ANTI-CD20 ANTIBODIES AND METHODS OF USE

FIELD OF THE INVENTION

The invention relates to antibodies capable of binding to CD20, methods of using the antibodies, and methods for treatment of diseases associated with CD20-expressing cells.

BACKGROUND OF THE INVENTION

The CD20 molecule (also called human B-lymphocyte-restricted differentiation antigen or Bp35) is a hydrophobic transmembrane protein with a molecular weight of approximately 35 kD located on pre-B and mature B lymphocytes (Valentine *et al.* (1989) *J. Biol. Chem.* 264(19):11282-11287; and Einfield *et al.* (1988) *EMBO J.* 7(3):311-717). CD20 is found on the surface of greater than 90% of B cells from peripheral blood or lymphoid organs and is expressed during early pre-B cell development and remains until plasma cell differentiation. CD20 is present on both normal B cells as well as malignant B cells. In particular, CD20 is expressed on greater than 90% of B cell non-Hodgkin's lymphomas (NHL) (Anderson *et al.* (1984) *Blood* 63(6):1424-1433), but is not found on hematopoietic stem cells, pro-B cells, normal plasma cells, or other normal tissue (Tedder *et al.* (1985) *J. Immunol.* 135(2):973-979).

The 85 amino acid carboxyl-terminal region of the CD20 protein is located within the cytoplasm. The length of this region contrasts with that of other B cell-specific surface structures such as IgM, IgD, and IgG heavy chains or histocompatibility antigens class II α or β chains, which have relatively short intracytoplasmic regions of 3, 3, 28, 15, and 16 amino acids, respectively (Komaromy *et al.* (1983) *NAR* 11:6775-6785). Of the last 61 carboxyl-terminal amino acids, 21 are acidic residues, whereas only 2 are basic, indicating that this region has a strong net negative charge. The GenBank Accession No. is NP_690605.

It is thought that CD20 might be involved in regulating an early step(s) in the activation and differentiation process of B cells (Tedder *et al.* (1986) *Eur. J. Immunol.* 16:881-887) and could function as a calcium ion channel (Tedder *et al.* (1990) *J. Cell. Biochem.* 14D:195).

Despite uncertainty about the actual function of CD20 in promoting proliferation and/or differentiation of B cells, it provides an important target for antibody-mediated therapy to control or kill B cells involved in cancers and autoimmune disorders. In particular, the expression of CD20 on tumor cells, e.g., NHL, makes it an important target for antibody-mediated therapy to specifically target therapeutic agents against CD20-positive neoplastic cells. However, while the results obtained to date clearly establish CD20 as a useful target for immunotherapy, they also show that currently available murine and chimeric antibodies do not constitute ideal therapeutic agents.

BRIEF SUMMARY

Compositions and methods are provided for treating diseases associated with CD20, including lymphomas, autoimmune diseases, and transplant rejections. Compositions include anti-CD20 antibodies capable of binding to a human CD20 antigen located on the surface of a human CD20-expressing cell, wherein the antibody has increased complement-dependent cell-mediated cytotoxicity (CDC) in comparison to rituximab. Compositions also include antigen-binding fragments, variants, and derivatives of the monoclonal antibodies, cell lines producing these antibody compositions, and isolated nucleic acid molecules encoding the amino acid sequences of the antibodies. The disclosure further includes pharmaceutical compositions comprising the anti-CD20 antibodies of the disclosure, or antigen-binding fragments, variants, or derivatives thereof, in a pharmaceutically acceptable carrier.

The monoclonal antibodies disclosed herein have a strong affinity for CD20 and are characterized by improved CDC function in comparison to rituximab. The antibodies of the disclosure mediate killing of the cells expressing CD20. The antibodies of the disclosure are capable of specifically binding to a human CD20 antigen expressed on the surface of a human cell and are characterized by having at

least one optimized complementarity-determining region (CDR) in the anti-CD20 heavy chain and/or the anti-CD20 light chain. The optimized CDR has been modified and comprises a sequence set forth in any one of SEQ ID NOS:1-8. Particular antibody sequences are disclosed having changes in at least one CDR of the anti-CD20 heavy chain, and in the CDR3 of the light chain. Compositions of the disclosure comprise anti-CD20 antibodies, and antigen-binding antibody fragments, variants, and derivatives thereof, comprising at least one optimized CDR.

In a first aspect, there is provided an immunoglobulin that specifically binds CD20 and exhibits increased complement-dependent cytotoxicity (CDC) as compared to rituximab, wherein said immunoglobulin comprises:

a) a variable heavy (V_H) domain comprising:

i) a complementarity-determining region 1 (CDR1) comprising the amino acid sequence set forth in SEQ ID NO:7 or the amino acid residues 25 through 35 of SEQ ID NO: 12;

ii) a complementarity-determining region 2 (CDR2) comprising the amino acid sequence set forth in SEQ ID NO:5 or 6 or the amino acid residues 50 through 66 of SEQ ID NO:12; and

iii) a complementarity-determining region 3 (CDR3) comprising an amino acid sequence selected from the group consisting of SEQ ID NOS:1-4; and

b) a variable light (V_L) domain comprising:

i) a CDR1 comprising the amino acid sequence set forth in amino acid residues 24 through 33 of SEQ ID NO:10;

ii) a CDR2 comprising the amino acid sequence set forth in SEQ ID NO:9; and

iii) a CDR3 comprising the amino acid sequence set forth in SEQ ID NO:8 or the amino acid residues 88 through 96 of SEQ ID NO:10.

sequences of the antibodies. The disclosure further includes pharmaceutical compositions comprising the anti-CD20 antibodies of the disclosure, or antigen-
antibodies of the disclosure mediate killing of the cells expressing CD20. The antibodies of the disclosure are capable of specifically binding to a human CD20
disclosure comprise anti-CD20 antibodies, and antigen-binding antibody fragments,

In one embodiment of the disclosure, methods of the treatment comprise
Lymphomas that can be treated or prevented by a method of the disclosure include
of the disclosure include systemic lupus erythematosus (SLE), rheumatoid arthritis,
sequences of the antibodies. The disclosure further includes pharmaceutical compositions comprising the anti-CD20 antibodies of the disclosure, or antigen-
antibodies of the disclosure mediate killing of the cells expressing CD20. The antibodies of the disclosure are capable of specifically binding to a human CD20
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In one embodiment of the disclosure, methods of the treatment comprise

In a first aspect, there is provided an immunoglobulin that specifically binds CD20 and exhibits increased complement-dependent cytotoxicity (CDC) as compared to rituximab, wherein said immunoglobulin comprises:

a) a variable heavy (V_H) domain comprising:

i) a complementarity-determining region 1 (CDR1) comprising the amino acid sequence set forth in SEQ ID NO:7 or the amino acid residues 25 through 35 of SEQ ID NO: 12;

ii) a complementarity-determining region 2 (CDR2) comprising the amino acid sequence set forth in SEQ ID NO:5 or 6 or the amino acid residues 50 through 66 of SEQ ID NO:12; and

iii) a complementarity-determining region 3 (CDR3) comprising an amino acid sequence selected from the group consisting of SEQ ID NOS:1-4; and

b) a variable light (V_L) domain comprising:

i) a CDR1 comprising the amino acid sequence set forth in amino acid residues 24 through 33 of SEQ ID NO:10;

ii) a CDR2 comprising the amino acid sequence set forth in SEQ ID NO:9; and

iii) a CDR3 comprising the amino acid sequence set forth in SEQ ID NO:8 or the amino acid residues 88 through 96 of SEQ ID NO:10.

iii) the aspartic acid (Asp) residue at the position corresponding to residue 110 of SEQ ID NO:14;

e) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:15, wherein said V_H domain comprises the following residues:

i) the phenylalanine (Phe) residue at the position corresponding to residue 31 of SEQ ID NO:15;

ii) the alanine (Ala) residue at the position corresponding to residue 103 of SEQ ID NO:15;

iii) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:15; and

iv) the asparagine (Asn) residue at the position corresponding to residue 110 of SEQ ID NO:15;

f) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:16, wherein said V_H domain comprises the following residues:

i) the alanine (Ala) residue at the position corresponding to residue 57 of SEQ ID NO:16;

ii) the alanine (Ala) residue at the position corresponding to residue 103 of SEQ ID NO:16;

iii) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:16; and

iv) the asparagine (Asn) residue at the position corresponding to residue 110 of SEQ ID NO:16;

g) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:17, wherein said V_H domain comprises the following residues:

i) the leucine (Leu) residue at the position corresponding to residue 57 of SEQ ID NO:17;

ii) the alanine (Ala) residue at the position corresponding to residue 103 of SEQ ID NO:17;

iii) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:17; and

iv) the asparagine (Asn) residue at the position corresponding to residue 110 of SEQ ID NO:17; and

h) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:18, wherein said V_H domain comprises the following residues:

i) the alanine (Ala) residue at the position corresponding to residue 103 of SEQ ID NO:18;

ii) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:18;

iii) the glycine (Gly) residue at the position corresponding to residue 109 of SEQ ID NO:18; and

iv) the asparagine (Asn) residue at the position corresponding to residue 110 of SEQ ID NO:18.

In a third aspect, there is provided an immunoglobulin comprising a variable heavy (V_H) domain and a variable light (V_L) domain, wherein said V_H domain comprises a polypeptide having an amino acid sequence selected from the group consisting of SEQ NOS:12-18, and wherein said V_L domain comprises a polypeptide having an amino acid sequence set forth in SEQ ID NO:10 or SEQ ID NO:11, wherein said immunoglobulin exhibits increased complement-dependent cytotoxicity (CDC) as compared to rituximab.

In a fourth aspect, there is provided a composition comprising the immunoglobulin as defined above, and a carrier.

In a fifth aspect, there is provided an isolated polynucleotide comprising a nucleic acid encoding a variable heavy (V_H) domain of an immunoglobulin heavy chain, wherein said V_H domain is selected from the group consisting of:

a) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:12, wherein said V_H domain comprises the following residues:

i) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:12; and

ii) the asparagine (Asn) residue at the position corresponding to residue 110 of SEQ ID NO:12;

b) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:13, wherein said V_H domain comprises the following residues:

i) the alanine (Ala) residue at the position corresponding to residue 103 of SEQ ID NO:13;

ii) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:13; and

iii) the asparagine (Asn) residue at the position corresponding to residue 110 of SEQ ID NO:13;

c) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:14, wherein said V_H domain comprises the following residues:

i) the alanine (Ala) residue at the position corresponding to residue 103 of SEQ ID NO:14;

ii) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:14; and

iii) the aspartic acid (Asp) residue at the position corresponding to residue 110 of SEQ ID NO:14;

d) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:15, wherein said V_H domain comprises the following residues:

i) the phenylalanine (Phe) residue at the position corresponding to residue 31 of SEQ ID NO:15;

ii) the alanine (Ala) residue at the position corresponding to residue 103 of SEQ ID NO:15;

iii) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:15; and

iv) the asparagine (Asn) residue at the position corresponding to residue 110 of SEQ ID NO:15;

e) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:16, wherein said V_H domain comprises the following residues:

i) the alanine (Ala) residue at the position corresponding to residue 57 of SEQ ID NO:16;

ii) the alanine (Ala) residue at the position corresponding to residue 103 of SEQ ID NO:16;

iii) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:16; and

iv) the asparagine (Asn) residue at the position corresponding to residue 110 of SEQ ID NO:16;

f) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:17, wherein said V_H domain comprises the following residues:

i) the leucine (Leu) residue at the position corresponding to residue 57 of SEQ ID NO:17;

ii) the alanine (Ala) residue at the position corresponding to residue 103 of SEQ ID NO:17;

iii) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:17; and

iv) the asparagine (Asn) residue at the position corresponding to residue 110 of SEQ ID NO:17;

g) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:18, wherein said V_H domain comprises the following residues:

i) the alanine (Ala) residue at the position corresponding to residue 103 of SEQ ID NO:18;

ii) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:18; and

iii) the glycine (Gly) residue at the position corresponding to residue 109 of SEQ ID NO:18; and

iv) the asparagine (Asn) residue at the position corresponding to residue 110 of SEQ ID NO:18; and

h) a V_H domain comprising an amino acid sequence selected from the group consisting of SEQ ID NOs:12-18;

wherein an immunoglobulin comprising said V_H domain specifically binds to CD20 and exhibits increased complement-dependent cytotoxicity (CDC) as compared to rituximab.

In a sixth aspect, there is provided a composition comprising the polynucleotide as defined above.

In a seventh aspect, there is provided a vector comprising the polynucleotide as defined above.

In an eighth aspect, there is provided a vector comprising the polynucleotide as defined above, and a polynucleotide comprising a nucleic acid encoding a variable light (V_L) domain of an immunoglobulin light chain.

In a ninth aspect, there is provided a vector comprising a polynucleotide encoding a variable heavy (V_H) domain of an immunoglobulin heavy chain and a polynucleotide encoding a variable light (V_L) domain of an immunoglobulin light chain, wherein said V_H domain comprises:

a) a complementarity-determining region 1 (CDR1) comprising the amino acid sequence set forth in SEQ ID NO:7or the amino acid residues 25 through 35 of SEQ ID NO: 12;

b) a complementarity-determining region 2 (CDR2) comprising the amino acid sequence set forth in SEQ ID NO:5 or SEQ ID NO:6 or the amino acid residues 50 through 66 of SEQ ID NO:12; and

c) a complementarity-determining region 3 (CDR3) comprising the amino acid sequence set forth in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4;

wherein said V_L domain comprises:

a) a CDR1 comprising the amino acid sequence set forth in amino acid residues 24 through 33 of SEQ ID NO:10;

b) a CDR2 comprising the amino acid sequence set forth in SEQ ID NO:9; and

c) a CDR3 comprising the amino acid sequence set forth in SEQ ID NO:8 or the amino acid residues 88 through 96 of SEQ ID NO:10

wherein an immunoglobulin comprising said V_H domain and said V_L domain specifically binds to CD20 and exhibits increased complement-dependent cytotoxicity (CDC) as compared to rituximab.

In a tenth aspect, there is provided a composition comprising the vector as defined above.

In an eleventh aspect, there is provided a composition comprising the immunoglobulin of the first aspect.

In a twelfth aspect, there is provided a host cell comprising the polynucleotide as defined above.

In a thirteenth aspect, there is provided a host cell comprising the vector as defined above.

In a fourteenth aspect, there is provided a method of producing an immunoglobulin that specifically binds to CD20, comprising culturing the host cell as defined above, and recovering said immunoglobulin.

In a fifteenth aspect, there is provided an immunoglobulin produced by the method of the fourteenth aspect.

In a sixteenth aspect, there is provided the use of an effective amount of a composition comprising the immunoglobulin as defined above in the manufacture of a medicament for treating a cancer in a subject, wherein said cancer is associated with CD20-expressing cells in said subject.

In a seventeenth aspect, there is provided the use of an effective amount of the immunoglobulin as defined above in the manufacture of a medicament for inhibiting growth or differentiation of a normal human B cell, wherein said medicament is to be contacted with said B cell.

In an eighteenth aspect, there is provided the use of an effective amount of the immunoglobulin as defined above in the manufacture of a medicament for inhibiting growth of cancer cells of B cell lineage, wherein said medicament is to be contacted with said cancer cells.

In a nineteenth aspect, there is provided the use of an effective amount of the immunoglobulin as defined above in the manufacture of a medicament for treating an autoimmune disease or inflammatory disease in a subject, wherein said disease is associated with CD20-expressing cells in said subject.

In a twentieth aspect, there is provided a pharmaceutical composition comprising the immunoglobulin as defined above.

