

[00172] It was observed that the humanized 1F5 antibodies had significantly decreased off-rates relative to rituximab (Figure 25). As shown in Figure 25, it is clear that the humanized 1F5 antibodies show roughly a 2-3 fold increase in half-life relative to rituximab.

[00173] The results of off-rate studies are summarized in Table 3. Although antibody Variants D and F showed some variation in half-life in the two studies, overall the half-lives were similar. All humanized 1F5 antibodies had significantly decreased off rates as compared to rituximab (i.e., the humanized 1F5 antibodies bound ~2-3 times longer than rituximab).

Table 3. Half-life values for anti-CD20 antibodies

	chimeric	Variant D	Variant E	Variant F	Variant G	RTX
Study 1	59	44	67	72	67	24
Study 2	57	59	63	64	65	24

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Average	58	52	65	68	66	24
---------	----	----	----	----	----	----

[00174] Figure 26 shows a bar graph of the half-lives obtained from the anti-CD20 antibodies in Study 1.

B-Cell Depletion in Whole Blood

5 [00175] The B-cell depleting activity of the humanized 1F5 antibodies was evaluated. Briefly, test antibodies were added to 100 ul fresh whole blood at specified concentrations and incubated for 4 hours at 37°C. The frequency of B cells was determined using CD19, a B-cell specific antibody that binds cells independently of CD20 receptor expression. Two studies were performed, one in which CD16 was not
10 blocked with an anti-CD16 antibody prior to exposure to test antibody and one in which CD16 was pre-blocked.

[00176] In the first set of experiments without anti-CD16, the cytotoxicity of the humanized 1F5 antibodies was observed to be greater than that of rituximab (Figure 27). That is, the humanized 1F5 antibodies deplete B cells more effectively than
15 rituximab. Figure 27 shows the results of the first set of experiments for the chimeric 1F5, variants D-G, rituximab, and an isotype control (i.e., a control IgG1, G0 antibody with no B-cell antigen binding properties).

[00177] In the second set of experiments, CD16 receptor on effector cells was pre-blocked with anti-CD16 antibody. When humanized antibody variants E and G were
20 then tested in the B-cell depletion assay, it was shown that the B-cell depleting activity of the anti-CD20 antibodies was inhibited by blocking CD16 on effector cells with an anti-CD16 antibody (Figure 28). The effect of anti-CD16 was to reduce B-cell depleting activity almost completely, implicating ADCC as the major mode of action for the humanized 1F5 antibodies. Anti-CD16 antibody inhibited B-cell
25 depletion for variants E and G similarly to rituximab.

Conclusion

[00178] I. The humanized 1F5 antibodies have much lower CDC activity than rituximab.

[00179] II. The rate at which all of the humanized 1F5 antibodies dissociated from
30 cells (i.e., the off-rate) was significantly reduced relative to the off-rate of rituximab.

[00180] III. All of the humanized 1F5 antibodies were more efficient than rituximab in depleting B-cells from whole blood.

[00181] IV. For the two humanized 1F5 antibody variants tested, B-cell depletion in whole blood was almost completely inhibited by treatment with a CD16-blocking antibody.

[00182] While the invention has been described in detail and with reference to
5 specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made without departing from the spirit and scope of the invention.

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What is claimed is:

1. A humanized antibody comprising:

(a) a light chain comprising a light chain variable region comprising an
 5 amino acid sequence selected from the group consisting of:

(1) Glu Ile Val Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
 Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Ser Ser Leu
 Ser Phe Met His Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro
 Lys Xaa₄₅ Xaa₄₆ Ile Tyr Ala Thr Ser Asn Leu Ala Ser Gly
 10 Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Xaa₇₀
 Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr
 Tyr Xaa₈₆ Cys His Gln Trp Ser Ser Asn Pro Leu Thr Phe Gly
 Gly Gly Thr Lys Val Glu Ile Lys (SEQ ID NO: 1)

wherein

15 Xaa₄₅ is Pro or Leu;
 Xaa₄₆ is Trp or Leu;
 Xaa₇₀ is Tyr or Phe; and
 Xaa₈₆ is Tyr or Phe; and

(2) Glu Ile Val Leu Thr Gln Ser Pro Gly Thr Leu Ser Leu Ser
 20 Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Ser Ser
 Leu Ser Phe Met His Trp Tyr Gln Gln Lys Pro Gly Gln Ala
 Pro Arg Xaa₄₅ Xaa₄₆ Ile Tyr Ala Thr Ser Asn Leu Ala Ser
 Gly Ile Pro Ala Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp
 Xaa₇₀ Thr Leu Thr Ile Ser Arg Leu Glu Pro Glu Asp Phe Ala
 25 Val Tyr Phe Cys His Gln Trp Ser Ser Asn Pro Leu Thr Phe
 Gly Gln Gly Thr Lys Val Glu Leu Lys (SEQ ID NO: 2)

wherein

Xaa₄₅ is Pro or Leu;
 Xaa₄₆ is Trp or Leu; and
 30 Xaa₇₀ is Tyr or Phe; and

(b) a heavy chain comprising a heavy chain variable region comprising the
 following amino acid sequence:

5 Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala
 Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
 Asn Met His Trp Val Arg Gln Ala Pro Gly Gln Arg Leu Glu Trp Ile
 Gly Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr Asn Gln Lys Phe
 Lys Gly Arg Xaa₆₈ Thr Xaa₇₀ Thr Xaa₇₂ Asp Xaa₇₄ Ser Ala Asn Thr
 Ala Tyr Met Glu Leu Ser Ser Leu Lys Ser Glu Asp Thr Ala Val Tyr
 Tyr Cys Ala Arg Ser His Tyr Gly Ser Asn Tyr Val Asp Tyr Phe Asp
 Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser (SEQ ID NO: 3)

wherein

- 10 Xaa₆₈ is Val or Ala;
 Xaa₇₀ is Val or Leu;
 Xaa₇₂ is Arg or Ala; and
 Xaa₇₄ is Thr or Lys;

wherein the humanized antibody binds to human CD-20.

15

2. The humanized antibody of claim 1, wherein the heavy chain variable region comprises the amino acid sequence of SEQ ID NO: 13.
3. The humanized antibody of claim 1, wherein the light chain variable region
 20 comprises the amino acid sequence of SEQ ID NO: 54.
4. The humanized antibody of claim 1, wherein the light chain variable region comprises the amino acid sequence of SEQ ID NO: 49.
- 25 5. The humanized antibody of claim 1, wherein the light chain variable region comprises the amino acid sequence of SEQ ID NO: 59.
6. The humanized antibody of claim 1, wherein the light chain variable region comprises the amino acid sequence of SEQ ID NO: 55.
- 30 7. The humanized antibody of claim 1, wherein the heavy chain variable region comprises the amino acid sequence of SEQ ID NO: 13 and the light chain

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variable region comprises an amino acid sequence selected from the group consisting of SEQ ID NOS: 49, 54, 55, and 59.

8. The humanized antibody of claim 1, wherein the heavy chain variable
5 region comprises an amino acid sequence selected from the group consisting of
SEQ ID NOS: 13 and 48.

9. The humanized antibody of claim 1, wherein the light chain variable region
comprises an amino acid sequence selected from the group consisting of SEQ ID
10 NOS: 28, 49, 50, 51, 52, 53, 54, 42, 55, 56, 57, 58 and 59.

10. The humanized antibody of claim 8, wherein the light chain variable region
comprises an amino acid sequence selected from the group consisting of SEQ ID
NOS: 28, 49, 50, 51, 52, 53, 54, 42, 55, 56, 57, 58 and 59.

15

11. The humanized antibody of claim 1, wherein the antibody binds to human
CD20 with an affinity similar to that of chimeric antibody c1F5.

12. The humanized antibody of claim 1, wherein the antibody binds to human
20 CD20 with an affinity greater than that of chimeric antibody c1F5.

13. The humanized antibody of claim 1, wherein the antibody binds to human
CD20 with an off-rate lower than that of chimeric antibody c1F5.

25 14. The humanized antibody of claim 1, wherein the light chain further
comprises a human light chain constant region.

15. The humanized antibody of claim 14, wherein the light chain comprises an
amino acid sequence selected from the group consisting of SEQ ID NOS: 60, 61,
30 62, and 63.

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16. The humanized antibody of claim 1, wherein the heavy chain further comprises a human heavy chain constant region.
17. The humanized antibody of claim 16, wherein the heavy chain comprises
5 the amino acid sequence of SEQ ID NO: 64.
18. The humanized antibody of claim 14, wherein the heavy chain further comprises a human heavy chain constant region.
- 10 19. The humanized antibody of claim 18, wherein the heavy chain comprises the amino acid sequence of SEQ ID NO: 64 and the light chain comprises an amino acid sequence selected from the group consisting of SEQ ID NOS: 60, 61, 62, and 63.
- 15 20. The humanized antibody of claim 19, wherein the heavy chain comprises the amino acid sequence of SEQ ID NO: 64 and the light chain comprises the amino acid sequence of SEQ ID NO: 60.
21. The humanized antibody of claim 19, wherein the heavy chain comprises
20 the amino acid sequence of SEQ ID NO: 64 and the light chain comprises the amino acid sequence of SEQ ID NO: 61.
22. The humanized antibody of claim 19, wherein the heavy chain comprises the amino acid sequence of SEQ ID NO: 64 and the light chain comprises the
25 amino acid sequence of SEQ ID NO: 62.
23. The humanized antibody of claim 19, wherein the heavy chain comprises the amino acid sequence of SEQ ID NO: 64 and the light chain comprises the amino acid sequence of SEQ ID NO: 63.
30
24. A pharmaceutical composition comprising the humanized antibody of any of the preceding claims and a pharmaceutically acceptable excipient.