In one embodiment of the disclosure, methods of the treatment comprise administering to a patient a therapeutically effective dose of a pharmaceutical composition comprising suitable anti-CD20 antibodies, or antigen-binding fragments, variants, or derivatives thereof. Diseases associated with CD20-expressing cells include autoimmune diseases, cancers, and organ and tissue graft rejections. Lymphomas that can be treated or prevented by a method of the disclosure include non-Hodgkin's lymphomas (high-grade lymphomas, intermediate grade lymphomas, and low grade lymphomas), Hodgkin's disease, acute lymphoblastic leukemias, myelomas, chronic lymphocytic leukemias, and myeloblastic leukemias.

Particular autoimmune diseases contemplated for treatment using the methods of the disclosure include systemic lupus erythematosus (SLE), rheumatoid arthritis, Crohn's disease, psoriasis, autoimmune thrombocytopenic purpura, multiple sclerosis, ankylosing spondylitis, myasthenia gravis, and pemphigus vulgaris. Such antibodies may also be used to prevent rejection of organ and tissue grafts by suppressing autoimmune responses, to treat lymphomas by targeting and killing B-lymphocytes, and to deliver toxins to CD20-bearing cells in a specific manner.

BRIEF DESCRIPTION OF THE DRAWINGS/FIGURES

Figure 1 sets forth the amino acid sequences for the H1286 variable heavy domain (SEQ ID NO:29) and L373 variable light domain (SEQ ID NO:10) for the original humanized murine anti-CD20 monoclonal antibody (mAb 1097). The residues making up the complementarity-determining regions (CDR1, CDR2, and CDR3) for each variable domain are underlined.

Figure 2 shows the substitutions made within CDR3 of the H1286 variable heavy domain of the humanized murine anti-CD20 mAb 1097 (this CDR3 is designated "271") to produce the 1236 (SEQ ID NO:1), 1237 (SEQ ID NO:2), and 1238 (SEQ ID NO:3) optimized CDR3s (see Example 1). The 271 CDR3 sequence (SEQ ID NO:30) is equivalent to that for the CDR3 of the variable domain within the heavy chain of the chimeric murine/human anti-CD20 antibody known as C2B8 (rituximab).

Figure 3 sets forth the amino acid sequences for the optimized H1569 (SEQ ID NO:12), H1570 (SEQ ID NO:13), and H1571 (SEQ ID NO:14) variable heavy domains. H1569 comprises the optimized 1236 CDR3 (SEQ ID NO:1); H1570 comprises the optimized 1237 CDR3 (SEQ ID NO:2), and H1571 comprises the optimized 1238 CDR3 (SEQ ID NO:3). Each of these optimized variable heavy domains was paired with the L373 variable light domain (SEQ ID NO:10) to produce optimized mAb 1236, mAb 1237, and mAb 1238. The residues making up CDR1, CDR2, and CDR3, respectively, for each variable domain are underlined.

Figure 4 sets forth the coding sequences for the optimized H1569 (SEQ ID NO:19), H1570 (SEQ ID NO:20), and H1571 (SEQ ID NO:21) variable heavy domains.

Figure 5 sets forth the amino acid (SEQ ID NO:10) and coding (SEQ ID NO:23) sequences for the L373 variable light domain. The residues and coding sequences for CDR1, CDR2, and CDR3, respectively, are underlined.

Figures 6A-6D show results of binding specificity for optimized humanized mAb 1236, mAb1237, and mAb 1238 as compared to mAb 271 (having the identical sequence to rituximab). Binding specificity of these optimized mAbs for CHO cells expressing CD20 (Figure 6A), $CD20^+$ EB1 cells (Figure 6B), $CD20^-$ CHO vector (Figure 6C), and $CD20^-$ NSO (Figure 6D) was assessed by Flurometric Microvolume Assay Technology (FMAT). See Example 2. NSO is a mouse myeloma cell line that does not express human CD20.

Figure 7 shows results of a murine 2H7 blocking assay to determine epitope recognition of optimized mAbs 1236, 1237, and 1238 as compared to mAb 271. The optimized mAbs all recognize the same epitope as mAb 271. See Example 2.

Figure 8 shows results of a CDC-mAb off-rate assay to determine complement dependent cytotoxicity (CDC) of optimized mAbs 1236, 1237, and 1238 as compared to mAb 271. The optimized mAbs have increased CDC functional activity relative to that observed for mAb 271. See Example 2.

Figure 9 shows results of an ADCC assay to determine antibody-dependent cellular cytotoxicity (ADCC) activity of optimized mAbs 1236, 1237, and 1238 against Daudi target cells as compared to mAb 271. The optimized mAbs have ADCC activity that is at least equivalent to that observed for mAb 271. See Example 2.

Figure 10 shows results of a direct apoptosis assay (independent of CDC and ADCC activity) to determine apoptosis activity of optimized mAbs 1236, 1237, and 1238 against Ramos (NHL) cells as compared to mAb 271 and mAb 11, which served as the isotype control. The optimized mAbs are as effective at inducing apoptosis as mAb 271. See Example 2.

Figure 11 sets forth the amino acid sequences for the H1639 variable heavy domain (SEQ ID NO:16) and L373 variable light domain (SEQ ID NO:10) for optimized mAb 1589. The residues making up the complementarity-determining regions (CDR1, CDR2, and CDR3) for each variable domain are underlined.

Figure 12 sets forth the coding sequences for the H1639 variable heavy domain (SEQ ID NO:22) and L373 variable light domain (SEQ ID NO:23) within the heavy and light chains, respectively, of optimized mAb 1589. Codons for the CDR1, CDR2, and CDR3, respectively, of the variable heavy and variable light domains are underlined.

Figure 13 shows results of a murine 2H7 blocking assay to determine epitope recognition of optimized mAbs 1588, 1589, 1590, 1652, and 1692 as compared to mAb 271, negative control antibody mAb 11, optimized mAb 1237, and rituximab (Rituxan®). With the exception of the negative control, all tested mAbs recognize the identical or overlapping epitopes. See Example 4.

Figure 14 shows results of a CDC assay to determine CDC activity of optimized mAbs 1588, 1589, 1590, 1652, and 1692 against target Daudi (NHL) cells as compared to that observed for mAb 271, 11, and optimized mAb 1237. All optimized mAbs generally mediate greater CDC functional activity than does mAb 271. See Example 4.

Figures 15A and 15B show results of a CDC assay to determine CDC activity of optimized mAbs 1588, 1589, 1590, 1652, and 1692 against two target B-CLL cell lines, EHEB cells (Figure 15A) and MEC-1 cells (Figure 15B), as compared to that observed for mAb 271, mAb 11, and optimized mAb 1237. All optimized mAbs have increased CDC functional activity relative to mAb 271. See Example 4.

Figure 16 shows results of a CDC-mAb off-rate assay to determine CDC activity of optimized mAbs 1588, 1590, 1652, and 1692 as compared to mAb 271 and optimized mAb 1237. All optimized mAbs have increased CDC functional activity relative to that observed for mAb 271. See Example 4.

Figures 17A and 17B show results of a non-radioactive CDC assay to determine CDC activity of optimized mAbs 1588, 1589, 1590, 1652, and 1692 against two target NHL cell lines, Daudi cells (Figure 17A) and WIL2-S cells (Figure 17B), as compared to that observed for mAb 271, optimized mAb 1237, and negative control mAb 11 (designated "NEG" in these figures). See Example 4.

Figures 18A and 18B show results of a non-radioactive CDC assay to determine CDC activity of optimized mAbs 1588, 1589, 1590, 1652, and 1692 against two target B-CLL cell lines, EHEB cells (Figure 18A) and MEC-1 cells (Figure 18B), as compared to that observed for mAb 271, optimized mAb 1237, and negative control mAb 11 (designated "NEG" in these figures). See Example 4.

Figures 19A and 19B show results of a non-radioactive CDC assay to determine CDC activity of optimized mAbs 1588, 1589, 1590, 1652, and 1692 against two target EBV-transformed B cell lines ("normal" B cells), SS BLCL cells (Figure 19A) and MelK BLCL cells (Figure 19B), as compared to that observed for mAb 271, optimized mAb 1237, and negative control mAb 11 (designated "NEG" in these figures). See Example 4.

Figure 20 shows results of an ADCC assay to determine ADCC activity of optimized mAbs 1588, 1589, 1590, 1652, and 1692 against Daudi (NHL) cells as compared to mAb 271, optimized mAb 1237, and control IgG. The optimized mAbs have ADCC activity that is similar to that observed for mAb 271. See Example 4.

Figure 21 shows results of an ADCC assay to determine ADCC activity of optimized mAbs 1588, 1589, 1590, 1652, and 1692 against MEC-1 (B-CLL) cells as compared to mAb 271, optimized mAb 1237, and control IgG. The optimized mAbs have ADCC activity that is similar to that observed for mAb 271. See Example 4.

Figure 22 shows a direct apoptosis assay (independent of CDC and ADCC activity) to determine apoptosis activity of optimized mAbs 1236, 1237, and 1238 against Ramos (NHL) cells as compared to mAb 271 and mAb 11, which served as the isotype control. The optimized mAbs are as effective at inducing apoptosis as mAb 271. See Example 4.

Figure 23 shows results of a whole-blood assay to determine cell killing activity of optimized mAbs 1588, 1589, 1590, 1652, and 1692 against Daudi (NHL) cells as compared to mAb 271 and optimized mAb 1237. The optimized mAbs have equivalent or better lysis against these cells as compared to mAb 271. See Example 4.

Figures 24A and 24B show results of a whole-blood assay to determine cell killing activity of optimized mAbs 1588, 1589, 1590, 1652, and 1692 against two target B-CLL cell lines, EHEB cells (Figure 24A) and MEC-1 cells (Figure 24B) as compared to mAb 271 and optimized mAb 1237. The optimized mAbs have equivalent or better lysis against these cells as compared to mAb 271. See Example 4.

Figure 25 shows results of an ADCC whole-blood assay to determine ADCC activity of optimized mAbs 1588, 1589, 1590, 1652, and 1692 against the EBV-transformed B cell line MelK BLCL ("normal" B cells) as compared to mAb 271 and optimized mAb 1237. The optimized mAbs have equivalent or better lysis against these cells as compared to mAb 271. See Example 4.

Figure 26 shows results of an *in vivo* test of optimized humanized anti-mouse CD20 monoclonal antibody 1589 to determine the activity of mAb 1589 in a Daudi cell xenograft model. The mAb 1589 antibody is effective at prolonging the survival of mice with human Daudi cell xenografts. See Example 5.

DETAILED DESCRIPTION OF THE INVENTION

I. DEFINITIONS

It is to be noted that the term "a" or "an" entity refers to one or more of that entity; for example, "an anti-CD20 antibody" is understood to represent one or more anti-CD20 antibodies. As such, the terms "a" (or "an"), "one or more," and "at least one" can be used interchangeably herein.

As used herein, the term "tumor" refers to all neoplastic cell growth and proliferation, whether malignant or benign, and all pre-cancerous cells and tissues.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include, but are not limited to lymphoma and leukemia.

As used herein, the term "polypeptide" is intended to encompass a singular "polypeptide" as well as plural "polypeptides," and refers to a molecule composed of monomers (amino acids) linearly linked by amide bonds (also known as peptide bonds). The term "polypeptide" refers to any chain or chains of two or more amino acids, and does not refer to a specific length of the product. Thus, peptides, dipeptides, tripeptides, oligopeptides, "protein," "amino acid chain," or any other term used to refer to a chain or chains of two or more amino acids, are included within the definition of "polypeptide," and the term "polypeptide" may be used instead of, or interchangeably with any of these terms. The term "polypeptide" is also intended to refer to the products of post-expression modifications of the polypeptide, including without limitation glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, or modification by non-naturally occurring amino acids. A polypeptide may be derived from a natural biological source or produced by recombinant technology, but is not necessarily translated from a designated nucleic acid sequence. It may be generated in any manner, including by chemical synthesis.

A polypeptide of the invention may be of a size of about 3 or more, 5 or more, 10 or more, 20 or more, 25 or more, 50 or more, 75 or more, 100 or more, 200 or more, 500 or more, 1,000 or more, or 2,000 or more amino acids. Polypeptides may have a defined three-dimensional structure, although they do not necessarily have such structure. Polypeptides with a defined three-dimensional structure are referred to as folded, and polypeptides that do not possess a defined three-dimensional structure, but rather can adopt a large number of different conformations, are referred to as unfolded. As used herein, the term glycoprotein refers to a *protein* coupled to at least one carbohydrate moiety that is attached to the protein via an oxygen-containing or a nitrogen-containing side chain of an amino acid residue, *e.g.*, a serine residue or an asparagine residue.

By an "isolated" polypeptide or a fragment, variant, or derivative thereof is intended a polypeptide that is not in its natural milieu. No particular level of purification is required. For example, an isolated polypeptide can be removed from its native or natural environment. Recombinantly produced polypeptides and proteins expressed in host cells are considered isolated for purpose of the invention, as are native or recombinant polypeptides that have been separated, fractionated, or partially or substantially purified by any suitable technique.

Also included as polypeptides of the present invention are fragments, derivatives, analogs, or variants of the foregoing polypeptides, and any combination thereof. The terms "fragment," "variant," "derivative," and "analog" when referring to anti-CD20 antibodies or antibody polypeptides of the present invention include any polypeptides that retain at least some of the antigen-binding properties of the corresponding antibody or antibody polypeptide of the invention. Fragments of polypeptides of the present invention include proteolytic fragments, as well as deletion fragments, in addition to specific antibody fragments discussed elsewhere herein. Variants of anti-CD20 antibodies and antibody polypeptides of the present invention include fragments as described above, and also polypeptides with altered amino acid sequences due to amino acid substitutions, deletions, or insertions. Variants may occur naturally or be non-naturally occurring. Non-naturally occurring variants may be produced using art-known mutagenesis techniques. Variant polypeptides may comprise conservative or non-conservative amino acid

substitutions, deletions, or additions. Derivatives of anti-CD20 antibodies and antibody polypeptides of the present invention, are polypeptides that have been altered so as to exhibit additional features not found on the reference antibody or antibody polypeptide of the invention. Examples include fusion proteins. Variant polypeptides may also be referred to herein as "polypeptide analogs." As used herein a "derivative" of an anti-CD20 antibody or antibody polypeptide refers to a subject polypeptide having one or more residues chemically derivatized by reaction of a functional side group. Also included as "derivatives" are those peptides that contain one or more naturally occurring amino acid derivatives of the twenty standard amino acids. For example, 4-hydroxyproline may be substituted for proline; 5-hydroxylysine may be substituted for lysine; 3-methylhistidine may be substituted for histidine; homoserine may be substituted for serine; and ornithine may be substituted for lysine.

The term "polynucleotide" is intended to encompass a singular nucleic acid as well as plural nucleic acids, and refers to an isolated nucleic acid molecule or construct, *e.g.*, messenger RNA (mRNA) or plasmid DNA (pDNA). A polynucleotide may comprise a conventional phosphodiester bond or a non-conventional bond (*e.g.*, an amide bond, such as found in peptide nucleic acids (PNA)). The term "nucleic acid" refers to any one or more nucleic acid segments, *e.g.*, DNA or RNA fragments, present in a polynucleotide. By "isolated" nucleic acid or polynucleotide is intended a nucleic acid molecule, DNA or RNA, that has been removed from its native environment. For example, a recombinant polynucleotide encoding an anti-CD20 antibody contained in a vector is considered isolated for the purposes of the present invention. Further examples of an isolated polynucleotide include recombinant polynucleotides maintained in heterologous host cells or purified (partially or substantially) polynucleotides in solution. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of polynucleotides of the present invention. Isolated polynucleotides or nucleic acids according to the present invention further include such molecules produced synthetically. In addition, a polynucleotide or a nucleic acid may be or may include a regulatory element such as a promoter, ribosome binding site, or a transcription terminator.

As used herein, a "coding region" is a portion of nucleic acid that consists of codons translated into amino acids. Although a "stop codon" (TAG, TGA, or TAA) is not translated into an amino acid, it may be considered to be part of a coding region, but any flanking sequences, for example promoters, ribosome binding sites, transcriptional terminators, introns, and the like, are not part of a coding region. Two or more coding regions of the present invention can be present in a single polynucleotide construct, *e.g.*, on a single vector, or in separate polynucleotide constructs, *e.g.*, on separate (different) vectors. Furthermore, any vector may contain a single coding region, or may comprise two or more coding regions, *e.g.*, a single vector may separately encode an immunoglobulin heavy chain variable region and an immunoglobulin light chain variable region. In addition, a vector, polynucleotide, or nucleic acid of the invention may encode heterologous coding regions, either fused or unfused to a nucleic acid encoding an anti-CD20 antibody or fragment, variant, or derivative thereof. Heterologous coding regions include without limitation specialized elements or motifs, such as a secretory signal peptide or a heterologous functional domain.

In certain embodiments, the polynucleotide or nucleic acid is DNA. In the case of DNA, a polynucleotide comprising a nucleic acid that encodes a polypeptide normally may include a promoter and/or other transcription or translation control elements operably associated with one or more coding regions. An operable association is when a coding region for a gene product, *e.g.*, a polypeptide, is associated with one or more regulatory sequences in such a way as to place expression of the gene product under the influence or control of the regulatory sequence(s). Two DNA fragments (such as a polypeptide coding region and a promoter associated therewith) are "operably associated" if induction of promoter function results in the transcription of mRNA encoding the desired gene product and if the nature of the linkage between the two DNA fragments does not interfere with the ability of the expression regulatory sequences to direct the expression of the gene product or interfere with the ability of the DNA template to be transcribed. Thus, a promoter region would be operably associated with a nucleic acid encoding a polypeptide if the promoter was capable of effecting transcription of that nucleic acid. The promoter may be a cell-specific promoter that directs substantial transcription of the DNA only

in predetermined cells. Other transcription control elements, besides a promoter, for example enhancers, operators, repressors, and transcription termination signals, can be operably associated with the polynucleotide to direct cell-specific transcription. Suitable promoters and other transcription control regions are disclosed herein.

A variety of transcription control regions are known to those skilled in the art. These include, without limitation, transcription control regions that function in vertebrate cells, such as, but not limited to, promoter and enhancer segments from cytomegaloviruses (the immediate early promoter, in conjunction with intron-A), simian virus 40 (the early promoter), and retroviruses (such as Rous sarcoma virus). Other transcription control regions include those derived from vertebrate genes such as actin, heat shock protein, bovine growth hormone and rabbit ß-globin, as well as other sequences capable of controlling gene expression in eukaryotic cells. Additional suitable transcription control regions include tissue-specific promoters and enhancers as well as lymphokine-inducible promoters (*e.g.*, promoters inducible by interferons or interleukins).

Similarly, a variety of translation control elements are known to those of ordinary skill in the art. These include, but are not limited to, ribosome binding sites, translation initiation and termination codons, and elements derived from picornaviruses (particularly an internal ribosome entry site, or IRES, also referred to as a CITE sequence).

In other embodiments, a polynucleotide of the present invention is RNA, for example, in the form of messenger RNA (mRNA).

Polynucleotide and nucleic acid coding regions of the present invention may be associated with additional coding regions that encode secretory or signal peptides, which direct the secretion of a polypeptide encoded by a polynucleotide of the present invention. According to the signal hypothesis, proteins secreted by mammalian cells have a signal peptide or secretory leader sequence that is cleaved from the mature protein once export of the growing protein chain across the rough endoplasmic reticulum has been initiated. Those of ordinary skill in the art are aware that polypeptides secreted by vertebrate cells generally have a signal peptide fused to the N-terminus of the polypeptide, which is cleaved from the complete or "full length" polypeptide to produce a secreted or "mature" form of the polypeptide. In certain

embodiments, the native signal peptide, *e.g.*, an immunoglobulin heavy chain or light chain signal peptide is used, or a functional derivative of that sequence that retains the ability to direct the secretion of the polypeptide that is operably associated with it. Alternatively, a heterologous mammalian signal peptide, or a functional derivative thereof, may be used. For example, the wild-type leader sequence may be substituted with the leader sequence of human tissue plasminogen activator (TPA) or mouse ß-glucuronidase.

The present invention is directed to certain anti-CD20 antibodies, or antigen-binding fragments, variants, or derivatives thereof. Unless specifically referring to full-sized antibodies such as naturally occurring antibodies, the term "anti-CD20 antibodies" encompasses full-sized antibodies as well as antigen-binding fragments, variants, analogs, or derivatives of such antibodies, *e.g.*, naturally occurring antibody or immunoglobulin molecules or engineered antibody molecules or fragments that bind antigen in a manner similar to antibody molecules.

The terms "antibody" and "immunoglobulin" are used interchangeably herein. An antibody or immunoglobulin comprises at least the variable domain of a heavy chain, and normally comprises at least the variable domains of a heavy chain and a light chain. Basic immunoglobulin structures in vertebrate systems are relatively well understood. *See, e.g.*, Harlow *et al.* (1988) *Antibodies: A Laboratory Manual* (2nd ed.; Cold Spring Harbor Laboratory Press).

As will be discussed in more detail below, the term "immunoglobulin" comprises various broad classes of polypeptides that can be distinguished biochemically. Those skilled in the art will appreciate that heavy chains are classified as gamma, mu, alpha, delta, or epsilon, (γ, μ, α, δ, ε) with some subclasses among them (*e.g.*, γ1-γ4). It is the nature of this chain that determines the "class" of the antibody as IgG, IgM, IgA, IgD, or IgE, respectively. The immunoglobulin subclasses (isotypes) *e.g.*, IgG_1, IgG_2, IgG_3, IgG_4, IgA_1, etc., are well characterized and are known to confer functional specialization. Modified versions of each of these classes and isotypes are readily discernable to the skilled artisan in view of the instant disclosure and, accordingly, are within the scope of the instant invention. Although all immunoglobulin classes are clearly within the scope of the present invention, the following discussion will generally be directed to the IgG class of immunoglobulin

molecules. With regard to IgG, a standard immunoglobulin molecule comprises two identical light chain polypeptides of molecular weight approximately 23,000 Daltons, and two identical heavy chain polypeptides of molecular weight 53,000-70,000 Daltons. The four chains are typically joined by disulfide bonds in a "Y" configuration wherein the light chains bracket the heavy chains starting at the mouth of the "Y" and continuing through the variable region.

Light chains are classified as either kappa or lambda (κ, 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 or non-covalent linkages when the immunoglobulins are generated either by hybridomas, B cells, or genetically engineered host cells. In the heavy chain, the amino acid sequences run from an N-terminus at the forked ends of the Y configuration to the C-terminus at the bottom of each chain.

Both the light and heavy chains are divided into regions of structural and functional homology referred to as the "constant region" and the "variable region." The terms "constant" and "variable" are used functionally. In this regard, it will be appreciated that the variable domains of both the light (V_L) and heavy (V_H) 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 N-terminal portion is a variable region and at the C-terminal portion is a constant region; the C_H3 and C_L domains actually comprise the carboxy-terminus of the heavy and light chain, respectively.

As indicated above, the variable region allows the antibody to selectively recognize and specifically bind epitopes on antigens. That is, the V_L domain and V_H domain, or subset of the complementarity determining regions (CDRs) within these variable domains, of an antibody combine to form the variable region that defines a three dimensional antigen binding site. This quaternary antibody structure forms the antigen binding site present at the end of each arm of the Y. More specifically, the

antigen binding site is defined by three CDRs on each of the V_H and V_L chains. In some instances, *e.g.*, certain immunoglobulin molecules derived from camelid species or engineered based on camelid immunoglobulins, a complete immunoglobulin molecule may consist of heavy chains only, with no light chains. *See, e.g.*, Hamers-Casterman *et al.* (1993) *Nature* 363:446-448.

In naturally occurring antibodies, the six "complementarity determining regions" or "CDRs" present in each antigen binding domain are short, non-contiguous sequences of amino acids that are specifically positioned to form the antigen binding domain as the antibody assumes its three dimensional configuration in an aqueous environment. The remainder of the amino acids in the antigen binding domains, referred to as "framework" regions, show less inter-molecular variability. The framework regions largely adopt a β-sheet conformation and the CDRs form loops that connect, and in some cases form part of, the β-sheet structure. Thus, framework regions act to form a scaffold that provides for positioning the CDRs in correct orientation by inter-chain, non-covalent interactions. The antigen binding domain formed by the positioned CDRs defines a surface complementary to the epitope on the immunoreactive antigen. This complementary surface promotes the non-covalent binding of the antibody to its cognate epitope. The amino acids comprising the CDRs and the framework regions, respectively, can be readily identified for any given heavy or light chain variable domain by one of ordinary skill in the art, since they have been precisely defined (*see*, "Sequences of Proteins of Immunological Interest," Kabat *et al.* (1983) U.S. Department of Health and Human Services; and Chothia and Lesk (1987) *J. Mol. Biol.*, 196:901-917, which are incorporated herein by reference in their entireties).

In the case where there are two or more definitions of a term that is used and/or accepted within the art, the definition of the term as used herein is intended to include all such meanings unless explicitly stated to the contrary. A specific example is the use of the term "complementarity determining region" ("CDR") to describe the non-contiguous antigen combining sites found within the variable region of both heavy and light chain polypeptides. This particular region has been described by Kabat *et al.* (1983) U.S. Dept. of Health and Human Services, "Sequences of Proteins of Immunological Interest" and by Chothia and Lesk (1987) *J. Mol. Biol.* 196:901-

917, which are incorporated herein by reference, where the definitions include overlapping or subsets of amino acid residues when compared against each other. Nevertheless, application of either definition to refer to a CDR of an antibody or variants thereof is intended to be within the scope of the term as defined and used herein. The appropriate amino acid residues that encompass the CDRs as defined by each of the above cited references are set forth below in Table I as a comparison. The exact residue numbers that encompass a particular CDR will vary depending on the sequence and size of the CDR. Those skilled in the art can routinely determine which residues comprise a particular CDR given the variable region amino acid sequence of the antibody.

Table 1. CDR Definitions[1]

	Kabat	Chothia
V_H CDR1	31-35	26-32
V_H CDR2	50-65	52-58
V_H CDR3	95-102	95-102
V_L CDR1	24-34	26-32
V_L CDR2	50-56	50-52
V_L CDR3	89-97	91-96

[1]Numbering of all CDR definitions in Table 1 is according to the numbering conventions set forth by Kabat *et al.* (see below).

Kabat *et al.* also defined a numbering system for variable domain sequences that is applicable to any antibody. One of ordinary skill in the art can unambigously assign this system of "Kabat numbering" to any variable domain sequence, without reliance on any experimental data beyond the sequence itself. As used herein, "Kabat numbering" refers to the numbering system set forth by Kabat *et al.* (1983) U.S. Dept. of Health and Human Services, "Sequence of Proteins of Immunological Interest." Unless otherwise specified, references to the numbering of specific amino acid residue positions in an anti-CD20 antibody or antigen-binding fragment, variant, or derivative thereof of the present invention are according to the Kabat numbering system.

In camelid species, the heavy chain variable region, referred to as V_HH, forms the entire antigen-binding domain. The main differences between camelid V_HH variable regions and those derived from conventional antibodies (V_H) include (a) more hydrophobic amino acids in the light chain contact surface of V_H as compared to the corresponding region in V_HH, (b) a longer CDR3 in V_HH, and (c) the frequent occurrence of a disulfide bond between CDR1 and CDR3 in V_HH.

Antibodies or antigen-binding fragments, variants, or derivatives thereof of the invention include, but are not limited to, polyclonal, monoclonal, multispecific, human, humanized, primatized, or chimeric antibodies, single chain antibodies, epitope-binding fragments, *e.g.*, Fab, Fab' and $F(ab')_2$, Fd, Fvs, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv), fragments comprising either a V_L or V_H domain, fragments produced by a Fab expression library, and anti-idiotypic (anti-Id) antibodies (including, *e.g.*, anti-Id antibodies to anti-CD20 antibodies disclosed herein). ScFv molecules are known in the art and are described, *e.g.*, in U.S. Patent No. 5,892,019. Immunoglobulin or antibody molecules of the invention can be of any type (*e.g.*, IgG, IgE, IgM, IgD, IgA, and IgY), class (*e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2, etc.), or subclass of immunoglobulin molecule.

Antibody fragments, including single-chain antibodies, may comprise the variable region(s) alone or in combination with the entirety or a portion of the following: hinge region, C_H1, C_H2, and C_H3 domains. Also included in the invention are antigen-binding fragments also comprising any combination of variable region(s) with a hinge region, C_H1, C_H2, and C_H3 domains. Antibodies or immunospecific fragments thereof for use in the diagnostic and therapeutic methods disclosed herein may be derived from any animal origin including birds and mammals. Preferably, the antibodies are derived from human, murine, donkey, rabbit, goat, guinea pig, camel, llama, horse, or chicken antibodies. In another embodiment, the variable region may be condricthoid in origin (*e.g.*, from sharks). As used herein, "human" antibodies include antibodies having the amino acid sequence of a human immunoglobulin and include antibodies isolated from human immunoglobulin libraries or from animals

transgenic for one or more human immunoglobulins and that do not express endogenous immunoglobulins, as described *infra* and, for example in, U.S. Patent No. 5,939,598 by Kucherlapati *et al.*

As used herein, the term "heavy chain portion" includes amino acid sequences derived from an immunoglobulin heavy chain. A polypeptide comprising a heavy chain portion comprises at least one of: a C_H1 domain, a hinge (*e.g.*, upper, middle, and/or lower hinge region) domain, a C_H2 domain, a C_H3 domain, or a variant or fragment thereof. For example, a binding polypeptide for use in the invention may comprise a polypeptide chain comprising a C_H1 domain; a polypeptide chain comprising a C_H1 domain, at least a portion of a hinge domain, and a C_H2 domain; a polypeptide chain comprising a C_H1 domain and a C_H3 domain; a polypeptide chain comprising a C_H1 domain, at least a portion of a hinge domain, and a C_H3 domain, or a polypeptide chain comprising a C_H1 domain, at least a portion of a hinge domain, a C_H2 domain, and a C_H3 domain. In another embodiment, a polypeptide of the invention comprises a polypeptide chain comprising a C_H3 domain. Further, a binding polypeptide for use in the invention may lack at least a portion of a C_H2 domain (*e.g.*, all or part of a C_H2 domain). As set forth above, it will be understood by one of ordinary skill in the art that these domains (*e.g.*, the heavy chain portions) may be modified such that they vary in amino acid sequence from the naturally occurring immunoglobulin molecule.

In certain anti-CD20 antibodies, or antigen-binding fragments, variants, or derivatives thereof disclosed herein, the heavy chain portions of one polypeptide chain of a multimer are identical to those on a second polypeptide chain of the multimer. Alternatively, heavy chain portion-containing monomers of the invention are not identical. For example, each monomer may comprise a different target binding site, forming, for example, a bispecific antibody.

The heavy chain portions of a binding polypeptide for use in the diagnostic and treatment methods disclosed herein may be derived from different immunoglobulin molecules. For example, a heavy chain portion of a polypeptide may comprise a C_H1 domain derived from an IgG1 molecule and a hinge region derived from an IgG3 molecule. In another example, a heavy chain portion can comprise a

hinge region derived, in part, from an IgG1 molecule and, in part, from an IgG3 molecule. In another example, a heavy chain portion can comprise a chimeric hinge derived, in part, from an IgG1 molecule and, in part, from an IgG4 molecule.

As used herein, the term "light chain portion" includes amino acid sequences derived from an immunoglobulin light chain. Preferably, the light chain portion comprises at least one of a V_L or C_L domain.