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25. A pharmaceutical composition comprising the humanized antibody of claim 7 and a pharmaceutically acceptable excipient.
- 5 26. The humanized antibody of claim 1, wherein the antibody is produced in a host cell selected from the group consisting of a mammalian cell, a yeast cell, and a plant cell.
27. A humanized antibody comprising a light chain comprising a light chain
10 variable region comprising the following amino acid sequence:
Glu Ile Val Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
Val Thr
Ile Thr Cys Arg Ala Ser Ser Ser Leu Ser Phe Met His Trp Tyr Gln Gln Lys
Pro Gly
15 Lys Ala Pro Lys Xaa₄₅ Xaa₄₆ Ile Tyr Ala Thr Ser Asn Leu Ala Ser Gly Val
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Xaa₇₀ Thr Leu Thr Ile
Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Xaa₈₆ Cys His Gln Trp Ser
Ser Asn Pro Leu Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys (SEQ ID
NO: 1)
20 wherein
Xaa₄₅ is Pro or Leu;
Xaa₄₆ is Trp or Leu;
Xaa₇₀ is Tyr or Phe; and
Xaa₈₆ is Tyr or Phe;
25 wherein the humanized antibody binds to human CD-20.
28. The humanized antibody of claim 27, wherein the light chain variable region has the amino acid sequence of SEQ ID NO: 54.
- 30 29. The humanized antibody of claim 27, wherein the light chain variable region has the amino acid sequence of SEQ ID NO: 49.

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30. The humanized antibody of claim 27, wherein the light chain variable region comprises an amino acid sequence selected from the group consisting of SEQ ID NOS: 28, 49, 50, 51, 52, 53, and 54.

5 31. The humanized antibody of claim 27, wherein the antibody binds to human CD20 with an affinity similar to that of chimeric antibody c1F5.

32. The humanized antibody of claim 27, wherein the antibody binds to human CD20 with an affinity greater than that of chimeric antibody c1F5.

10

33. The humanized antibody of claim 27, wherein the antibody binds to human CD20 with an off-rate lower than that of chimeric antibody c1F5.

34. A humanized antibody comprising a light chain comprising a light chain
15 variable region comprising the following amino acid sequence:

Glu Ile Val Leu Thr Gln Ser Pro Gly Thr Leu Ser Leu Ser Pro Gly Glu Arg
Ala Thr

Leu Ser Cys Arg Ala Ser Ser Ser Leu Ser Phe Met His Trp Tyr Gln Gln Lys
Pro Gly

20 Gln Ala Pro Arg Xaa₄₅ Xaa₄₆ Ile Tyr Ala Thr Ser Asn Leu Ala Ser Gly Ile
Pro Ala Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Xaa₇₀ Thr Leu Thr Ile
Ser Arg Leu Glu Pro Glu Asp Phe Ala Val Tyr Phe Cys His Gln Trp Ser
Ser Asn Pro Leu Thr Phe Gly Gln Gly Thr Lys Val Glu Leu Lys (SEQ ID
NO: 2)

25 wherein

Xaa₄₅ is Pro or Leu;

Xaa₄₆ is Trp or Leu; and

Xaa₇₀ is Tyr or Phe;

wherein the humanized antibody binds to human CD-20.

30

35. The humanized antibody of claim 34, wherein the light chain variable region comprises the amino acid sequence of SEQ ID NO: 59.

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36. The humanized antibody of claim 34, wherein the light chain variable region comprises the amino acid sequence of SEQ ID NO: 55.
- 5 37. The humanized antibody of claim 34, wherein the light chain variable region comprises an amino acid sequence selected from the group consisting of SEQ ID NOS: 42, 55, 56, 57, 58 and 59.
38. The humanized antibody of claim 34, wherein the antibody binds to human
10 CD20 with an affinity similar to that of chimeric antibody c1F5.
39. The humanized antibody of claim 34, wherein the antibody binds to human CD20 with an affinity greater than that of chimeric antibody c1F5.
- 15 40. The humanized antibody of claim 34, wherein the antibody binds to human CD20 with an off-rate lower than that of chimeric antibody c1F5.
41. A humanized antibody comprising a heavy chain comprising a heavy chain variable region comprising the following amino acid sequence:
- 20 Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala Ser
Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Tyr Asn Met
His Trp Val Arg Gln Ala Pro Gly Gln Arg Leu Glu Trp Ile Gly Ala Ile Tyr
Pro Gly Asn Gly Asp Thr Ser Tyr Asn Gln Lys Phe Lys Gly Arg Xaa₆₈ Thr
Xaa₇₀ Thr Xaa₇₂ Asp Xaa₇₄ Ser Ala Asn Thr Ala Tyr Met Glu Leu Ser Ser
25 Leu Lys Ser Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg Ser His Tyr Gly
Ser Asn Tyr Val Asp Tyr Phe Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr
Val Ser Ser (SEQ ID NO: 3)
wherein
Xaa₆₈ is Val or Ala;
30 Xaa₇₀ is Val or Leu;
Xaa₇₂ is Arg or Ala; and
Xaa₇₄ is Thr or Lys;

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wherein the humanized antibody is capable of binding to human CD-20.

42. The humanized antibody of claim 41, wherein the antibody binds to human CD20 with an affinity similar to that of chimeric antibody c1F5.

5

43. The humanized antibody of claim 41, wherein the antibody binds to human CD20 with an affinity greater than that of chimeric antibody c1F5.

44. The humanized antibody of claim 41, wherein the antibody binds to human CD20 with an off-rate lower than that of chimeric antibody c1F5.

10

45. The humanized antibody of claim 41, wherein the heavy chain variable region comprises the amino acid sequence of SEQ ID NO: 13.

46. The humanized antibody of claim 41, wherein the heavy chain variable region comprises the amino acid sequence of SEQ ID NO: 48.

15

47. A method of treating a B-cell disorder in a subject comprising administering to a subject in need thereof a therapeutically effective amount of the antibody of claim 1, 27, 34, or 41.

20

48. A method of preventing a B-cell disorder in a subject comprising administering to the subject a prophylactically effective amount of the antibody of claim 1, 27, 34, or 41.

25

49. A humanized anti-CD20 antibody composition comprising the humanized antibody of claim 1, 27, 34, or 41, wherein at least 90% of the N-glycans present in said composition are GlcNAc₂Man₃GlcNAc₂ (G0).

50. An isolated nucleic acid comprising a nucleic acid sequence encoding the antibody of claim 1, 27, 34, or 41.

30

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51. A host cell comprising the isolated nucleic acid of claim 50.

Figure 1A

Region	DNA	SEQ ID NO
X62109 signal	ATGGACTGGACCTGGAGGATCCTCTTTTTGGTGGCAGCAGCCACAGGTGCCCCACTCC	4
U00570 FR1	caggtccagcttgtgcagtctggggctgaggtgaagaagcctggggcctcagtgaaaggttccctgcaaggcttctggatacaccttcact	5
IF5VH CDR1	AGTTACAATATGCAC	6
U00570 FR2	tgggtgcgccaggccccggacaaaggcttgagtggatagga	7
IF5VH CDR2	GCTATTTATCCAGGAAATGGTGATACTTCCTACAATCAGAAGTTCAAAGGC	8
U00570 FR3	agagtcaccgttaccaggacacatccgcgaacacagcctacatggagctgagcagcctaaaaatctgaagacacggctgtgtattactgtgcgaga	9
IF5VH CDR3	TCGCACTACGGTAGTAACCTACGTAGACTACTTTGACTAC	10
U00570 FR4	tggggccagggaaccctgggtcaccgtctcctca	11

Figure 1B

Kabat Number	1	2	3	4	5	6	7	8	9	10	11	SEQ ID NO
CDR	===H1== =====H2===== =====H3=====											
IF5VH	-QVQLRQPGAEIVKPGASVKMSCKASGYTFTSYNMH--WVRQTPGQGLEWIGAIYP--GNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYCARSHYGSNYVDY-----FDYWGQGT LVTVST											12
IF5VH CDR	SYNMH--AIYP--GNGDTSYNQKFKGSHYGSNYVDY-----FDY											77 78 79
IF5RHA	-QVQLVQSGAEVKKPGASVKVSKASGYTFTSYNMH--WVRQAPGQRLEWIGAIYP--GNGDTSYNQKFKGRVTVTRDTSANTAYMELSSLKSEDTAVYYCARSHYGSNYVDY-----FDYWGQGT LVTVSS											13
U00570 FR	-QVQLVQSGAEVKKPGASVKVSKASGYTFTWVRQAPGQRLEWIGRVTVTRDTSANTAYMELSSLKSEDTAVYYCARWGQGT LVTVSS											80 81 82 83
U00570	-QVQLVQSGAEVKKPGASVKVSKASGYTFTNYAIH--WVRQAPGQRLEWIGWINT--ASANTKCSQNFQGRVTVTRDTSANTAYMELSSLKSEDTAVYYCARCSGDTCTCYQG-----SDYWGQGT LVTVSS											14