Anti-CD20 antibodies, or antigen-binding fragments, variants, or derivatives thereof disclosed herein may be described or specified in terms of the epitope(s) or portion(s) of an antigen, *e.g.*, a target polypeptide (CD20) that they recognize or specifically bind. The portion of a target polypeptide that specifically interacts with the antigen binding domain of an antibody is an "epitope," or an "antigenic determinant." A target polypeptide may comprise a single epitope, but typically comprises at least two epitopes, and can include any number of epitopes, depending on the size, conformation, and type of antigen. Furthermore, it should be noted that an "epitope" on a target polypeptide may be or include non-polypeptide elements, *e.g.*, an epitope may include a carbohydrate side chain.

The minimum size of a peptide or polypeptide epitope for an antibody is thought to be about four to five amino acids. Peptide or polypeptide epitopes preferably contain at least seven, more preferably at least nine and most preferably between at least about 15 to about 30 amino acids. Since a CDR can recognize an antigenic peptide or polypeptide in its tertiary form, the amino acids comprising an epitope need not be contiguous, and in some cases, may not even be on the same peptide chain. In the present invention, peptide or polypeptide epitope recognized by anti-CD20 antibodies of the present invention contains a sequence of at least 4, at least 5, at least 6, at least 7, more preferably at least 8, at least 9, at least 10, at least 15, at least 20, at least 25, or between about 15 to about 30 contiguous or non-contiguous amino acids of CD20.

By "specifically binds," it is generally meant that an antibody binds to an epitope via its antigen binding domain, and that the binding entails some complementarity between the antigen binding domain and the epitope. According to this definition, an antibody is said to "specifically bind" to an epitope when it binds to that epitope, via its antigen binding domain more readily than it would bind to a

random, unrelated epitope. The term "specificity" is used herein to qualify the relative affinity by which a certain antibody binds to a certain epitope. For example, antibody "A" may be deemed to have a higher specificity for a given epitope than antibody "B," or antibody "A" may be said to bind to epitope "C" with a higher specificity than it has for related epitope "D."

By "preferentially binds," it is meant that the antibody specifically binds to an epitope more readily than it would bind to a related, similar, homologous, or analogous epitope. Thus, an antibody that "preferentially binds" to a given epitope would more likely bind to that epitope than to a related epitope, even though such an antibody may cross-react with the related epitope.

By way of non-limiting example, an antibody may be considered to bind a first epitope preferentially if it binds said first epitope with a dissociation constant (K_D) that is less than the antibody's K_D for the second epitope. In another non-limiting example, an antibody may be considered to bind a first antigen preferentially if it binds the first epitope with an affinity that is at least one order of magnitude less than the antibody's K_D for the second epitope. In another non-limiting example, an antibody may be considered to bind a first epitope preferentially if it binds the first epitope with an affinity that is at least two orders of magnitude less than the antibody's K_D for the second epitope.

In another non-limiting example, an antibody may be considered to bind a first epitope preferentially if it binds the first epitope with an off rate (k(off)) that is less than the antibody's k(off) for the second epitope. In another non-limiting example, an antibody may be considered to bind a first epitope preferentially if it binds the first epitope with an affinity that is at least one order of magnitude less than the antibody's k(off) for the second epitope. In another non-limiting example, an antibody may be considered to bind a first epitope preferentially if it binds the first epitope with an affinity that is at least two orders of magnitude less than the antibody's k(off) for the second epitope.

An antibody or antigen-binding fragment, variant, or derivative disclosed herein may be said to bind a target polypeptide disclosed herein or a fragment or variant thereof with an off rate (k(off)) of less than or equal to 5×10^{-2} sec^{-1}, 10^{-2} sec-^{1}, 5×10^{-3} sec^{-1}, or 10^{-3} sec^{-1}. More preferably, an antibody of the invention may

be said to bind a target polypeptide disclosed herein or a fragment or variant thereof with an off rate (k(off)) less than or equal to 5 X 10^{-4} sec^{-1}, 10^{-4} sec^{-1}, 5 X 10^{-5} sec^{-1}, or 10^{-5} sec^{-1}, 5 X 10^{-6} sec^{-1}, 10^{-6} sec^{-1}, 5 X 10^{-7} sec^{-1}, or 10^{-7} sec^{-1}.

An antibody or antigen-binding fragment, variant, or derivative thereof disclosed herein may be said to bind a target polypeptide disclosed herein or a fragment or variant thereof with an on rate (k(on)) of greater than or equal to 10^3 M^{-1} sec^{-1}, 5 X 10^3 M^{-1} sec^{-1}, 10^4 M^{-1} sec^{-1}, or 5 X 10^4 M^{-1} sec^{-1}. More preferably, an antibody of the invention may be said to bind a target polypeptide disclosed herein or a fragment or variant thereof with an on rate (k(on)) greater than or equal to 10^5 M^{-1} sec^{-1}, 5 X 10^5 M^{-1} sec^{-1}, 10^6 M^{-1} sec^{-1}, 5 X 10^6 M^{-1} sec^{-1}, or 10^7 M^{-1} sec^{-1}.

An antibody is said to competitively inhibit binding of a reference antibody to a given epitope if it preferentially binds to that epitope to the extent that it blocks, to some degree, binding of the reference antibody to the epitope. Competitive inhibition may be determined by any method known in the art, for example, competition ELISA assays. An antibody may be said to competitively inhibit binding of the reference antibody to a given epitope by at least 90%, at least 80%, at least 70%, at least 60%, or at least 50%.

As used herein, the term "affinity" refers to a measure of the strength of the binding of an individual epitope with the CDR of an immunoglobulin molecule. *See, e.g.*, Harlow *et al.* (1988) *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, 2nd ed.) pages 27-28. As used herein, the term "avidity" refers to the overall stability of the complex between a population of immunoglobulins and an antigen, that is, the functional combining strength of an immunoglobulin mixture with the antigen. *See, e.g.* , Harlow at pages 29-34. Avidity is related to both the affinity of individual immunoglobulin molecules in the population with specific epitopes, and also the valencies of the immunoglobulins and the antigen. For example, the interaction between a bivalent monoclonal antibody and an antigen with a highly repeating epitope structure, such as a polymer, would be one of high avidity.

Anti-CD20 antibodies or antigen-binding fragments, variants, or derivatives thereof of the invention may also be described or specified in terms of their cross-reactivity. As used herein, the term "cross-reactivity" refers to the ability of an antibody, specific for one antigen, to react with a second antigen; a measure of

relatedness between two different antigenic substances. Thus, an antibody is cross reactive if it binds to an epitope other than the one that induced its formation. The cross reactive epitope generally contains many of the same complementary structural features as the inducing epitope, and in some cases, may actually fit better than the original.

For example, certain antibodies have some degree of cross-reactivity, in that they bind related, but non-identical epitopes, *e.g.*, epitopes with at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 65%, at least 60%, at least 55%, and at least 50% identity (as calculated using methods known in the art and described herein) to a reference epitope. An antibody may be said to have little or no cross-reactivity if it does not bind epitopes with less than 95%, less than 90%, less than 85%, less than 80%, less than 75%, less than 70%, less than 65%, less than 60%, less than 55%, and less than 50% identity (as calculated using methods known in the art and described herein) to a reference epitope. An antibody may be deemed "highly specific" for a certain epitope, if it does not bind any other analog, ortholog, or homolog of that epitope.

Anti-CD20 antibodies or antigen-binding fragments, variants or derivatives thereof of the invention may also be described or specified in terms of their binding affinity to a polypeptide of the invention. Preferred binding affinities include those with a dissociation constant or Kd less than 5×10^{-2} M, 10^{-2} M, 5×10^{-3} M, 10^{-3} M, 5×10^{-4} M, 10^{-4} M, 5×10^{-5} M, 10^{-5} M, 5×10^{-6} M, 10^{-6} M, 5×10^{-7} M, 10^{-7} M, 5×10^{-8} M, 10^{-8} M, 5×10^{-9} M, 10^{-9} M, 5×10^{-10} M, 10^{-10} M, 5×10^{-11} M, 10^{-11} M, 5×10^{-12} M, 10^{-12} M, 5×10^{-13} M, 10^{-13} M, 5×10^{-14} M, 10^{-14} M, 5×10^{-15} M, or 10^{-15} M.

Anti-CD20 antibodies or antigen-binding fragments, variants or derivatives thereof of the invention may be "multispecific," *e.g.*, bispecific, trispecific, or of greater multispecificity, meaning that it recognizes and binds to two or more different epitopes present on one or more different antigens (*e.g.*, proteins) at the same time. Thus, whether an anti-CD20 antibody is "monospecific" or "multispecific," *e.g.*, "bispecific," refers to the number of different epitopes with which a binding polypeptide reacts. Multispecific antibodies may be specific for different epitopes of

a target polypeptide described herein or may be specific for a target polypeptide as well as for a heterologous epitope, such as a heterologous polypeptide or solid support material.

As used herein the term "valency" refers to the number of potential binding domains, *e.g.*, antigen binding domains, present in an anti-CD20 antibody, binding polypeptide, or antibody. Each binding domain specifically binds one epitope. When an anti-CD20 antibody, binding polypeptide, or antibody comprises more than one binding domain, each binding domain may specifically bind the same epitope, for an antibody with two binding domains, termed "bivalent monospecific," or to different epitopes, for an antibody with two binding domains, termed "bivalent bispecific." An antibody may also be bispecific and bivalent for each specificity (termed "bispecific tetravalent antibodies"). In another embodiment, tetravalent minibodies or domain deleted antibodies can be made.

Bispecific bivalent antibodies, and methods of making them, are described, for instance in U.S. Patent Nos. 5,731,168; 5,807,706; 5,821,333; and U.S. Patent Appl. Publ. Nos. 2003/020734 and 2002/0155537, the disclosures of all of which are incorporated by reference herein. Bispecific tetravalent antibodies, and methods of making them are described, for instance, in WO 02/096948 and WO 00/44788, the disclosures of both of which are incorporated by reference herein. See generally, PCT publications WO 93/17715; WO 92/08802; WO 91/00360; WO 92/05793; Tutt *et al.* (1991) *J. Immunol.* 147:60-69; U.S. Patent Nos. 4,474,893; 4,714,681; 4,925,648; 5,573,920; 5,601,819; Kostelny *et al.* (1992) *J. Immunol.* 148:1547-1553.

As previously indicated, the subunit structures and three dimensional configuration of the constant regions of the various immunoglobulin classes are well known. As used herein, the term "V_H domain" includes the amino terminal variable domain of an immunoglobulin heavy chain and the term "C_H1 domain" includes the first (most amino terminal) constant region domain of an immunoglobulin heavy chain. The C_H1 domain is adjacent to the V_H domain and is amino terminal to the hinge region of an immunoglobulin heavy chain molecule.

As used herein the term "C_H2 domain" includes the portion of a heavy chain molecule that extends, *e.g.*, from about residue 244 to residue 360 of an antibody using conventional numbering schemes (residues 244 to 360, Kabat numbering

system; and residues 231-340, EU numbering system; see Kabat EA *et al. op. cit.* The C_H2 domain is unique in that it is not closely paired with another domain. Rather, two N-linked branched carbohydrate chains are interposed between the two C_H2 domains of an intact native IgG molecule. It is also well documented that the C_H3 domain extends from the C_H2 domain to the C-terminal of the IgG molecule and comprises approximately 108 residues.

As used herein, the term "hinge region" includes the portion of a heavy chain molecule that joins the C_H1 domain to the C_H2 domain. This hinge region comprises approximately 25 residues and is flexible, thus allowing the two N-terminal antigen binding regions to move independently. Hinge regions can be subdivided into three distinct domains: upper, middle, and lower hinge domains (Roux *et al.* (1998) *J. Immunol.* 161:4083).

As used herein the term "disulfide bond" includes the covalent bond formed between two sulfur atoms. The amino acid cysteine comprises a thiol group that can form a disulfide bond or bridge with a second thiol group. In most naturally occurring IgG molecules, the C_H1 and C_L regions are linked by a disulfide bond and the two heavy chains are linked by two disulfide bonds at positions corresponding to 239 and 242 using the Kabat numbering system (position 226 or 229, EU numbering system).

As used herein, the term "chimeric antibody" will be held to mean any antibody wherein the immunoreactive region or site is obtained or derived from a first species and the constant region (which may be intact, partial or modified in accordance with the instant invention) is obtained from a second species. In preferred embodiments the target binding region or site will be from a non-human source (*e.g.*, mouse or primate) and the constant region is human.

As used herein, the term "engineered antibody" refers to an antibody in which the variable domain in either the heavy or light chain or both is altered by at least partial replacement of one or more CDRs from an antibody of known specificity and, if necessary, by partial framework region replacement and sequence changing. Although the CDRs may be derived from an antibody of the same class or even subclass as the antibody from which the framework regions are derived, it is envisaged that the CDRs will be derived from an antibody of different class and preferably from an antibody from a different species. An engineered antibody in

which one or more "donor" CDRs from a non-human antibody of known specificity is grafted into a human heavy or light chain framework region is referred to herein as a "humanized antibody." It may not be necessary to replace all of the CDRs with the complete CDRs from the donor variable domain to transfer the antigen binding capacity of one variable domain to another. Rather, it may only be necessary to transfer those residues that are necessary to maintain the activity of the target binding site.

It is further recognized that the framework regions within the variable domain in a heavy or light chain, or both, of a humanized antibody may comprise solely residues of human origin, in which case these framework regions of the humanized antibody are referred to as "fully human framework regions." Alternatively, one or more residues of the framework region(s) of the donor variable domain can be engineered within the corresponding position of the human framework region(s) of a variable domain in a heavy or light chain, or both, of a humanized antibody if necessary to maintain proper binding or to enhance binding to the CD20 antigen. A human framework region that has been engineered in this manner would thus comprise a mixture of human and donor framework residues, and is referred to herein as a "partially human framework region." Given the explanations set forth in, *e.g.*, U. S. Patent Nos. 5,585,089, 5,693,761, 5,693,762, and 6,180,370, it will be well within the competence of those skilled in the art, either by carrying out routine experimentation or by trial and error testing to obtain a functional engineered or humanized antibody.

For example, humanization of an anti-CD20 antibody can be essentially performed following the method of Winter and co-workers (Jones *et al.* (1986) *Nature* 321:522-525; Riechmann *et al.* (1988) *Nature* 332:323-327; Verhoeyen *et al.* (1988) *Science* 239:1534-1536), by substituting rodent or mutant rodent CDRs or CDR sequences for the corresponding sequences of a human anti-CD20 antibody. See also U.S. Patent Nos. 5,225,539; 5,585,089; 5,693,761; 5,693,762; 5,859,205; herein incorporated by reference. The resulting humanized anti-CD20 antibody would comprise at least one rodent or mutant rodent CDR within the fully human framework regions of the variable domain of the heavy and/or light chain of the humanized antibody. In some instances, residues within the framework regions of one or more

variable domains of the humanized anti-CD20 antibody are replaced by corresponding non-human (for example, rodent) residues (see, for example, U.S. Patent Nos. 5,585,089; 5,693,761; 5,693,762; and 6,180,370), in which case the resulting humanized anti-CD20 antibody would comprise partially human framework regions within the variable domain of the heavy and/or light chain.

Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance (e.g., to obtain desired affinity). In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDRs correspond to those of a non-human immunoglobulin and all or substantially all of the framework regions are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details see Jones *et al.* (1986) *Nature* 331:522-525; Riechmann *et al.* (1988) *Nature* 332:323-329; and Presta (1992) *Curr. Op. Struct. Biol.* 2:593-596; herein incorporated by reference. Accordingly, such "humanized" antibodies may include antibodies wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some framework residues are substituted by residues from analogous sites in rodent antibodies. See, for example, U.S. Patent Nos. 5,225,539; 5,585,089; 5,693,761; 5,693,762; 5,859,205. See also U.S. Patent No. 6,180,370, and International Publication No. WO 01/27160, where humanized antibodies and techniques for producing humanized antibodies having improved affinity for a predetermined antigen are disclosed.

As used herein, the term "properly folded polypeptide" includes polypeptides (*e.g.*, anti-CD20 antibodies) in which all of the functional domains comprising the polypeptide are distinctly active. As used herein, the term "improperly folded polypeptide" includes polypeptides in which at least one of the functional domains of the polypeptide is not active. In one embodiment, a properly folded polypeptide

comprises polypeptide chains linked by at least one disulfide bond and, conversely, an improperly folded polypeptide comprises polypeptide chains not linked by at least one disulfide bond.

As used herein, the term "engineered" includes manipulation of nucleic acid or polypeptide molecules by synthetic means (*e.g.*, by recombinant techniques, *in vitro* peptide synthesis, by enzymatic or chemical coupling of peptides or some combination of these techniques).

As used herein, the terms "linked," "fused," or "fusion" are used interchangeably. These terms refer to the joining together of two more elements or components, by whatever means, including chemical conjugation or recombinant means. An "in-frame fusion" refers to the joining of two or more polynucleotide open reading frames (ORFs) to form a continuous longer ORF, in a manner that maintains the correct translational reading frame of the original ORFs. Thus, a recombinant fusion protein is a single protein containing two or more segments that correspond to polypeptides encoded by the original ORFs (which segments are not normally so joined in nature). Although the reading frame is thus made continuous throughout the fused segments, the segments may be physically or spatially separated by, for example, in-frame linker sequence. For example, polynucleotides encoding the CDRs of an immunoglobulin variable region may be fused, in-frame, but be separated by a polynucleotide encoding at least one immunoglobulin framework region or additional CDR regions, as long as the "fused" CDRs are co-translated as part of a continuous polypeptide.

In the context of polypeptides, a "linear sequence" or a "sequence" is an order of amino acids in a polypeptide in an amino to carboxyl terminal direction in which residues that neighbor each other in the sequence are contiguous in the primary structure of the polypeptide.

The term "expression" as used herein refers to a process by which a gene produces a biochemical, for example, a polypeptide. The process includes any manifestation of the functional presence of the gene within the cell including, without limitation, gene knockdown as well as both transient expression and stable expression. It includes without limitation transcription of the gene into messenger RNA (mRNA), and the translation of such mRNA into polypeptide(s). If the final

desired product is a biochemical, expression includes the creation of that biochemical and any precursors. Expression of a gene produces a "gene product." As used herein, a gene product can be either a nucleic acid, *e.g.*, a messenger RNA produced by transcription of a gene, or a polypeptide which is translated from a transcript. Gene products described herein further include nucleic acids with post transcriptional modifications, *e.g.*, polyadenylation, or polypeptides with post translational modifications, *e.g.*, methylation, glycosylation, the addition of lipids, association with other protein subunits, proteolytic cleavage, and the like.

As used herein, the terms "treat" or "treatment" refer to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) an undesired physiological change or disorder, such as the progression of multiple sclerosis. Beneficial or desired clinical results include, but are not limited to, alleviation of symptoms, diminishment of extent of disease, stabilized (*i.e.*, not worsening) state of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. "Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment. Those in need of treatment include those already with the condition or disorder as well as those prone to have the condition or disorder or those in which the condition or disorder is to be prevented.

By "subject" or "individual" or "animal" or "patient" or "mammal," is meant any subject, particularly a mammalian subject, for whom diagnosis, prognosis, or therapy is desired. Mammalian subjects include humans, domestic animals, farm animals, and zoo, sports, or pet animals such as dogs, cats, guinea pigs, rabbits, rats, mice, horses, cattle, cows, and so on.

As used herein, phrases such as "a subject that would benefit from administration of an anti-CD20 antibody" and "an animal in need of treatment" includes subjects, such as mammalian subjects, that would benefit from administration of an anti-CD20 antibody used, *e.g.*, for detection of an anti-CD20 polypeptide (*e.g.*, for a diagnostic procedure) and/or for treatment, *i.e.*, palliation or

prevention of a disease, with an anti-CD20 antibody. As described in more detail herein, the anti-CD20 antibody can be used in unconjugated form or can be conjugated, *e.g.*, to a drug, prodrug, or an isotope.

II. TARGET POLYPEPTIDE DESCRIPTION

The B1 (CD20) molecule is a phosphoprotein of approximately 35,000 Daltons on the surface of human B lymphocytes that may serve a central role in the humoral immune response by regulating B-cell proliferation and differentiation. The DNA sequence that encodes the CD20 molecule was isolated and the amino acid sequence of CD20 has been determined. See, Tedder *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:208-212. Synonyms of CD20, as recognized in the art, include B-lymphocyte antigen CD20, B-lymphocyte surface antigen B1, Leu-16, Bp35, BM5, and LF5.

The antibodies of the invention have binding specificity to the CD20 human B-cell surface antigen. The antigen is a polypeptide or comprises a polypeptide bound by the 2H7 monoclonal antibody described in Clark *et al.* (1985) *Proc. Natl. Acad. Sci.* U.S.A. 82:1766-1770. This antigen is a phosphoprotein designated (Bp35(CD20)) and is only expressed on cells of the B cell lineage. Murine monoclonal antibodies to this antigen have been made before and are described in Clark *et al.*, *supra*; see also Stashenko *et al.* (1980) *J. Immunol.* 125:1678-1685.

III. ANTI-CD20 ANTIBODIES

In one embodiment, the present invention is directed to anti-CD20 antibodies, including antigen-binding fragments, variants, or derivatives thereof. The antibodies of the invention are anti-CD20 antibodies that have been optimized for enhanced activity. As used herein, the term "anti-CD20 antibody" is an antibody that specifically recognizes a cell surface non-glycosylated phosphoprotein of approximately 35,000 Daltons, typically designated as the human B-lymphocyte restricted differentiation antigen Bp35, commonly referred to as CD20. The antigen is expressed on greater than 90% of B-cell non-Hodgkin's lymphomas but is not found on hematopoietic stem cells, pro-B cells, normal plasma cells, or other normal

tissues. CD20 regulates an early step in the activation process for cell proliferation and differentiation. More specifically, the antibodies of the invention bind the same epitope as bound by the 2H7 monoclonal antibody described above.

The antibodies of the invention are optimized based on the monoclonal antibody (mAb) C2B8, a chimeric murine/human anti-CD20 antibody as disclosed in U.S. Patent No. 5,736,137 and (Reff *et al.* (1994) *Blood* 83:435-445), herein incorporated by reference. The antibody sequences of the invention comprise modified CDRs that when engineered into the variable domains of light and heavy chains of an anti-CD20 antibody result in enhanced CDC activity of the antibody while maintaining binding specificity and ability to mediate apoptosis as compared to rituximab.

As noted, the anti-CD20 antibodies of the invention exhibit enhanced CDC activity as compared to rituximab. Rituximab is a chimeric murine/human anti-CD20 monoclonal antibody (IDEC-C2B8; IDEC Pharmaceutical Corp., San Diego, California; commercially available under the tradename Rituxan®) containing human IgG1 and kappa constant regions with murine variable regions isolated from a murine anti-CD20 monoclonal antibody, IDEC-2B8 (Reff *et al.* (1994) *Blood* 83:435-445). Rituximab is used for treatment of relapsed B cell low-grade or follicular non-Hodgkin's lymphoma (NHL). While not bound to any mechanism of action, anti-CD20 antibodies bind to the CD20 antigen and mechanisms of cell lysis include complement-dependent cytotoxicity (CDC) and antibody-dependent cell mediated cytotoxicity (ADCC). Therefore, the discovery of anti-CD20 antibodies with superior CDC and/or ADCC activity, and/or increased binding affinity compared to rituximab (also referred to herein as Rituxan®) will potentially improve methods of cancer therapy for B cell lymphomas, particularly B cell non-Hodgkin's lymphoma. The antibodies are compared in assays in equivalent amounts. By "equivalent amount" of the anti-CD20 antibody of the invention and rituximab is intended the same dose is used for each antibody.

Binding affinity of these novel anti-CD20 antibodies for CD20 is increased by at least about 50%, about 75%, about 100%, about 125% relative to that observed for rituximab using a CDC off-rate assay. In a non-radioactive CDC assay, at least about

2 times, about 3 times, up to about 4 times more rituximab is needed to mediate the same level of cell killing on three different NHL lines than is needed using an anti-CD20 antibody of the invention.

The anti-CD20 antibodies of the invention comprise at least one optimized complementarity-determining region (CDR). By "optimized CDR" is intended that the CDR has been modified and optimized sequences selected based on the improved binding affinity and/or improved CDC activity that is imparted to an anti-CD20 antibody comprising the optimized CDR. The modifications involve replacement of amino acid residues within the CDR such that an anti-CD20 antibody retains specificity for the CD20 antigen and has improved binding affinity and/or improved CDC activity. CDC activity of an anti-CD20 antibody of the invention is improved as compared to rituxamab in a functional assay as described in U.S. Application Publication No. 2004/0167319 A1, herein incorporated by reference. The novel anti-CD20 antibodies of the invention and suitable antigen-binding fragments, variants, and derivatives thereof also exhibit ADCC and apoptosis activity that is at least similar to that exhibited by rituximab, as measured in standard assays, for example, those described in U.S. Application Publication No. 2004/0167319 A1. The optimized CDRs of the invention are utilized in V_H and V_L domains of the heavy and light chains, respectively, of anti-CD20 antibodies. Exemplary anti-CD20 antibodies of the invention comprise a V_H domain selected from the group consisting of SEQ ID NOS:12-18 (respectively designated H1569, H1570, H1571, H1638, H1639, H1640, H1670) and/or a V_L domain selected from SEQ ID NOS:10 and 11 (respectively designated L373 and L419).

In particular embodiments, the anti-CD20 antibodies of the invention comprise optimized CDRs. That is, the anti-CD20 antibodies of the invention comprise at least one optimized CDR amino acid sequence selected from the group consisting of SEQ ID NOS:1-8 or amino acid sequences having at least about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, or 100% sequence identity to a sequence selected from the group consisting of SEQ ID NOS:1-8. That is, the optimized CDRs comprise the sequences

set forth in SEQ ID NOS:1-8 and the sequences of SEQ ID NOS:1-8 having at least one, two, three, four, or five amino acid substitutions, depending upon the CDR involved.

Thus, in some embodiments, the anti-CD20 antibodies of the invention comprise a V_H domain having at least one optimized CDR selected from the group consisting of:

(a) a CDR1 comprising the amino acid sequence set forth in SEQ ID NO:7;

(b) a CDR1 comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID:7, where the CDR1 comprises the phenylalanine (Phe) residue at the position corresponding to residue 7 of SEQ ID NO:7;

(c) a CDR2 comprising the amino acid sequence set forth in SEQ ID NO:5 or SEQ ID NO:6;

(d) a CDR2 comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID:5 or SEQ ID NO:6, where the CDR2 comprises the alanine (Ala) or leucine (Leu) residue at the position corresponding to residue 8 of SEQ ID NO:5 or SEQ ID NO:6, respectively;

(e) a CDR3 comprising the amino acid sequence set forth in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4;

(f) a CDR3 comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:1, where the CDR3 comprises at least one residue selected from the group consisting of: (i) the asparagine (Asn) residue at the position corresponding to residue 9 of SEQ ID NO:1, and (ii) the asparagine (Asn) residue at the position corresponding to residue 12 of SEQ ID NO:1;

(g) a CDR3 comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:2, where the CDR3 comprises at least one residue selected from the group consisting of: (i) the alanine (Ala) residue at the position corresponding to residue 5 of SEQ ID NO:2, (ii) the asparagine (Asn) residue at the position corresponding to residue 9 of SEQ ID NO:2, and (iii) the asparagine (Asn) residue at the position corresponding to residue 12 of SEQ ID NO:2;

(h) a CDR3 comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:3, where the CDR3 comprises at least one residue selected from the group consisting of: (i) the alanine (Ala) residue at the position corresponding to residue 5 of SEQ ID NO:3, (ii) the asparagine (Asn) residue at the position corresponding to residue 9 of SEQ ID NO:3, and (iii) the aspartic acid (Asp) residue at the position corresponding to residue 12 of SEQ ID NO:3;

(i) a CDR3 comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:4, where the CDR3 comprises at least one residue selected from the group consisting of: (i) the alanine (Ala) residue at the position corresponding to residue 5 of SEQ ID NO:4, (ii) the asparagine (Asn) residue at the position corresponding to residue 9 of SEQ ID NO:4; (iii) the glycine (Gly) residue at the position corresponding to residue 11 of SEQ ID NO:4; and (iv) the asparagine (Asn) residue at the position corresponding to residue 12 of SEQ ID NO:4;

(j) a CDR1 comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID:7, where the CDR1 comprises at the position corresponding to residue 7 of SEQ ID NO:7 a residue that is a conservative amino acid substitution for phenylalanine (Phe);

(k) a CDR2 comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID:5, where the CDR2 comprises at the position corresponding to residue 8 of SEQ ID NO:5 a residue that is a conservative amino acid substitution for alanine (Ala);

(l) a CDR2 comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID:6, where the CDR2 comprises at the position corresponding to residue 8 of SEQ ID NO:6 a residue that is a conservative amino acid substitution for leucine (Leu);

(m) a CDR3 comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:1, where the CDR3 comprises at least one residue selected from the group consisting of: (i) at the position corresponding to residue 9 of SEQ ID NO:1, a residue that is a conservative

amino acid substitution for asparagine (Asn), and (ii) at the position corresponding to residue 12 of SEQ ID NO:1, a residue that is a conservative amino acid substitution for asparagine (Asn);

(n) a CDR3 comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:2, where the CDR3 comprises at least one residue selected from the group consisting of: (i) at the position corresponding to residue 5 of SEQ ID NO:2, a residue that is a conservative amino acid substitution for alanine (Ala), wherein the conservative amino acid substitution is not glycine, (ii) at the position corresponding to residue 9 of SEQ ID NO:2, a residue that is a conservative amino acid substitution for asparagine (Asn), and (iii) at the position corresponding to residue 12 of SEQ ID NO:2, a residue that is a conservative amino acid substitution for asparagine (Asn);

(o) a CDR3 comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:3, where the CDR3 comprises at least one residue selected from the group consisting of: (i) at the position corresponding to residue 5 of SEQ ID NO:3, a residue that is a conservative amino acid substitution for alanine (Ala), wherein the conservative amino acid substitution is not glycine, (ii) at the position corresponding to residue 9 of SEQ ID NO:3, a residue that is a conservative amino acid substitution for asparagine (Asn), and (iii) at the position corresponding to residue 12 of SEQ ID NO:3, a residue that is a conservative amino acid substitution for aspartic acid (Asp); and

(p) a CDR3 comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:4, where the CDR3 comprises at least one residue selected from the group consisting of: (i) at the position corresponding to residue 5 of SEQ ID NO:4, a residue that is a conservative amino acid substitution for alanine (Ala), wherein the conservative amino acid substitution is not glycine, (ii) at the position corresponding to residue 9 of SEQ ID NO:4, a residue that is a conservative amino acid substitution for asparagine (Asn), (iii) at the position corresponding to residue 11 of SEQ ID NO:4, a residue that is a conservative amino acid substitution for glycine (Gly), and (iv) at the position corresponding to residue 12 of SEQ ID NO:4, a residue that is a conservative amino acid substitution for asparagine (Asn).