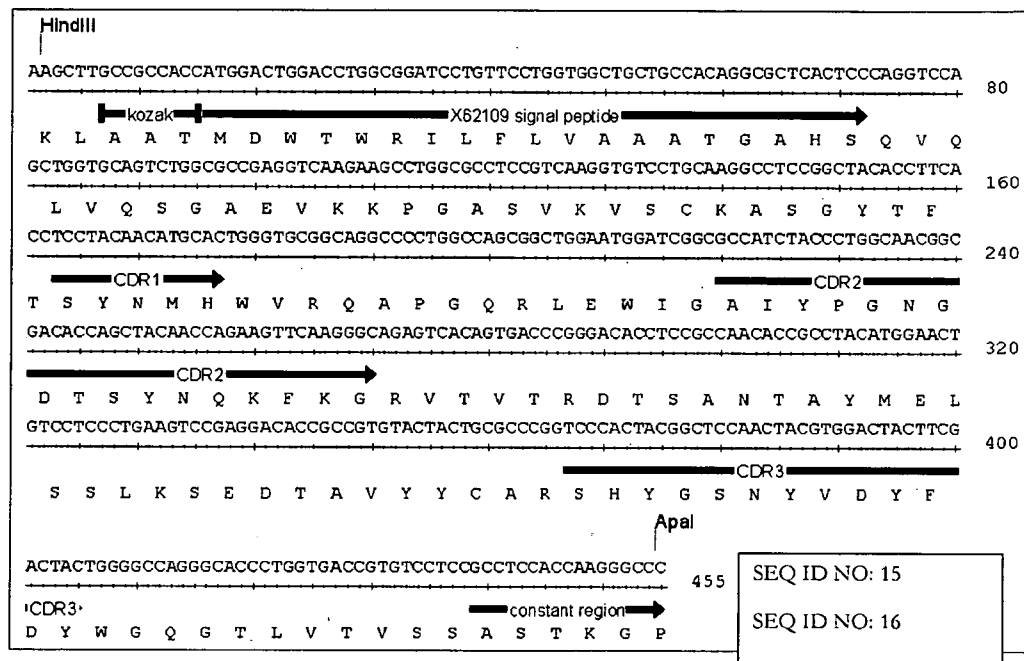


Figure 2



Figure 3

Figure 4A

Region	DNA	SEQ ID NO:
Z00013 signal	ATGGACATGAGGGTCCCCGCTCAGCTCCTGGGGCTCCTGCTGCTCTGGCTCCCAGGTGC CAGATGT	19
AB064140 FR1	gaaattgtgctgactcagtcctccatcctccctgtctgcatctgtaggagacagagtcac catcacttgc	20
1F5VK CDR1	AGGGCCAGCTCAAGTTTAAGTTTCATGCAC	21
AB064140 FR2	tggtatcagcaaaaaccagggaaagcccccctaagctcctgatctat	22
1F5VK CDR2	GCCACATCCAACCTGGCTTCT	23
AB064140 FR3	ggggtcccatcaaggttcagcggcagtggaatctgggacagatttcactctcaccatcag cagcctgcagcctgaagattttgcaacttattactgt	24
1F5VK CDR3	CATCAGTGGAGTAGTAACCCGCTCACG	25
AB064140 FR4	ttcggcggaggggaccaaggtggagatcaaa	26

Legend: Z00013 signal peptide, AB064140 FR and 1F5VK CDRs, intercalated to generate 1F5RKA11ss

Figure 4B

[illegible]

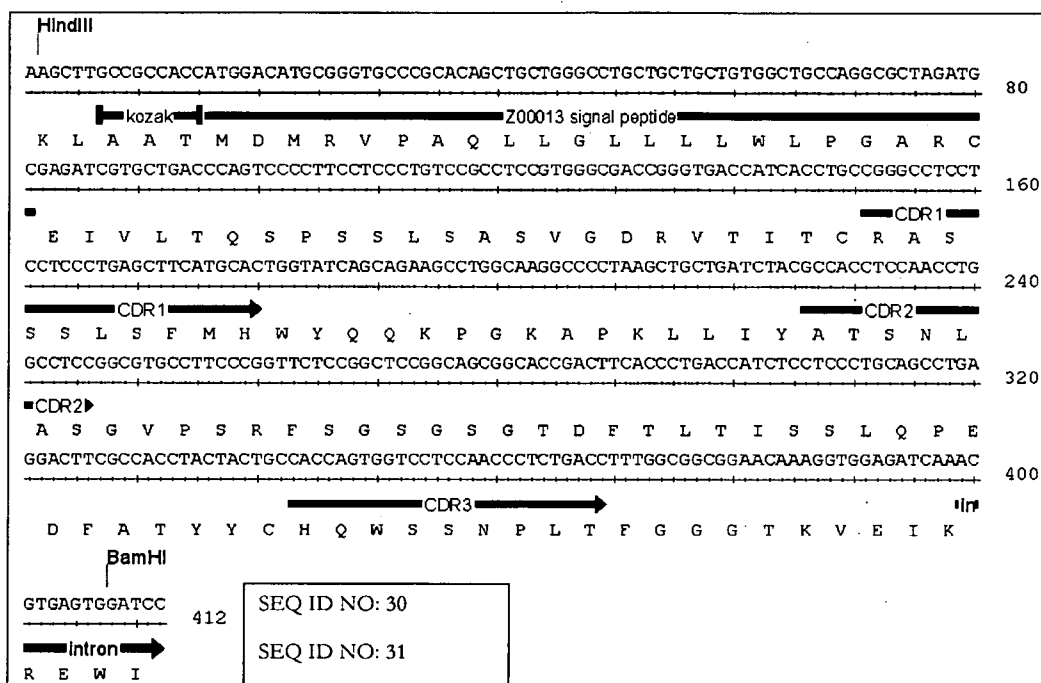


Figure 5

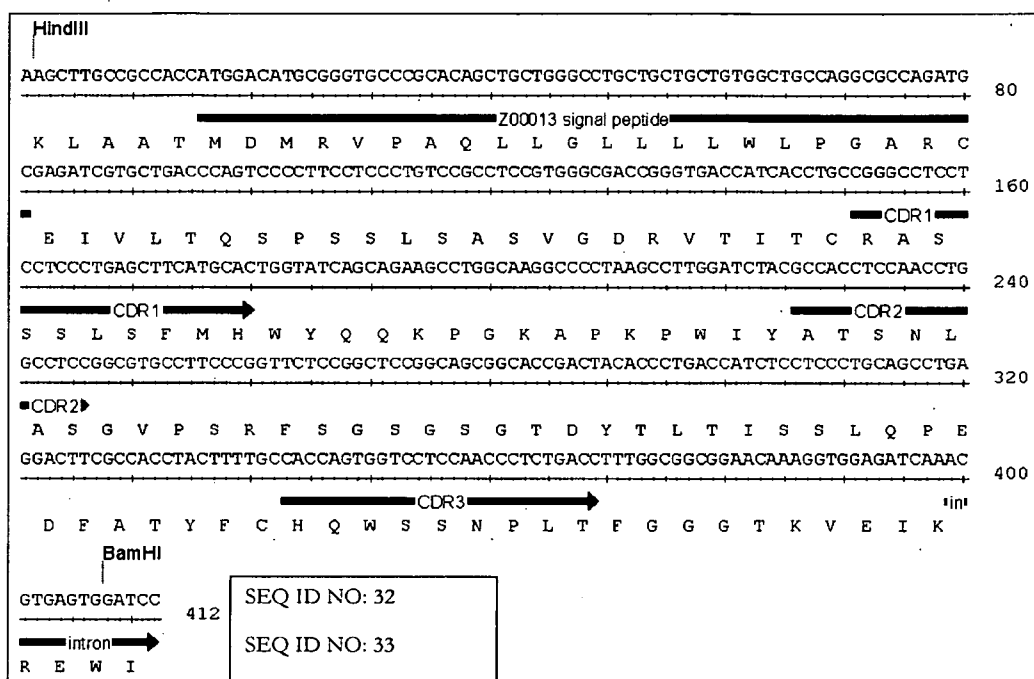


Figure 6

Region	DNA	SEQ ID NO:
X12686 signal	ATGGAAACCCAGCGCAGCTTCTCTTCCTCCTGCTACTCTGGCTCCCAGATACCACCG GA	34
AY263415 FR1	Gaaattgtgttgacgcagtcctccaggcaccctgtctttgtctccaggggaaagagcca ccctctcctgc	35
1F5VK CDR1	AGGGCCAGCTCAAGTTTAAGTTTCATGCAC	36
AY263415 FR2	tggtaccagcagaaacctggccagggtcccagggtcctcatctat	37
1F5VK CDR2	GCCACATCCAACCTGGCTTCT	38
AY263415 FR3	Ggcattcccagccaggttcagtgaggcagtggtctgggacagacttcactctcaccatca gcagactggagcctgaagattttgcagtgattttctgc	39
1F5VK CDR3	CATCAGTGGAGTAGTAACCCGCTCACG	40
AY263415 FR4	ttcggccaagggaaccaaggtggaactcaaa	41

Figure 7B

[illegible]