In other embodiments, the anti-CD20 antibodies of the invention comprise a V_L domain having at least one CDR selected from the group consisting of: (a) a CDR3 comprising the amino acid sequence set forth in SEQ ID NO:8; (b) a CDR3 comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:8, where the CDR3 comprises the glutamine (Gln) residue at the position corresponding to residue 4 of SEQ ID NO:8; and (c) a CDR3 comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:8, where the CDR3 comprises at the position corresponding to residue 4 of SEQ ID NO:8 a residue that is a conservative amino acid substitution for glutamine (Gln); and (d) a CDR2 comprising the sequence set forth in SEQ ID NO:9. In some of these embodiments, the anti-CD20 antibodies of the invention comprise a V_L domian having a CDR2 comprising the sequence set forth in SEQ ID NO:9 and a CDR3 selected from the group consisting of: (a) a CDR3 comprising the amino acid sequence set forth in SEQ ID NO:8; and (b) a CDR3 comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:8, where the CDR3 comprises the glutamine (Gln) residue at the position corresponding to residue 4 of SEQ ID NO:8.

In yet other embodiments, the anti-CD20 antibodies of the invention comprise a V_H domain having an optimized CDR selected from the group recited in items (a) through (p) *supra*; and a V_L domain having at least one CDR selected from the group consisting of: (a) a CDR3 comprising the amino acid sequence set forth in SEQ ID NO:8; (b) a CDR3 comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:8, where the CDR3 comprises the glutamine (Gln) residue at the position corresponding to residue 4 of SEQ ID NO:8; (c) a CDR3 comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:8, where the CDR3 comprises at the position corresponding to residue 4 of SEQ ID NO:8 a residue that is a conservative amino acid substitution for glutamine (Gln); and (d) a CDR2 comprising the amino acid sequence set forth in SEQ ID NO:9. In some of these embodiments, the anti-CD20 antibodies of the invention comprise a V_L domain having a CDR2 comprising the sequence set forth in SEQ ID NO:9 and a CDR3

selected from the group consisting of: (a) a CDR3 comprising the amino acid sequence set forth in SEQ ID NO:8; and (b) a CDR3 comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:8, where the CDR3 comprises the glutamine (Gln) residue at the position corresponding to residue 4 of SEQ ID NO:8.

In still other embodiments, the anti-CD20 antibodies of the invention comprise a V_H domain having an optimized CDR selected from the group recited in items (a) through (p) *supra*; and a V_L domain having the sequence set forth in SEQ ID NO:10 or SEQ ID NO:11.

In some embodiments, the anti-CD20 antibodies comprising at least one of the optimized CDRs of the invention are IgG1 kappa immunoglobulins. In such embodiments, the IgG1 kappa immunoglobulin can comprise a human IgG1 constant region within a heavy chain of the immunoglobulin and a human kappa constant region within a light chain of the immunoglobulin. In particular embodiments, the IgG1 kappa immunoglobulin comprises fully or partially human framework regions within the variable domain of the heavy chain and within the variable domain of the light chain. In other embodiments, the IgG1 kappa immunoglobulin comprises murine framework regions within the variable domain of the heavy chain and within the variable domain of the light chain.

In further embodiments of the invention, the anti-CD20 antibodies of the invention comprise a V_H domain having an amino acid sequence selected from the group consisting of SEQ ID NOS:12-18 and/or a V_L domain having an amino acid sequence selected from SEQ ID NOS:10 and 11, or amino acid sequences having at least about 80%, 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99% or 100% sequence identity to a sequence set forth in SEQ ID NOS:10-18.

In yet other embodiments of the invention, the anti-CD20 antibodies of the invention comprise a V_H domain, where the V_H domain is selected from the group consisting of:

(a) a V_H domain comprising an amino acid sequence having at least 90% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOS:12-18;

(b) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:12, where theV_H domain comprises at least one residue selected from the group consisting of: (i) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:12, and (ii) the asparagine (Asn) residue at the position corresponding to residue 110 of SEQ ID NO:12;

(c) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:13, where the V_H domain comprises at least one residue selected from the group consisting of: (i) the alanine (Ala) residue at the position corresponding to residue 103 of SEQ ID NO:13, (ii) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:13, and (iii) the asparagine (Asn) residue at the position corresponding to residue 110 of SEQ ID NO:13;

(d) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:14, where the V_H domain comprises at least one residue selected from the group consisting of: (i) the alanine (Ala) residue at the position corresponding to residue 103 of SEQ ID NO:14, (ii) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:14, and (iii) the aspartic acid (Asp) residue at the position corresponding to residue 110 of SEQ ID NO:14;

(e) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:15, where the V_H domain comprises at least one residue selected from the group consisting of: (i) the phenylalanine (Phe) residue at the position corresponding to residue 31 of SEQ ID NO:15; (ii) the alanine (Ala) residue at the position corresponding to residue 103 of SEQ ID NO:15; (iii) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:15, and (iv) the asparagine (Asn) residue at the position corresponding to residue 110 of SEQ ID NO:15;

(f) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:16, where the V_H domain comprises at least one residue selected from the group consisting of: (i) the alanine (Ala) residue at the position corresponding to residue 57 of SEQ ID

NO:16, (ii) the alanine (Ala) residue at the position corresponding to residue 103 of SEQ ID NO:16, (iii) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:16, and (iv) the asparagine (Asn) residue at the position corresponding to residue 110 of SEQ ID NO:16;

(g) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:17, where the V_H domain comprises at least one residue selected from the group consisting of: (i) the leucine (Leu) residue at the position corresponding to residue 57 of SEQ ID NO:17, (ii) the alanine (Ala) residue at the position corresponding to residue 103 of SEQ ID NO:17, (iii) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:17, and (iv) the asparagine (Asn) residue at the position corresponding to residue 110 of SEQ ID NO:17;

(h) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:18, where the V_H domain comprises at least one residue selected from the group consisting of: (i) the alanine (Ala) residue at the position corresponding to residue 103 of SEQ ID NO:18, (ii) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:18, (iii) the glycine (Gly) residue at the position corresponding to residue 109 of SEQ ID NO:18, and (iv) the asparagine (Asn) residue at the position corresponding to residue 110 of SEQ ID NO:18;

(i) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:12, where theV_H domain comprises at least one residue selected from the group consisting of: (i) at the position corresponding to residue 107 of SEQ ID NO:12, a residue that is a conservative amino acid substitution for asparagine (Asn), and (ii) at the position corresponding to residue 110 of SEQ ID NO:12, a residue that is a conservative amino acid substitution for asparagine (Asn);

(j) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:13, where the V_H domain comprises at least one residue selected from the group consisting of: (i) at the position corresponding to residue 103 of SEQ ID NO:13, a residue that is a conservative amino acid substitution for alanine (Ala), wherein the conservative

amino acid substitution is not glycine, (ii) at the position corresponding to residue 107 of SEQ ID NO:13, a residue that is a conservative amino acid substitution for asparagine (Asn), and (iii) at the position corresponding to residue 110 of SEQ ID NO:13, a residue that is a conservative amino acid substitution for asparagine (Asn);

(k) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:14, where the V_H domain comprises at least one residue selected from the group consisting of: (i) at the position corresponding to residue 103 of SEQ ID NO:14, a residue that is a conservative amino acid substitution for alanine (Ala), where the conservative amino acid substitution is not glycine, (ii) at the position corresponding to residue 107 of SEQ ID NO:14, a residue that is a conservative amino acid substitution for asparagine (Asn), and (iii) at the position corresponding to residue 110 of SEQ ID NO:14, a residue that is a conservative amino acid substitution for aspartic acid (Asp);

(l) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:15, where the V_H domain comprises at least one residue selected from the group consisting of: (i) at the position corresponding to residue 31 of SEQ ID NO:15, a residue that is a conservative amino acid substitution for phenylalanine (Phe), (ii) at the position corresponding to residue 103 of SEQ ID NO:15, a residue that is a conservative amino acid substitution for alanine (Ala), where the conservative amino acid substitution is not glycine, (iii) at the position corresponding to residue 107 of SEQ ID NO:15, a residue that is a conservative amino acid substitution for asparagine (Asn), and (iv) at the position corresponding to residue 110 of SEQ ID NO:15, a residue that is a conservative amino acid substitution for asparagine (Asn);

(m) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:16, where the V_H domain comprises at least one residue selected from the group consisting of: (i) at the position corresponding to residue 57 of SEQ ID NO:16, a residue that is a conservative amino acid substitution for alanine (Ala), (ii) at the position corresponding to residue 103 of SEQ ID NO:16, a residue that is a conservative amino acid substitution for alanine (Ala), wherein the conservative amino acid substitution is not glycine, (iii) at the position corresponding to residue 107 of SEQ

ID NO:16, a residue that is a conservative amino acid substitution for asparagine (Asn), and (iv) at the position corresponding to residue 110 of SEQ ID NO:16, a residue that is a conservative amino acid substitution for asparagine (Asn);

(n) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:17, where the V_H domain comprises at least one residue selected from the group consisting of: (i) at the position corresponding to residue 57 of SEQ ID NO:17, a residue that is a conservative amino acid substitution for leucine (Leu), (ii) at the position corresponding to residue 103 of SEQ ID NO:17, a residue that is a conservative amino acid substitution for alanine (Ala), where the conservative amino acid substitution is not glycine, (iii) at the position corresponding to residue 107 of SEQ ID NO:17, a residue that is a conservative amino acid substitution for asparagine (Asn), and (iv) at the position corresponding to residue 110 of SEQ ID NO:17, a residue that is a conservative amino acid substitution for asparagine (Asn); and

(o) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:18, where the V_H domain comprises at least one residue selected from the group consisting of: (i) at the position corresponding to residue 103 of SEQ ID NO:18, a residue that is a conservative amino acid substitution for alanine (Ala), where the conservative amino acid substitution is not glycine, (ii) at the position corresponding to residue 107 of SEQ ID NO:18, a residue that is a conservative amino acid substitution for asparagine (Asn), (iii) at the position corresponding to residue 109 of SEQ ID NO:18, a residue that is a conservative amino acid substitution for glycine (Gly), and (iv) at the position corresponding to residue 110 of SEQ ID NO:18, a residue that is a conservative amino acid substitution for asparagine (Asn).

In some of these embodiments, the anti-CD20 antibodies of the invention comprise a V_H domain selected from the group recited in items (a) through (o) *supra*; and comprise a variable light (V_L) domain, where the V_L domain is selected from the group consisting of:

(a) a V_L domain comprising the sequence set forth in SEQ ID NO:10 or SEQ ID NO:11;

(b) a V_L domain comprising an amino acid sequence having at least 90% sequence identity to the sequence set forth in SEQ ID NO:10 or SEQ ID NO:11;

(c) a V_L domain comprising an amino acid sequence having at least 85% sequence identity to the sequence set forth in SEQ ID NO:10, where the V_L domain comprises the CDR2 set forth in SEQ ID NO:9;

(d) a V_L domain comprising an amino acid sequence having at least 85% sequence identity to the sequence set forth in SEQ ID NO:11, where the V_L domain comprises at least one of: (i) the CDR2 set forth in SEQ ID NO:9, and (ii) the glutamine (Gln) residue at the position corresponding to residue 91 of SEQ ID NO:11; and

(e) a V_L domain comprising an amino acid sequence having at least 85% sequence identity to the sequence set forth in SEQ ID NO:11, where the V_L domain comprises at least one of: (i) the CDR2 set forth in SEQ ID NO:9, and (ii) at the position corresponding to residue 91 of SEQ ID NO:11, a conservative amino acid substitution for glutamine (Gln).

In some of these embodiments, the anti-CD20 antibodies of the invention comprise a V_H domain comprising the amino acid sequence set forth in SEQ ID NO:12 and a V_L domain comprising the amino acid sequence set forth in SEQ ID NO: 10. In other embodiments, the anti-CD20 antibodies of the invention comprise a V_H domain comprising the amino acid sequence set forth in SEQ ID NO:13 and a V_L domain comprising the amino acid sequence set forth in SEQ ID NO: 10. In certain other embodiments, the anti-CD20 antibodies of the invention comprise a V_H domain comprising the amino acid sequence set forth in SEQ ID NO:14 and a V_L domain comprising the amino acid sequence set forth in SEQ ID NO: 10. In still other embodiments, the anti-CD20 antibodies of the invention comprise a V_H domain comprising the amino acid sequence set forth in SEQ ID NO:15 and a V_L domain comprising the amino acid sequence set forth in SEQ ID NO: 10. In yet other embodiments, the anti-CD20 antibodies of the invention comprise a V_H domain comprising the amino acid sequence set forth in SEQ ID NO:16 and a V_L domain comprising the amino acid sequence set forth in SEQ ID NO: 10. In further embodiments, the anti-CD20 antibodies of the invention comprise a V_H domain comprising the amino acid sequence set forth in SEQ ID NO:17 and a V_L domain

comprising the amino acid sequence set forth in SEQ ID NO: 10. In still further embodiments, the anti-CD20 antibodies of the invention comprise a V_H domain comprising the amino acid sequence set forth in SEQ ID NO:18 and a V_L domain comprising the amino acid sequence set forth in SEQ ID NO: 10. In certain embodiments, the anti-CD20 antibodies of the invention comprise a V_H domain comprising the amino acid sequence set forth in SEQ ID NO:12 and a V_L domain comprising the amino acid sequence set forth in SEQ ID NO: 11. In other embodiments, the anti-CD20 antibodies of the invention comprise a V_H domain comprising the amino acid sequence set forth in SEQ ID NO:13 and a V_L domain comprising the amino acid sequence set forth in SEQ ID NO: 11. In certain other embodiments, the anti-CD20 antibodies of the invention comprise a V_H domain comprising the amino acid sequence set forth in SEQ ID NO:14 and a V_L domain comprising the amino acid sequence set forth in SEQ ID NO: 11. In still other embodiments, the anti-CD20 antibodies of the invention comprise a V_H domain comprising the amino acid sequence set forth in SEQ ID NO:15 and a V_L domain comprising the amino acid sequence set forth in SEQ ID NO: 11. In yet other embodiments, the anti-CD20 antibodies of the invention comprise a V_H domain comprising the amino acid sequence set forth in SEQ ID NO:16 and a V_L domain comprising the amino acid sequence set forth in SEQ ID NO: 11. In further embodiments, the anti-CD20 antibodies of the invention comprise a V_H domain comprising the amino acid sequence set forth in SEQ ID NO:17 and a V_L domain comprising the amino acid sequence set forth in SEQ ID NO: 11. In still further embodiments, the anti-CD20 antibodies of the invention comprise a V_H domain comprising the amino acid sequence set forth in SEQ ID NO:18 and a V_L domain comprising the amino acid sequence set forth in SEQ ID NO: 11.

In yet other embodiments, the anti-CD20 antibodies of the invention comprise a V_H domain comprising an amino acid sequence having at least 90% sequence identity to any one of the sequences set forth in SEQ ID NOS:12-18. In some of these embodiments, the anti-CD20 antibodies of the invention further comprise a V_L domain comprising the amino acid sequence set forth in SEQ ID NO:10 or SEQ ID NO:11.

In other embodiments, the anti-CD20 antibodies of the invention comprise the V_H domain set forth in SEQ ID NO:29 (designated H1286) or a V_H domain having at least about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or 100% sequence identity to the V_H domain set forth in SEQ ID NO:29. In some of these embodiments, these anti-CD20 antibodies also comprise a V_L domain selected from SEQ ID NOS:10 and 11 (respectively designated L373 and L419).

In yet other embodiments, the anti-CD20 antibodies of the invention comprise a V_H domain selected from the group consisting of: (a) the V_H domain set forth in SEQ ID NO:29, and (b) a V_H domain having at least about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or 100% sequence identity to the V_H domain set forth in SEQ ID NO:29; and a V_L domain having at least one CDR selected from the group consisting of: (a) a CDR3 comprising the amino acid sequence set forth in SEQ ID NO:8; (b) a CDR3 comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:8, where the CDR3 comprises the glutamine (Gln) residue at the position corresponding to residue 4 of SEQ ID NO:8; and (c) a CDR3 comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:8, where the CDR3 comprises at the position corresponding to residue 4 of SEQ ID NO:8 a residue that is a conservative amino acid substitution for glutamine (Gln). Such anti-CD20 antibodies can optionally comprise a CDR2 comprising the sequence set forth in SEQ ID NO:9.