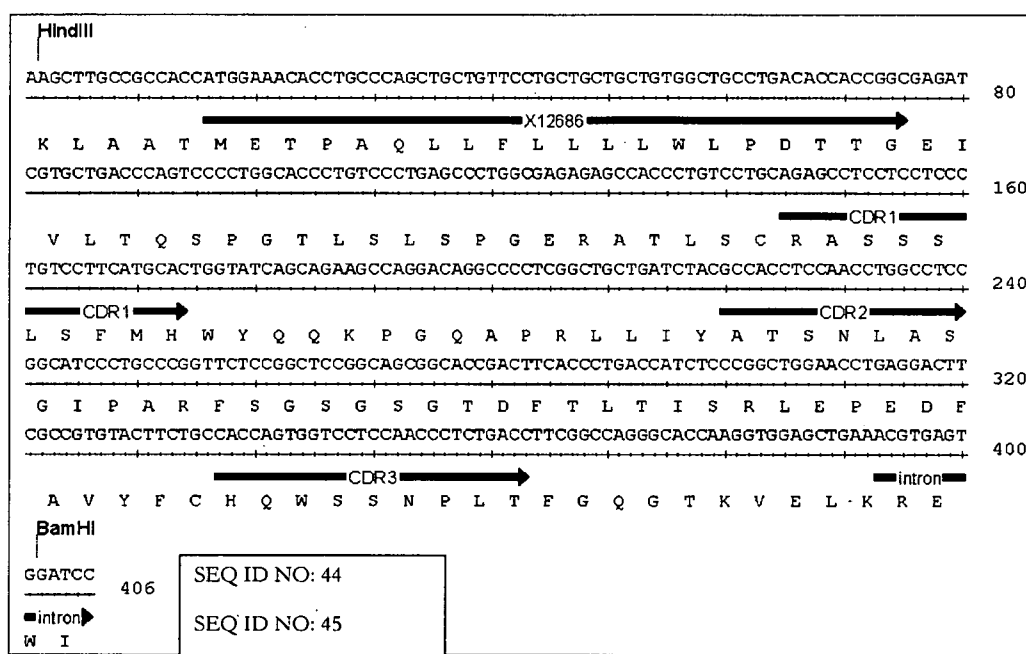


Figure 8

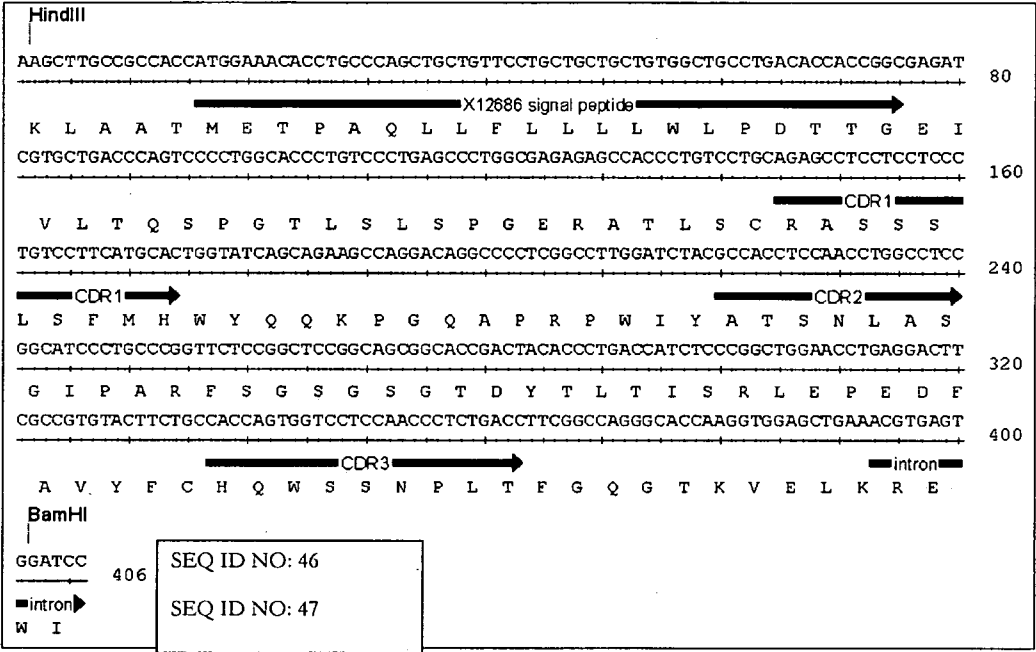


Figure 9

[illegible][illegible][illegible]

Figure 11A—1F5RKG11 protein sequence of mature V and C region only

EIVLTQSPSSLSASVGDRTITCRASSLSFMHWYQQKPGKAPKPLIYATSNLASGVPSRFSGSGSGTDFTLTISLQPEDFATYYCHQWSSNPLTFGGGTKVEIKRTVAAPSVFIFPPSDE
QLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDYSLSSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 60)

Figure 11B—1F5RKB11 protein sequence of mature V and C region only

EIVLTQSPSSLSASVGDRTITCRASSLSFMHWYQQKPGKAPKPLIYATSNLASGVPSRFSGSGSGTDYTLTISLQPEDFATYFCHQWSSNPLTFGGGTKVEIKRTVAAPSVFI
FPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDYSLSSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 61)

Figure 11C—1F5RKF12 protein sequence of mature V and C region only

EIVLTQSPGTLISLSPGERATLSCRASSLSFMHWYQQKPGQAPRPLIYATSNLASGIPARFSGSGSGTDFTLTISRLEPEDFAVYFCHQWSSNPLTFGQGTKVELKRTVAAPSVFIFPPSDE
QLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDYSLSSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 62)

Figure 11D—1FSRKB12 protein sequence of mature V and C region only

EIVLTQSPGTLSSLSPGERATLSCRASSSLSEMHNYQQKPGQAPRPWIYATSNLASGIPARFSGSGSGTDYTLTISRLEPEDFAVYFCHQWSSNPLTFGQGTKVELKRTVAAPSVFIFPPSDE
QLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSSTYLSLSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 63)

Figure 11E—1FSRHA protein sequence of mature V and C region only

QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYNMHWVRQAPGQRLIEWIGAIYPGNGDTSYNQKFKGRVTITRDT SANTAYMELSSLKSEDTAVYYCARSHYGSNYVDYFDYWGGT LVTVSS
ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCTPPCPAPELLGGPS
VFLFPPKPKDILMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKN
QVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFCSCVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 64)

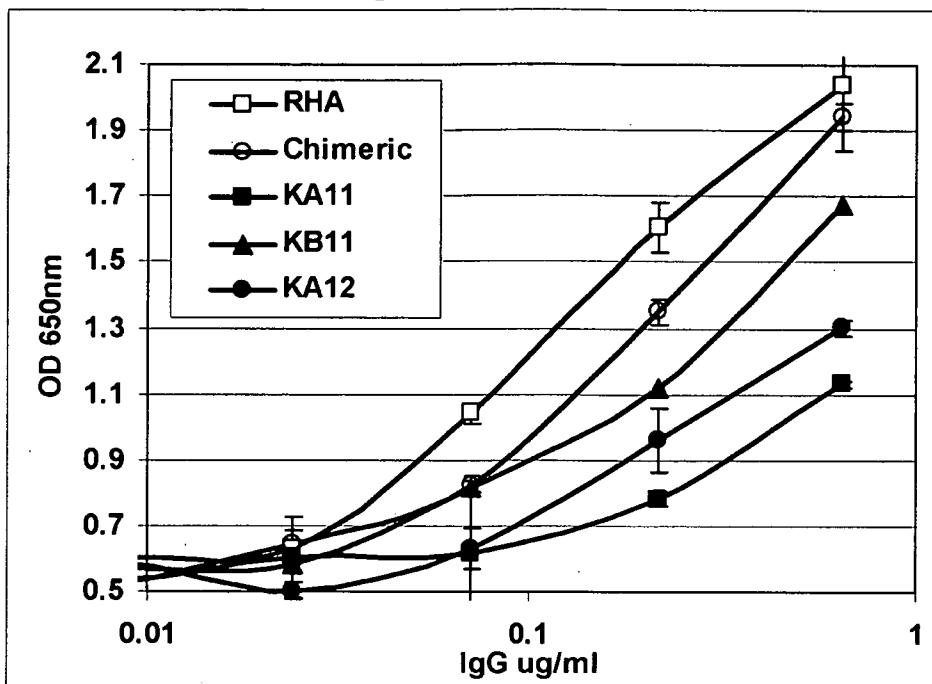
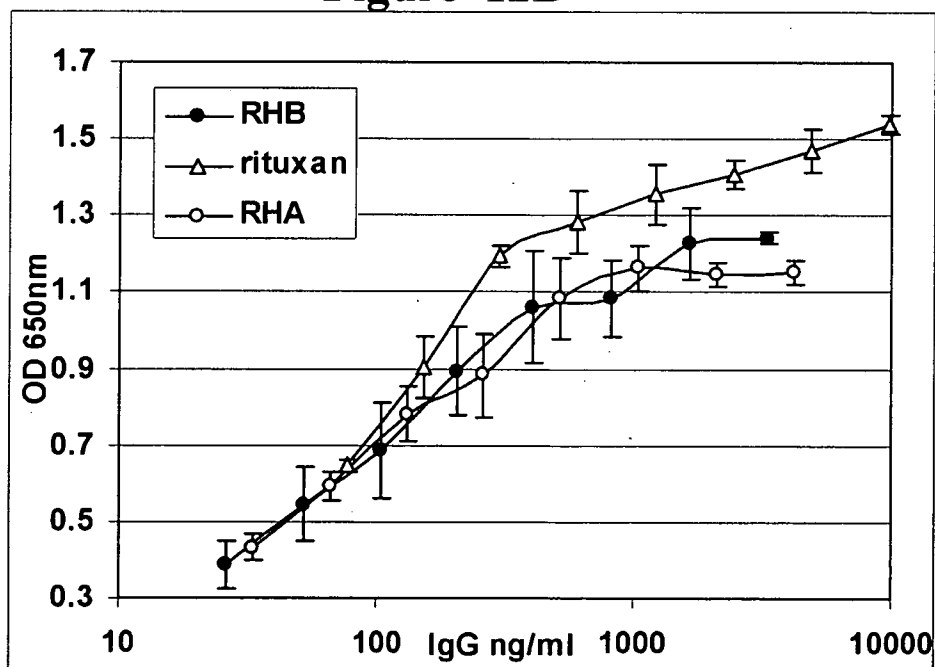
Figure 12A**Figure 12B**

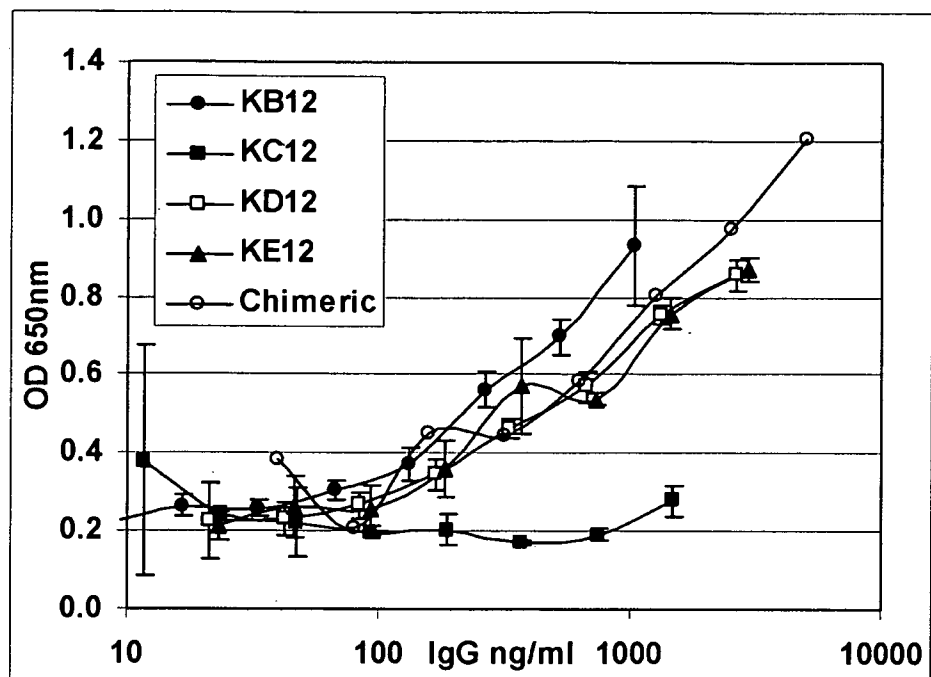
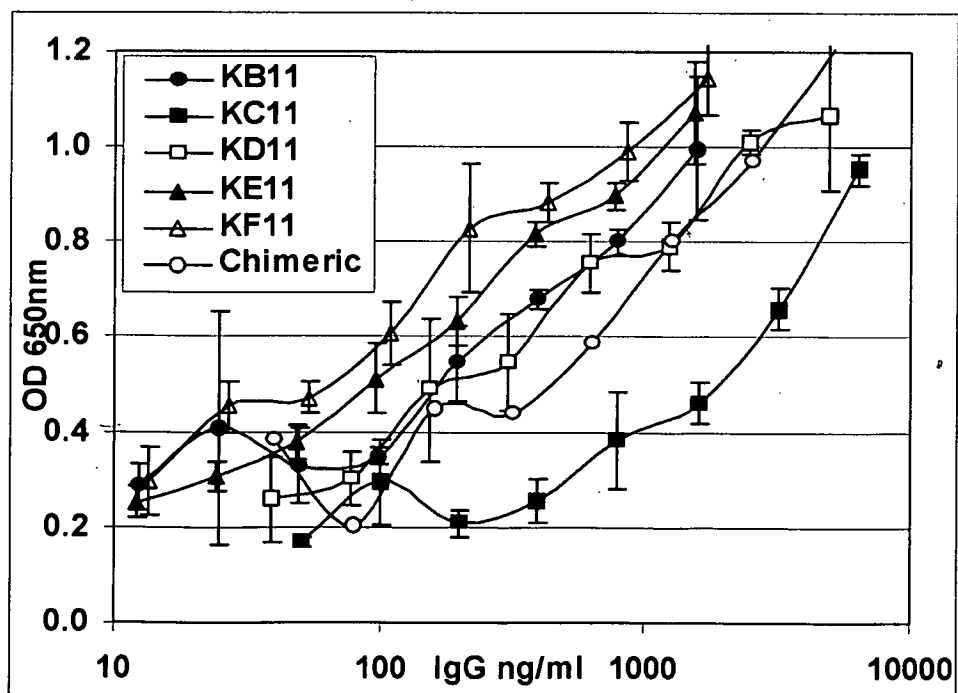
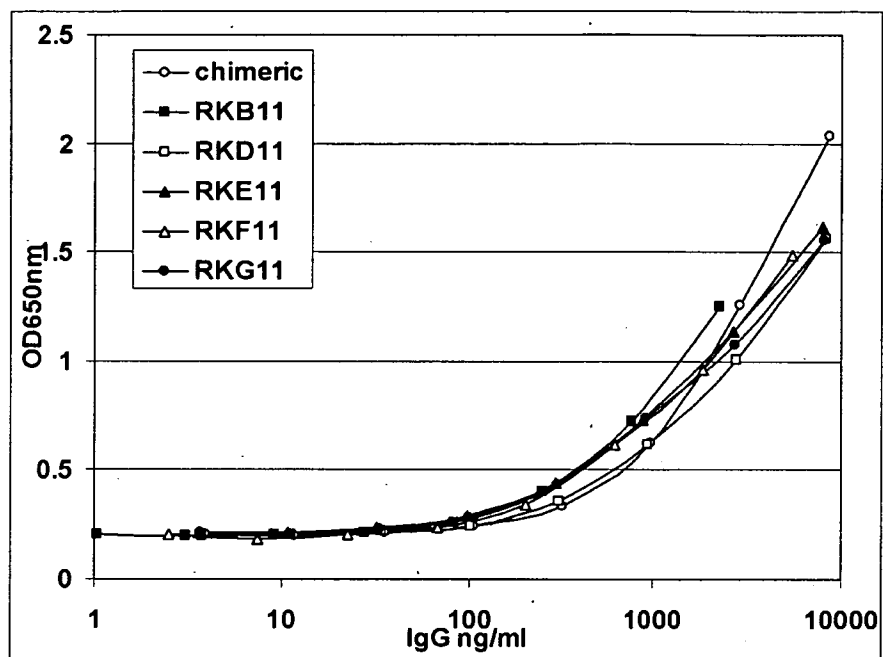
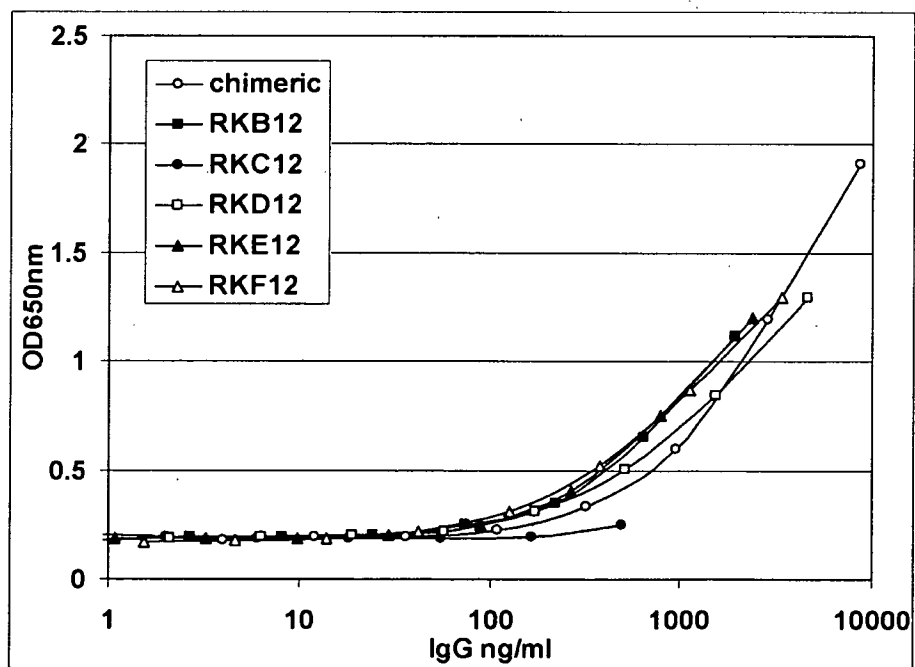
Figure 12C**Figure 12D**