In still other embodiments, the anti-CD20 antibodies of the invention comprise a V_H domain selected from the group consisting of: (a) the V_H domain set forth in SEQ ID NO:29, and (b) a V_H domain having at least about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or 100% sequence identity to the V_H domain set forth in SEQ ID NO:29; and a V_L domain having at least one CDR selected from the group consisting of: (a) a CDR3 comprising the amino acid sequence set forth in SEQ ID NO:8; (b) a CDR3 comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:8, where the CDR3

comprises the glutamine (Gln) residue at the position corresponding to residue 4 of SEQ ID NO:8; (c) a CDR3 comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:8, where the CDR3 comprises at the position corresponding to residue 4 of SEQ ID NO:8 a residue that is a conservative amino acid substitution for glutamine (Gln); and (d) a CDR2 comprising the amino acid sequence set forth in SEQ ID NO:9.

In some of these embodiments, the anti-CD20 antibodies of the invention comprise a V_H domain selected from the group consisting of: (a) the V_H domain set forth in SEQ ID NO:29, and (b) a V_H domain having at least about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or 100% sequence identity to the V_H domain set forth in SEQ ID NO:29; and a variable light (V_L) domain, where the V_L domain is selected from the group consisting of: (a) a V_L domain comprising the sequence set forth in SEQ ID NO:10 or SEQ ID NO:11; (b) a V_L domain comprising an amino acid sequence having at least 90% sequence identity to the sequence set forth in SEQ ID NO:10 or SEQ ID NO:11; (c) a V_L domain comprising an amino acid sequence having at least 85% sequence identity to the sequence set forth in SEQ ID NO:10, where the V_L domain comprises the CDR2 set forth in SEQ ID NO:9; (d) a V_L domain comprising an amino acid sequence having at least 85% sequence identity to the sequence set forth in SEQ ID NO:11, where the V_L domain comprises at least one of: (i) the CDR2 set forth in SEQ ID NO:9, and (ii) the glutamine (Gln) residue at the position corresponding to residue 91 of SEQ ID NO:11; and (e) a V_L domain comprising an amino acid sequence having at least 85% sequence identity to the sequence set forth in SEQ ID NO:11, where the V_L domain comprises at least one of: (i) the CDR2 set forth in SEQ ID NO:9, and (ii) at the position corresponding to residue 91 of SEQ ID NO:11, a conservative amino acid substitution for glutamine (Gln).

Anti-CD20 antibodies sequences are known in the art. See, for example, U.S. Patent Nos. 5,736,137; 5,776,456; 5,843,439; 5,500,362; 5,677,180; 5,693,493; 5,721,108; 5,736,137; 6,120,767; 5,843,685; 5,576,184; 6,399,061; and, U.S. Patent Application Publication No. 2004/0167319; all of which are herein incorporated by reference. It is recognized that the modifications described here can be made to any

of the anti-CD20 antibodies known in the art or combinations thereof. Thus murine anti-CD20 antibodies, chimeric anti-CD20 antibodies, and humanized anti-CD20 antibodies comprising at least one of the optimized CDRs described herein are contemplated by the present invention. Anti-CD20 antibodies engineered with these modifications and combinations can be tested for the enhanced activity by assays known in the art and described herein. Methods for measuring anti-CD20 antibody binding specificity include, but are not limited to, standard competitive binding assays, assays for monitoring immunoglobulin secretion by B cells, B cell proliferation assays, Banchereau-Like B cell proliferation assays, T cell helper assays for antibody production, co-stimulation of B cell proliferation assays, and assays for up-regulation of B cell activation markers. See, for example, such assays disclosed in WO 00/75348 and U.S. Patent No. 6,087,329. For CDC, ADCC, and apoptosis assays, see, for example, Subbramanian *et al.* (2002) *J. Clin. Microbiol.* 40:2141-2146; Ahman *et al.* (1994) *J. Immunol. Methods* 36:243-254; Brezicka *et al.* (2000) *Cancer Immunol. Immunother.* 49:235-242; Gazzano-Santoro *et al.* (1997) *J. Immunol. Methods* 202:163-171; Prang *et al.* (2005) *British J. Cancer* 92:342-349; Shan *et al.* (1998) *Blood* 92:3756-3771; Ghetie *et al.* (2001) *Blood* 97:1392-1398; and, Mathas *et al.* (2000) *Cancer Research* 60:7170-7176; all of which are herein incorporated by reference.

Particular anti-CD20 antibodies of the invention include a V_H domain having an amino acid sequence selected from one of SEQ ID NOS:12-18 or variants thereof paired with a V_L domain having an amino acid sequence of SEQ ID NO:10 or 11 or variants thereof, in any combination. Anti-CD20 antibodies of the invention include antibodies comprising a V_H domain having an amino acid sequence selected from the group consisting of SEQ ID NOS:12-18 and a V_L domain having an amino acid sequence selected from SEQ ID NO:10 or SEQ ID NO:11.

Suitable biologically active variants of the anti-CD20 antibodies can be used in the methods of the present invention. Such variants will retain the desired binding properties of the parent anti-CD20 antibody. Methods for making antibody variants are generally available in the art.

For example, amino acid sequence variants of an anti-CD20 antibody, an antibody region, for example the CDRs (SEQ ID NOS:1-8), or an antibody variable domain of a heavy or light chain, for example the V_H domain set forth in any one of SEQ ID NOS:12-18 or the V_L domain set forth in SEQ ID NO:10 or SEQ ID NO:11, described herein, can be prepared by mutations in the cloned DNA sequence encoding the amino acid sequence of interest. Methods for mutagenesis and nucleotide sequence alterations are well known in the art. See, for example, Walker and Gaastra, eds. (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York); Kunkel (1985) *Proc. Natl. Acad. Sci. USA* 82:488-492; Kunkel *et al.* (1987) *Methods Enzymol.* 154:367-382; Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor, New York); U.S. Patent No. 4,873,192; and the references cited therein; herein incorporated by reference. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the polypeptide of interest may be found in the model of Dayhoff *et al.* (1978) in *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, D.C.), pp. 345-352, herein incorporated by reference in its entirety. The model of Dayhoff *et al.* uses the Point Accepted Mutation (PAM) amino acid similarity matrix (PAM 250 matrix) to determine suitable conservative amino acid substitutions. Conservative substitutions, such as exchanging one amino acid with another having similar properties, may be preferred. Examples of conservative amino acid substitutions as taught by the PAM 250 matrix of the Dayhoff *et al.* model include, but are not limited to, Gly⇔Ala, Val⇔Ile⇔Leu, Asp⇔Glu, Lys⇔Arg, Asn⇔Gln, and Phe⇔Trp⇔Tyr.

In constructing variants of the anti-CD20 antibody polypeptides of interest, modifications are made such that variants continue to possess the desired properties, i.e., being capable of specifically binding to a human CD20 antigen expressed on the surface of a human cell, and having enhanced function, particularly increased CDC activity and/or increased binding affinity for the CD20 antigen, as described herein. Obviously, any mutations made in the DNA encoding the variant polypeptide must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure. See EP Patent Application Publication No. 75,444.

In addition, the constant region of an anti-CD20 antibody can be mutated to alter effector function in a number of ways. For example, see U.S. Patent No. 6,737,056B1 and U.S. Patent Application Publication No. 2004/0132101A1, which disclose Fc mutations that optimize antibody binding to Fc receptors.

Preferably, variants of a reference anti-CD20 antibody have amino acid sequences that have at least about 80%, about 85%, about 88%, about 90%, about 91%, about 92%, about 93%, about 94%, or about 95% sequence identity to the amino acid sequence for the reference anti-CD20 antibody molecule or to a shorter portion of the reference antibody molecule. More preferably, the molecules share at least about 96%, about 97%, about 98%, or about 99% sequence identity. When discussed herein, whether any particular polypeptide, including the CDRs, V_H domains, and V_L domains disclosed herein, is at least about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or even about 100% identical to another polypeptide can be determined using methods and computer programs/software known in the art such as, but not limited to, the BESTFIT program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). BESTFIT uses the local homology algorithm of Smith and Waterman (1981) *Adv. Appl. Math.* 2:482-489, to find the best segment of homology between two sequences. When using BESTFIT or any other sequence alignment program to determine whether a particular sequence is, for example, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference polypeptide sequence and that gaps in homology of up to 5% of the total number of amino acids in the reference sequence are allowed.

For purposes of the present invention, percent sequence identity is determined using the Smith-Waterman homology search algorithm using an affine gap search with a gap open penalty of 12 and a gap extension penalty of 2, BLOSUM matrix of 62. The Smith-Waterman homology search algorithm is taught in Smith and Waterman (1981) *Adv. Appl. Math.* 2:482-489. A variant may, for example, differ

from the reference anti-CD20 antibody by as few as 1 to 15 amino acid residues, as few as 1 to 10 amino acid residues, such as 6-10, as few as 5, as few as 4, 3, 2, or even 1 amino acid residue.

With respect to optimal alignment of two amino acid sequences, the contiguous segment of the variant amino acid sequence may have additional amino acid residues or deleted amino acid residues with respect to the reference amino acid sequence. The contiguous segment used for comparison to the reference amino acid sequence will include at least 20 contiguous amino acid residues, and may be 30, 40, 50, or more amino acid residues. Corrections for sequence identity associated with conservative residue substitutions or gaps can be made (see Smith-Waterman homology search algorithm).

When any two polypeptide sequences are optimally aligned for comparison, it is recognized that residues appearing opposite of one another within the alignment occupy positions within their respective polypeptides that correspond to one another. Such positions are referred to herein as "corresponding positions" and the residues residing at corresponding positions are referred to as "corresponding residues" or residues that "correspond" to one another. Thus, for example, where a polypeptide of interest is optimally aligned to a reference polypeptide sequence having, for example, 10 residues, the residue within the polypeptide of interest appearing opposite residue 5 of the reference sequence is referred to as the "residue at the position corresponding to residue 5" of the reference sequence.

The precise chemical structure of a polypeptide capable of specifically binding CD20 and retaining the desired increase in CDC activity and/or increased binding affinity for the CD20 antigen depends on a number of factors. As ionizable amino and carboxyl groups are present in the molecule, a particular polypeptide may be obtained as an acidic or basic salt, or in neutral form. All such preparations that retain their biological activity when placed in suitable environmental conditions are included in the definition of anti-CD20 antibodies as used herein. Further, the primary amino acid sequence of the polypeptide may be augmented by derivatization using sugar moieties (glycosylation) or by other supplementary molecules such as lipids, phosphate, acetyl groups and the like. It may also be augmented by conjugation with saccharides. Certain aspects of such augmentation are accomplished through post-

translational processing systems of the producing host; other such modifications may be introduced *in vitro*. In any event, such modifications are included in the definition of an anti-CD20 antibody used herein so long as the desired properties of the anti-CD20 antibody are not destroyed. It is expected that such modifications may quantitatively or qualitatively affect the activity, either by enhancing or diminishing the activity of the polypeptide, in the various assays. Further, individual amino acid residues in the chain may be modified by oxidation, reduction, or other derivatization, and the polypeptide may be cleaved to obtain fragments that retain activity. Such alterations that do not destroy the desired properties (i.e., binding specificity for CD20, and increased CDC activity and/or increased binding affinity for the CD20 antigen) do not remove the polypeptide sequence from the definition of anti-CD20 antibodies of interest as used herein.

The art provides substantial guidance regarding the preparation and use of polypeptide variants. In preparing the anti-CD20 antibody variants, one of skill in the art can readily determine which modifications to the native protein nucleotide or amino acid sequence will result in a variant that is suitable for use as a therapeutically active component of a pharmaceutical composition used in the methods of the present invention.

In certain anti-CD20 antibodies, the Fc portion may be mutated to decrease effector function using techniques known in the art. For example, the deletion or inactivation (through point mutations or other means) of a constant region domain may reduce Fc receptor binding of the circulating modified antibody thereby increasing tumor localization. In other cases it may be that constant region modifications consistent with the instant invention moderate complement binding and thus reduce the serum half life and nonspecific association of a conjugated cytotoxin. Yet other modifications of the constant region may be used to modify disulfide linkages or oligosaccharide moieties that allow for enhanced localization due to increased antigen specificity or antibody flexibility. The resulting physiological profile, bioavailability and other biochemical effects of the modifications, such as tumor localization, biodistribution and serum half-life, may easily be measured and quantified using well known immunological techniques without undue experimentation.

Anti-CD20 antibodies of the invention also include derivatives that are modified, *e.g.*, by the covalent attachment of any type of molecule to the antibody such that covalent attachment does not prevent the antibody from specifically binding to its cognate epitope. For example, but not by way of limitation, the antibody derivatives include antibodies that have been modified, *e.g.*, by glycosylation, acetylation, pegylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, etc. Any of numerous chemical modifications may be carried out by known techniques, including, but not limited to specific chemical cleavage, acetylation, formylation, metabolic synthesis of tunicamycin, etc. Additionally, the derivative may contain one or more non-classical amino acids.

A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a side chain with a similar charge. Families of amino acid residues having side chains with similar charges have been defined in the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Alternatively, mutations can be introduced randomly along all or part of the coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for biological activity to identify mutants that retain activity (*e.g.*, the ability to bind an anti-CD20 polypeptide).

For example, it is possible to introduce mutations only in framework regions or only in CDR regions of an antibody molecule. Introduced mutations may be silent or neutral missense mutations, *i.e.*, have no, or little, effect on an antibody's ability to bind antigen. These types of mutations may be useful to optimize codon usage, or improve a hybridoma's antibody production. Alternatively, non-neutral missense mutations may alter an antibody's ability to bind antigen. The location of most silent and neutral missense mutations is likely to be in the framework regions, while the location of most non-neutral missense mutations is likely to be in CDR, though this is

not an absolute requirement. One of skill in the art would be able to design and test mutant molecules with desired properties such as no alteration in antigen binding activity or alteration in binding activity (*e.g.*, improvements in antigen binding activity or change in antibody specificity). Following mutagenesis, the encoded protein may routinely be expressed and the functional and/or biological activity of the encoded protein, (*e.g.*, ability to immunospecifically bind at least one epitope of a CD20 polypeptide) can be determined using techniques described herein or by routinely modifying techniques known in the art.

IV. POLYNUCLEOTIDES ENCODING ANTI-CD20 ANTIBODIES

The present invention also provides for nucleic acid molecules encoding anti-CD20 antibodies of the invention, or antigen-binding fragments, variants, or derivatives thereof.

In one embodiment, the present invention provides an isolated polynucleotide comprising, consisting essentially of, or consisting of a nucleic acid encoding an immunoglobulin heavy chain variable domain (V_H domain), where at least one of the CDRs of the V_H domain has an amino acid sequence that is at least about 80%, about 85%, about 90%, about 95% , about 96%, about 97%, about 98%, about 99%, or identical to any one of SEQ ID NOS:1-7.