Figure 13A**Figure 13B**

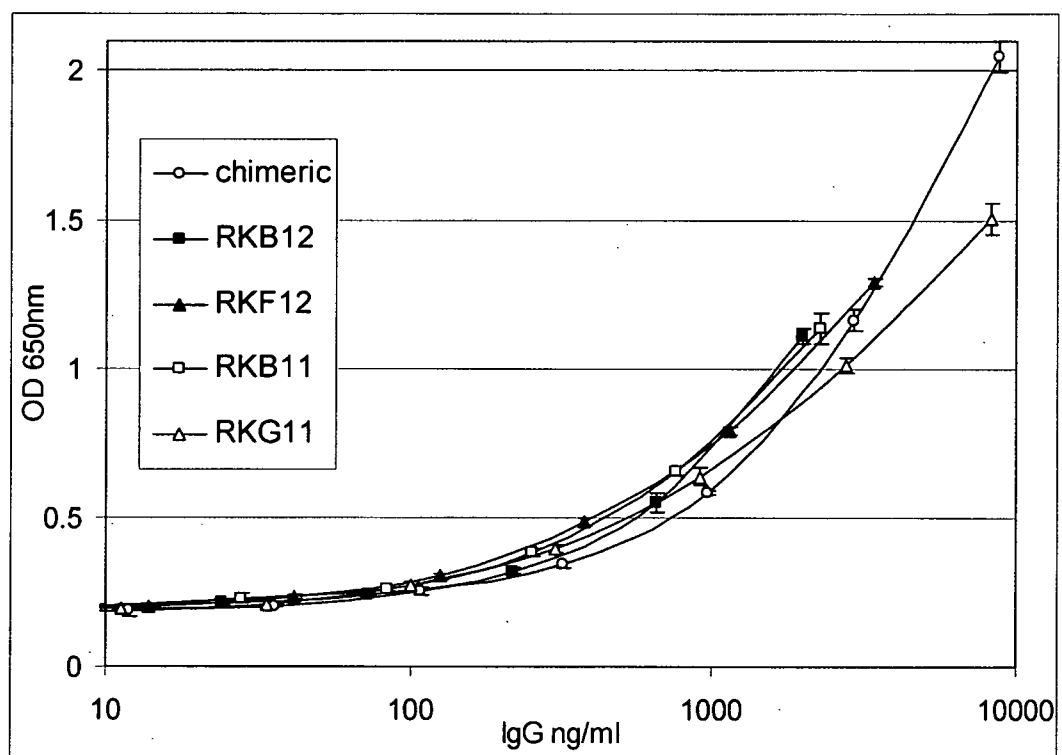


Figure 14

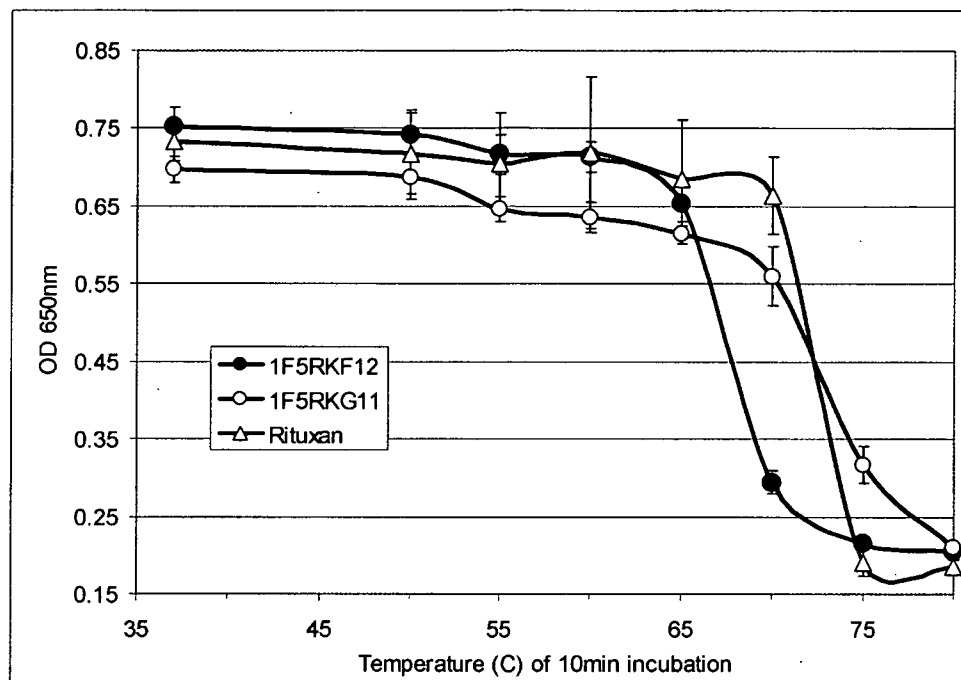
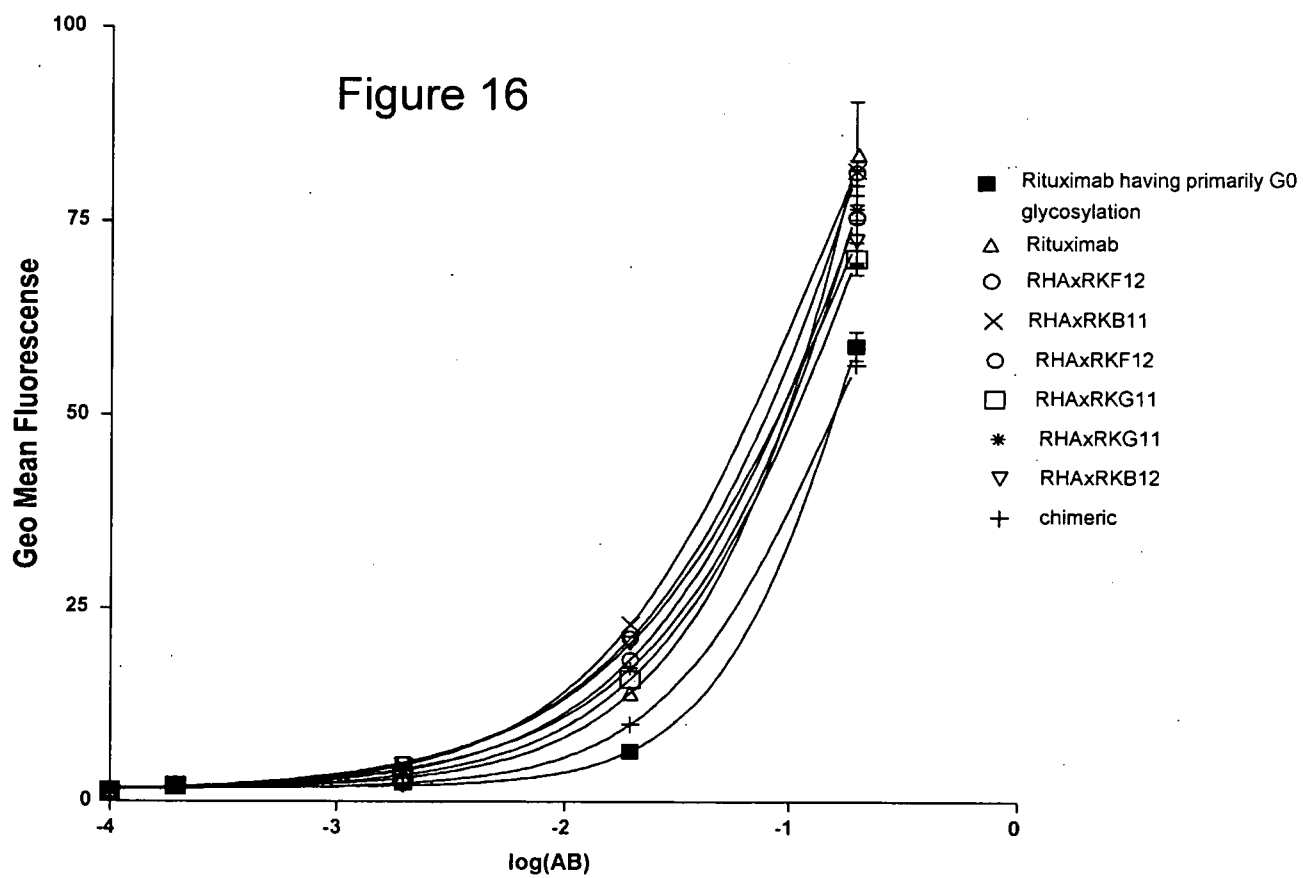


Figure 15



Protein and DNA sequence of pre-optimised c1F5VK construct

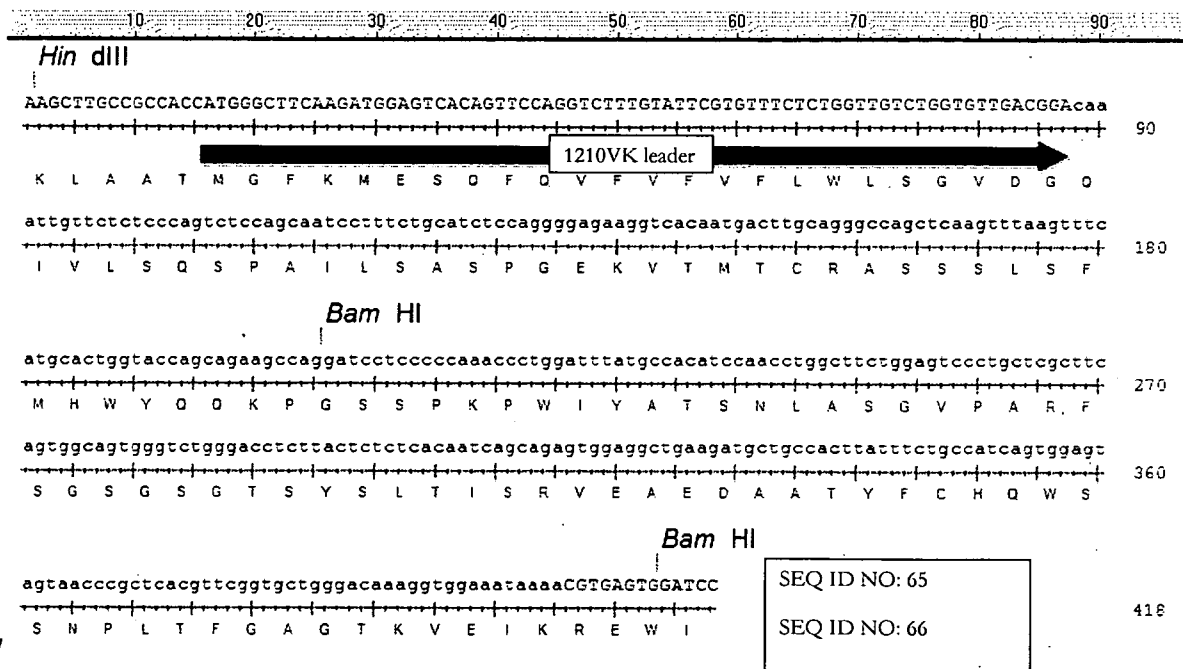


Figure 17

DNA sequence of optimised chimeric c1F5VK construct

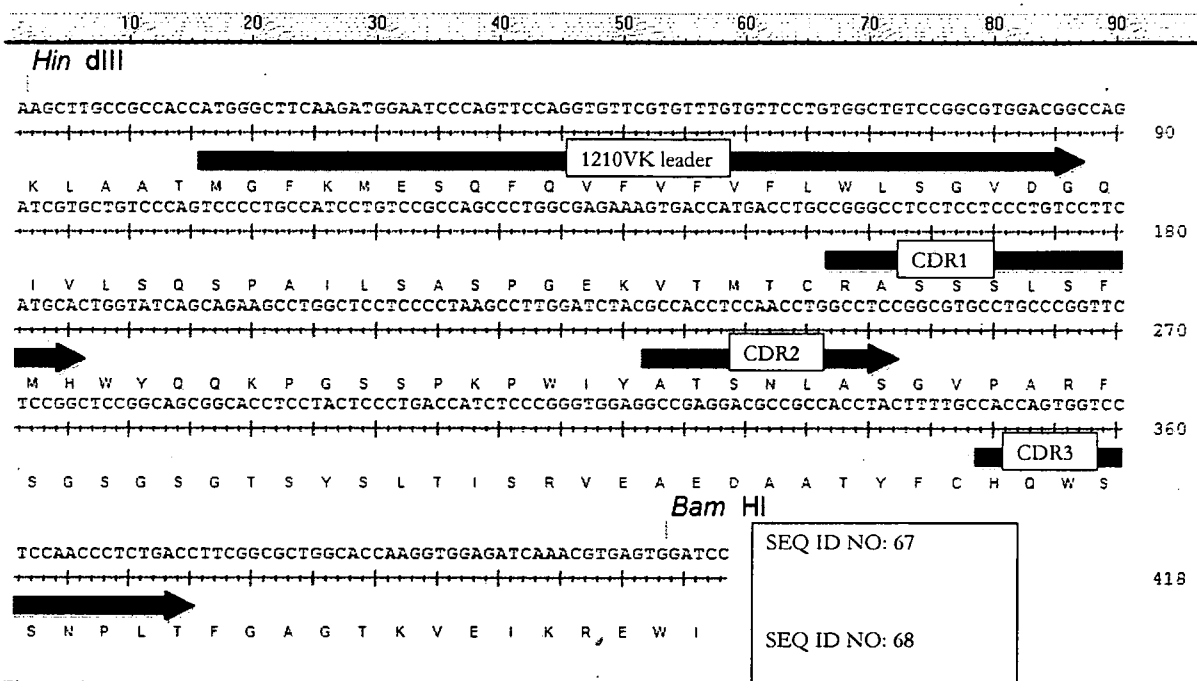


Figure 18

Protein and DNA sequence of pre-optimised chimeric c1F5VH construct

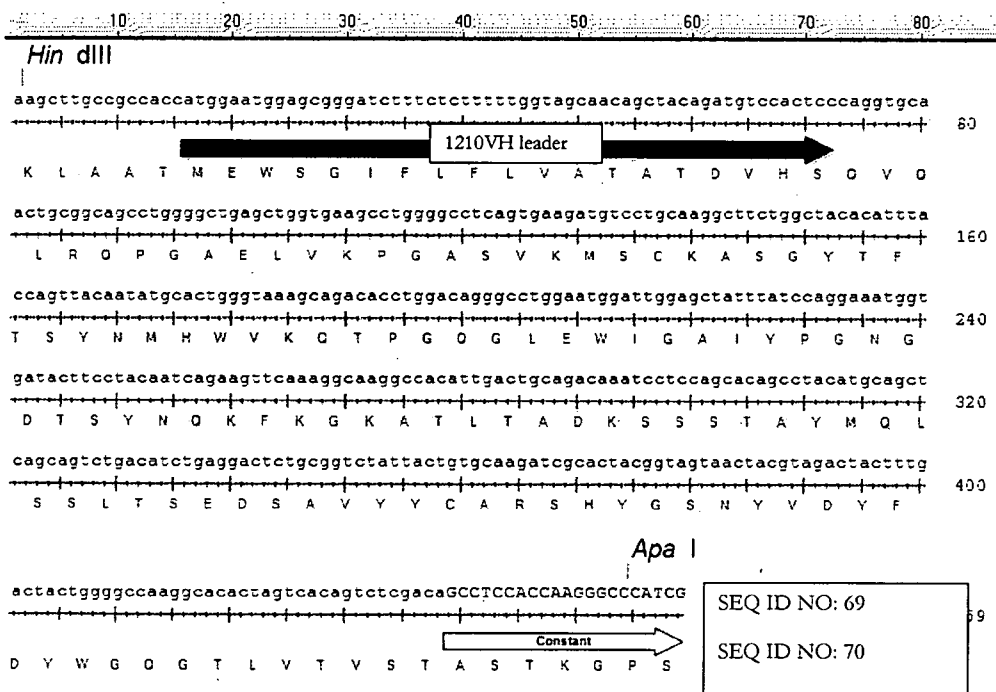


Figure 19

Protein and DNA sequence of optimised chimeric c1F5VH construct

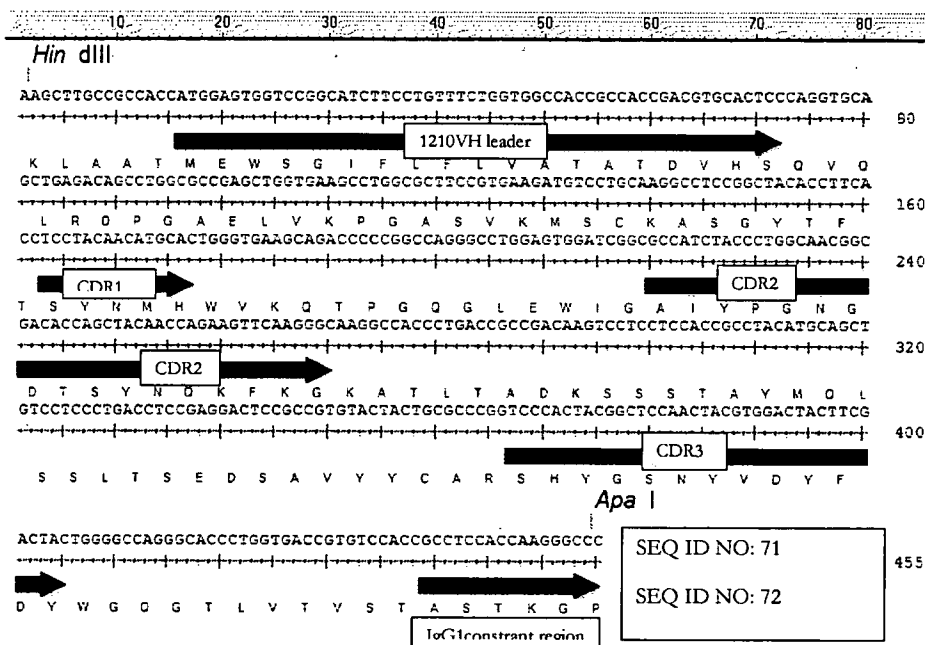


Figure 20

Figure 21

N-GLYCAN STRUCTURE

G0 or GnGn or
GlcNAc₂Man₃GlcNAc₂

LEGEND

■ N-acetylglucosamine

○ Mannose

chimeric c1F5VH protein sequence of mature V and C region only

QVQLRQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGQGLEWIGAIYPGNGDTSYNQKFKGKATLTADKSSSTAY
MQLSSLTSEDSAVYYCARSHYGSNYVDYFDYWGGTLVTVSTASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT
VSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELL
GGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLN
GKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTP
PVLDSGGSFFLYSKLTVDKSRWQQGNVFCVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 73)

Figure 22

chimeric c1F5VK protein sequence of mature V and C region only

QIVLSQSPAILSASPGEKVTMTCRASSLSFMHWYQQKPGSSPKPWIYATSNLASGVPARFSGSGSGTSYSLTISRVEAE
DAATYFCHQWSSNPLTFGAGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQE
SVTEQDSKDYSLSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 74)

Figure 23

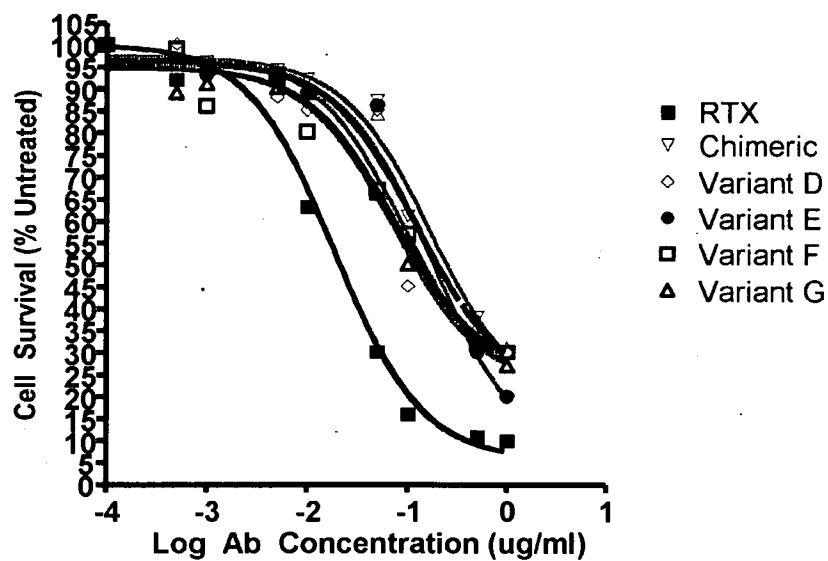
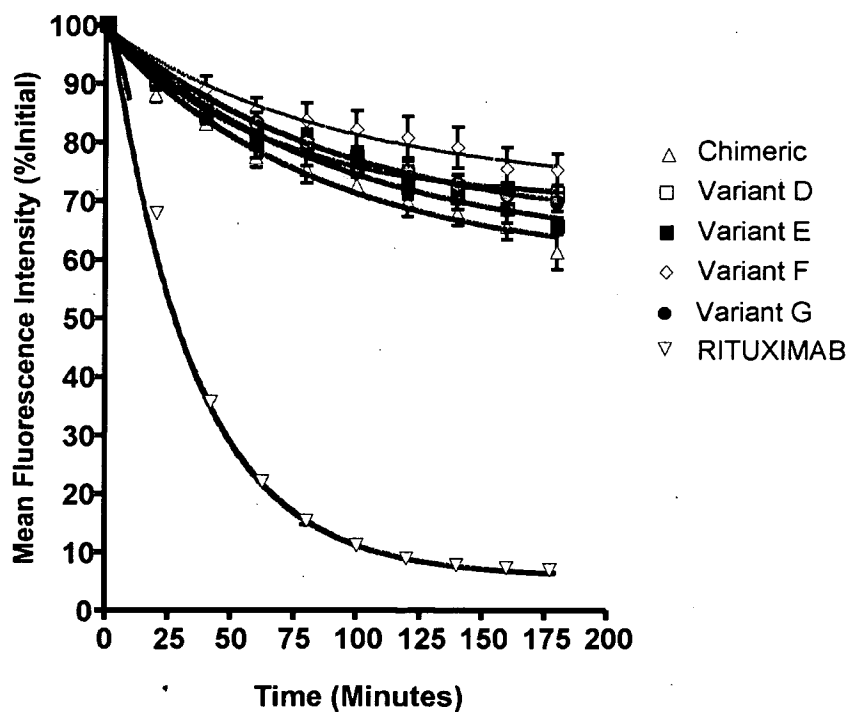
Figure 24: Complement dependent cytotoxicity of anti-CD20 antibodies**Figure 25:** Dissociation of anti-CD20 antibodies from Raji cells

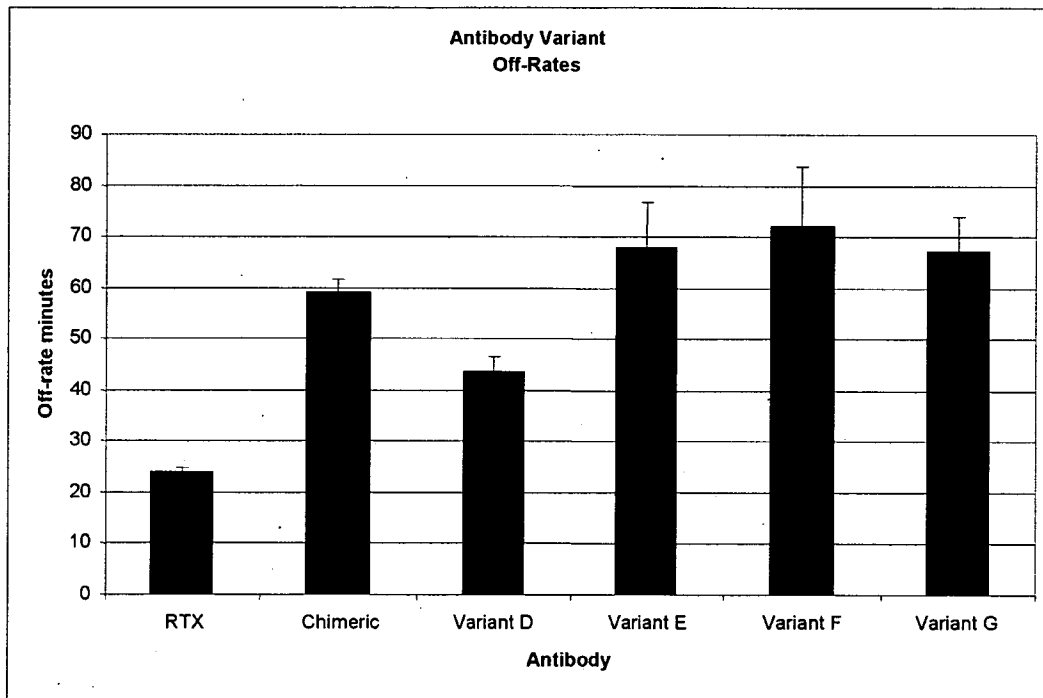
Figure 26

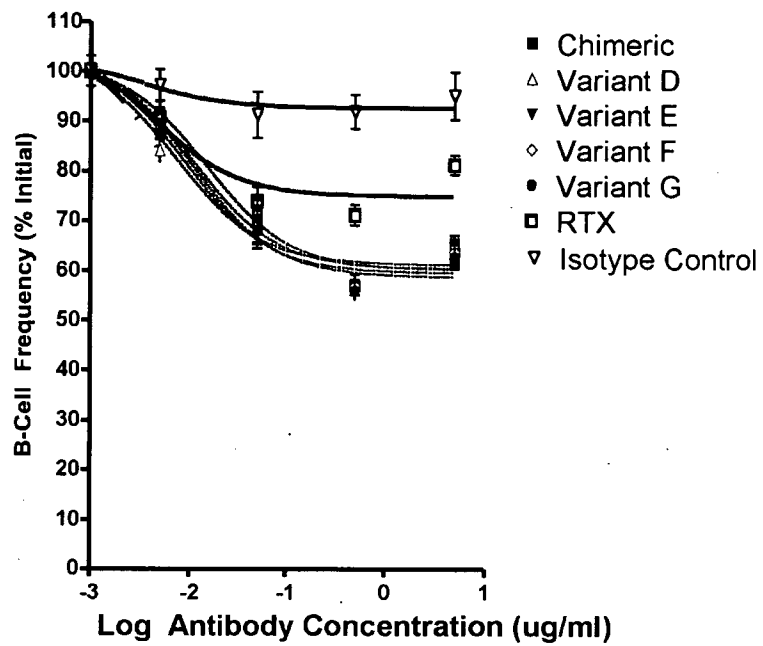
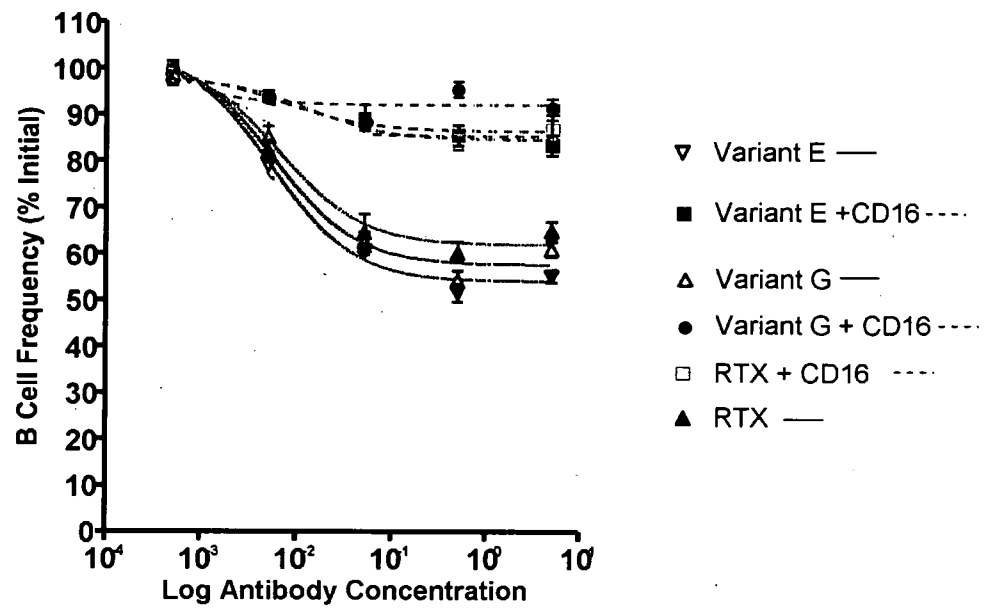
Figure 27: B-cell depleting activity of anti-CD20 antibodies

Figure 28: Inhibition of B-cell depletion by anti-CD16 antibody

PATENT COOPERATION TREATY

PCT

DECLARATION OF NON-ESTABLISHMENT OF INTERNATIONAL SEARCH REPORT

(PCT Article 17(2)(a), Rules 13ter.1(c) and (d) and 39)

Applicant's or agent's file reference 13790-90	IMPORTANT DECLARATION	Date of mailing (<i>day/month/year</i>) 17 MAY 2010
International application No. PCT/US10/00449	International filing date (<i>day/month/year</i>) 16 February 2010	(Earliest) Priority Date (<i>day/month/year</i>) 16 February 2009
International Patent Classification (IPC) or both national classification and IPC C12P 21/06 (2009.01); 514/2		
Applicant BIOLEX THERAPEUTICS, INC.		

This International Searching Authority hereby declares, according to Article 17(2)(a), that **no international search report will be established** on the international application for the reasons indicated below.

1. ☐ The subject matter of the international application relates to:
 - a. ☐ scientific theories
 - b. ☐ mathematical theories
 - c. ☐ plant varieties
 - d. ☐ animal varieties
 - e. ☐ essentially biological processes for the production of plants and animals, other than microbiological processes and the products of such processes
 - f. ☐ schemes, rules or methods of doing business
 - g. ☐ schemes, rules or methods of performing purely mental acts
 - h. ☐ schemes, rules or methods of playing games
 - i. ☐ methods for treatment of the human body by surgery or therapy
 - j. ☐ methods for treatment of the animal body by surgery or therapy
 - k. ☐ diagnostic methods practised on the human or animal body
 - l. ☐ mere presentations of information
 - m. ☐ computer programs for which this International Searching Authority is not equipped to search prior art
2. ☐ The failure of the following parts of the international application to comply with prescribed requirements prevents a meaningful search from being carried out:

☐ the description
☐ the claims
☐ the drawings
3. ☒ A meaningful search could not be carried out without the sequence listing; the applicant did not, within the prescribed time limit:

☐ furnish a sequence listing on paper complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it.
☒ furnish a sequence listing in electronic form complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it.
☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rule 13ter.1(a) or (b).
4. ☐ A meaningful search could not be carried out without the tables related to the sequence listings; the applicant did not, within the prescribed time limit, furnish such tables in electronic form complying with the technical requirements provided for in Annex C-bis of the Administrative Instructions, and such tables were not available to the International Searching Authority in a form and manner acceptable to it.
5. Further comments: Applicant failed to submit a valid CRF in response to the ISA/225 of 08 April 2010.

Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774
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