In other embodiments, the present invention provides an isolated polynucleotide comprising, consisting essentially of, or consisting of a nucleic acid encoding an immunoglobulin V_H domain, where at least one of the CDRs of the V_H domain is selected from the group consisting of: (a) a CDR1 comprising the amino acid sequence set forth in SEQ ID NO:7; (b) a CDR1 comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID:7, where the CDR1 comprises the phenylalanine (Phe) residue at the position corresponding to residue 7 of SEQ ID NO:7; (c) a CDR2 comprising the amino acid sequence set forth in SEQ ID NO:5 or SEQ ID NO:6; (d) a CDR2 comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID:5 or SEQ ID NO:6, where the CDR2 comprises the alanine (Ala) or leucine (Leu) residue at the position corresponding to residue 8 of SEQ ID NO:5 or SEQ ID NO:6, respectively; (e) a CDR3 comprising the

amino acid sequence set forth in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4; (f) a CDR3 comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:1, where the CDR3 comprises at least one residue selected from the group consisting of: (i) the asparagine (Asn) residue at the position corresponding to residue 9 of SEQ ID NO:1, and (ii) the asparagine (Asn) residue at the position corresponding to residue 12 of SEQ ID NO:1; (g) a CDR3 comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:2, where the CDR3 comprises at least one residue selected from the group consisting of (i) the alanine (Ala) residue at the position corresponding to residue 5 of SEQ ID NO:2, (ii) the asparagine (Asn) residue at the position corresponding to residue 9 of SEQ ID NO:2, and (iii) the asparagine (Asn) residue at the position corresponding to residue 12 of SEQ ID NO:2; (h) a CDR3 comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:3, where the CDR3 comprises at least one residue selected from the group consisting of: (i) the alanine (Ala) residue at the position corresponding to residue 5 of SEQ ID NO:3, (ii) the asparagine (Asn) residue at the position corresponding to residue 9 of SEQ ID NO:3, and (iii) the aspartic acid (Asp) residue at the position corresponding to residue 12 of SEQ ID NO:3; and (i) a CDR3 comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:4, where the CDR3 comprises at least one residue selected from the group consisting of: (i) the alanine (Ala) residue at the position corresponding to residue 5 of SEQ ID NO:4, (ii) the asparagine (Asn) residue at the position corresponding to residue 9 of SEQ ID NO:4, (iii) the glycine (Gly) residue at the position corresponding to residue 11 of SEQ ID NO:4, and (iv) the asparagine (Asn) residue at the position corresponding to residue 12 of SEQ ID NO:4; wherein an anti-CD20 antibody comprising the encoded V_H domain specifically or preferentially binds to CD20.

In some embodiments, the present invention provides an isolated polynucleotide comprising, consisting essentially of, or consisting of a nucleic acid encoding an immunoglobulin V_H domain, where at least one of the CDRs of the V_H domain is selected from the group consisting of: (a) a CDR1 comprising the sequence

set forth in SEQ ID NO:7; (b) a CDR2 comprising the sequence set forth in SEQ ID NO:5 or SEQ ID NO:6; and (c) a CDR3 comprising the sequence set forth in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4. In particular embodiments, the isolated polynucleotide encoding an immunoglobulin V_H domain comprises at least one of the CDR-encoding sequences selected from the group consisting of SEQ ID NO:24 (encoding optimized CDR3 of SEQ ID NO:1), SEQ ID NO:25 (encoding optimized CDR3 of SEQ ID NO:2), SEQ ID NO:26 (encoding optimized CDR3 of SEQ ID NO:3), and SEQ ID NO:27 (encoding optimized CDR2 of SEQ ID NO:5).

In a further embodiment, the present invention includes an isolated polynucleotide comprising, consisting essentially of, or consisting of a nucleic acid encoding a V_H domain that has an amino acid sequence that is at least about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or 100% identical to a reference V_H domain polypeptide sequence selected from the group consisting of SEQ ID NOS:12-18 and 29, wherein an anti-CD20 antibody comprising the encoded V_H domain specifically or preferentially binds to CD20.

In yet other embodiments, the present invention includes an isolated polynucleotide comprising, consisting essentially of, or consisting of a nucleic acid encoding a V_H domain selected from the group consisting of: (a) a V_H domain comprising an amino acid sequence having at least 90% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOS:12-18 and 29; (b) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:12, where theV_H domain comprises at least one residue selected from the group consisting of: (i) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:12, and (ii) the asparagine (Asn) residue at the position corresponding to residue 110 of SEQ ID NO:12; (c) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:13, where the V_H domain comprises at least one residue selected from the group consisting of: (i) the alanine (Ala) residue at the position corresponding to residue 103 of SEQ ID NO:13, (ii) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:13, and (iii) the asparagine (Asn) residue at the

position corresponding to residue 110 of SEQ ID NO:13; (d) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:14, where the V_H domain comprises at least one residue selected from the group consisting of: (i) the alanine (Ala) residue at the position corresponding to residue 103 of SEQ ID NO:14, (ii) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:14, and (iii) the aspartic acid (Asp) residue at the position corresponding to residue 110 of SEQ ID NO:14; (e) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:15, where the V_H domain comprises at least one residue selected from the group consisting of: (i) the phenylalanine (Phe) residue at the position corresponding to residue 31 of SEQ ID NO:15; (ii) the alanine (Ala) residue at the position corresponding to residue 103 of SEQ ID NO:15; (iii) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:15, and (iv) the asparagine (Asn) residue at the position corresponding to residue 110 of SEQ ID NO:15; (f) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:16, where the V_H domain comprises at least one residue selected from the group consisting of: (i) the alanine (Ala) residue at the position corresponding to residue 57 of SEQ ID NO:16, (ii) the alanine (Ala) residue at the position corresponding to residue 103 of SEQ ID NO:16, (iii) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:16, and (iv) the asparagine (Asn) residue at the position corresponding to residue 110 of SEQ ID NO:16; (g) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:17, where the V_H domain comprises at least one residue selected from the group consisting of: (i) the leucine (Leu) residue at the position corresponding to residue 57 of SEQ ID NO:17, (ii) the alanine (Ala) residue at the position corresponding to residue 103 of SEQ ID NO:17, (iii) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:17, and (iv) the asparagine (Asn) residue at the position corresponding to residue 110 of SEQ ID NO:17; and (h) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:18, where the V_H domain comprises at least one

residue selected from the group consisting of: (i) the alanine (Ala) residue at the position corresponding to residue 103 of SEQ ID NO:18, (ii) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:18, (iii) the glycine (Gly) residue at the position corresponding to residue 109 of SEQ ID NO:18, and (iv) the asparagine (Asn) residue at the position corresponding to residue 110 of SEQ ID NO:18.

In a further embodiment, the present invention includes an isolated polynucleotide comprising, consisting essentially of, or consisting of a nucleic acid encoding a V_H domain, where the nucleic acid has a sequence that has at least about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or 100% sequence identity to a nucleotide sequence selected from the group consisting of SEQ ID NOS:19-22 and wherein an anti-CD20 antibody comprising the encoded V_H domain specifically or preferentially binds to anti-CD20.

In other embodiments, the present invention includes an isolated polynucleotide comprising, consisting essentially of, or consisting of a nucleic acid encoding an immunoglobulin light chain variable domain (V_L domain), where the V_L domain comprises a CDR with an amino acid sequence that is at least about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% or 100% identical to the CDR sequence set forth in SEQ ID NO:8.

In further embodiments, the present invention includes an isolated polynucleotide comprising, consisting essentially of, or consisting of a nucleic acid encoding a V_L domain of an immunoglobulin light chain, where the V_L domain comprises at least one CDR selected from the group consisting of: (a) a CDR3 comprising the amino acid sequence set forth in SEQ ID NO:8; (b) a CDR3 comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:8, where the CDR3 comprises the glutamine (Gln) residue at the position corresponding to residue 4 of SEQ ID NO:8 and (c) a CDR2 having the amino acid sequence set forth in SEQ ID NO:9. In some of these embodiments, the present invention includes an isolated polynucleotide comprising, consisting essentially of, or consisting of a nucleic acid encoding a V_L domain of an immunoglobulin light chain, where the V_L domain comprises a CDR2

having the amino acid sequence set forth in SEQ ID NO:9 and a CDR3 selected from the group consisting of: (a) a CDR3 comprising the amino acid sequence set forth in SEQ ID NO:8; and (b) a CDR3 comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:8, where the CDR3 comprises the glutamine (Gln) residue at the position corresponding to residue 4 of SEQ ID NO:8.

In yet a further embodiment, the present invention includes an isolated polynucleotide comprising, consisting essentially of, or consisting of a nucleic acid encoding a V_L domain that has an amino acid sequence that is at least about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, or 100% identical to a reference V_L domain polypeptide sequence set forth in SEQ ID NO:11, wherein an anti-CD20 antibody comprising the encoded V_L domain specifically or preferentially binds to CD20.

In still other embodiments, the present invention includes an isolated polynucleotide comprising, consisting essentially of, or consisting of a nucleic acid encoding a V_L domain selected from the group consisting of: (a) a V_L domain comprising the sequence set forth in SEQ ID NO:10 or SEQ ID NO:11; (b) a V_L domain comprising an amino acid sequence having at least 90% sequence identity to the sequence set forth in SEQ ID NO:10 or SEQ ID NO:11; (c) a V_L domain comprising an amino acid sequence having at least 85% sequence identity to the sequence set forth in SEQ ID NO:10, where the V_L domain comprises the CDR2 set forth in SEQ ID NO:9; and (d) a V_L domain comprising an amino acid sequence having at least 85% sequence identity to the sequence set forth in SEQ ID NO:11, where the V_L domain comprises at least one of: (i) the CDR2 set forth in SEQ ID NO:9, and (ii) the glutamine (Gln) residue at the position corresponding to residue 91 of SEQ ID NO:11.

Any of the polynucleotides described above may further include additional nucleic acids, encoding, *e.g.*, a signal peptide to direct secretion of the encoded polypeptide, antibody constant regions as described herein, or other heterologous polypeptides as described herein. Also, as described in more detail elsewhere herein, the present invention includes compositions comprising the polynucleotides comprising one or more of the polynucleotides described above. In one embodiment,

the invention includes compositions comprising a first polynucleotide and second polynucleotide wherein said first polynucleotide encodes a V_H domain as described herein and wherein said second polynucleotide encodes a V_L domain as described herein. Specifically a composition which comprises, consists essentially of, or consists of a V_H domain-encoding polynucleotide, as set forth in any one of SEQ ID NOS:19-22, and a V_L domain-encoding polynucleotide, for example, a polynucleotide encoding the V_L domain as set forth in SEQ ID NO:10 or SEQ ID NO:11.

The present invention also includes fragments of the polynucleotides of the invention, as described elsewhere. Additionally polynucleotides that encode fusion polypolypeptides, Fab fragments, and other derivatives, as described herein, are also contemplated by the invention.

The polynucleotides may be produced or manufactured by any method known in the art. For example, if the nucleotide sequence of the antibody is known, a polynucleotide encoding the antibody may be assembled from chemically synthesized oligonucleotides (*e.g.*, as described in Kutmeier *et al.* (1994) *BioTechniques* 17:242), which, briefly, involves the synthesis of overlapping oligonucleotides containing portions of the sequence encoding the antibody, annealing and ligating of those oligonucleotides, and then amplification of the ligated oligonucleotides by PCR.

Alternatively, a polynucleotide encoding an anti-CD20 antibody, or antigen-binding fragment, variant, or derivative thereof, may be generated from nucleic acid from a suitable source. If a clone containing a nucleic acid encoding a particular antibody is not available, but the sequence of the antibody molecule is known, a nucleic acid encoding the antibody may be chemically synthesized or obtained from a suitable source (*e.g.*, an antibody cDNA library, or a cDNA library generated from, or nucleic acid, preferably poly A+RNA, isolated from, any tissue or cells expressing the antibody or other anti-CD20 antibody, such as hybridoma cells selected to express an antibody) by PCR amplification using synthetic primers hybridizable to the 3' and 5' ends of the sequence or by cloning using an oligonucleotide probe specific for the particular gene sequence to identify, *e.g.*, a cDNA clone from a cDNA library that encodes the antibody or other anti-CD20 antibody. Amplified nucleic acids generated by PCR may then be cloned into replicable cloning vectors using any method well known in the art.

Once the nucleotide sequence and corresponding amino acid sequence of the anti-CD20 antibody, or antigen-binding fragment, variant, or derivative thereof is determined, its nucleotide sequence may be manipulated using methods well known in the art for the manipulation of nucleotide sequences, *e.g.*, recombinant DNA techniques, site directed mutagenesis, PCR, etc. (see, for example, the techniques described in Sambrook *et al.* (1990) *Molecular Cloning, A Laboratory Manual* (2nd ed.; Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) and Ausubel *et al.*, eds. (1998) *Current Protocols in Molecular Biology* (John Wiley & Sons, NY), which are both incorporated by reference herein in their entireties), to generate antibodies having a different amino acid sequence, for example to create amino acid substitutions, deletions, and/or insertions.

A polynucleotide encoding an anti-CD20 antibody, or antigen-binding fragment, variant, or derivative thereof, can be composed of any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. For example, a polynucleotide encoding anti-CD20 antibody, or antigen-binding fragment, variant, or derivative thereof can be composed of single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, a polynucleotide encoding an anti-CD20 antibody, or antigen-binding fragment, variant, or derivative thereof can be composed of triple-stranded regions comprising RNA or DNA or both RNA and DNA. A polynucleotide encoding an anti-CD20 antibody, or antigen-binding fragment, variant, or derivative thereof, may also contain one or more modified bases or DNA or RNA backbones modified for stability or for other reasons. "Modified" bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications can be made to DNA and RNA; thus, "polynucleotide" embraces chemically, enzymatically, or metabolically modified forms.

An isolated polynucleotide encoding a non-natural variant of a polypeptide derived from an immunoglobulin (*e.g.*, an immunoglobulin heavy chain portion or light chain portion) can be created by introducing one or more nucleotide

substitutions, additions or deletions into the nucleotide sequence of the immunoglobulin such that one or more amino acid substitutions, additions or deletions are introduced into the encoded protein. Mutations may be introduced by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis. Preferably, conservative amino acid substitutions are made at one or more non-essential amino acid residues.

V. FUSION PROTEINS AND ANTIBODY CONJUGATES

As discussed in more detail elsewhere herein, anti-CD20 antibodies of the invention, or antigen-binding fragments, variants, or derivatives thereof, may further be recombinantly fused to a heterologous polypeptide at the N- or C-terminus or chemically conjugated (including covalent and non-covalent conjugations) to polypeptides or other compositions. For example, anti-CD20 antibodies may be recombinantly fused or conjugated to molecules useful as labels in detection assays and effector molecules such as heterologous polypeptides, drugs, radionuclides, or toxins. See, *e.g.*, PCT publications WO 92/08495; WO 91/14438; WO 89/12624; U.S. Patent No. 5,314,995; and EP 396,387.

Anti-CD20 antibodies of the invention, or antigen-binding fragments, variants, or derivatives thereof, include derivatives that are modified, *i.e.*, by the covalent attachment of any type of molecule to the antibody such that covalent attachment does not prevent the antibody binding CD20. For example, but not by way of limitation, the antibody derivatives include antibodies that have been modified, *e.g.*, by glycosylation, acetylation, pegylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, etc. Any of numerous chemical modifications may be carried out by known techniques, including, but not limited to specific chemical cleavage, acetylation, formylation, metabolic synthesis of tunicamycin, etc. Additionally, the derivative may contain one or more non-classical amino acids.

Anti-CD20 antibodies of the invention, or antigen-binding fragments, variants, or derivatives thereof, can be composed of amino acids joined to each other by peptide bonds or modified peptide bonds, *i.e.*, peptide isosteres, and may contain amino acids other than the 20 gene-encoded amino acids. Anti-CD20 antibodies may

be modified by natural processes, such as posttranslational processing, or by chemical modification techniques that are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature. Modifications can occur anywhere in the anti-CD20 antibody, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini, or on moieties such as carbohydrates. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given anti-CD20 antibody. Also, a given anti-CD20 antibody may contain many types of modifications. Anti-CD20 antibodies may be branched, for example, as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched, and branched cyclic anti-CD20 antibodies may result from posttranslation natural processes or may be made by synthetic methods. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, pegylation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. (See, for instance, *Proteins - Structure and Molecular Properties*, T. E. Creighton, W. H. Freeman and Company, NY; 2nd ed. (1993); Johnson, ed. (1983) *Posttranslational Covalent Modification of Proteins* (Academic Press, NY), pgs. 1-12; Seifter *et al.* (1990) *Meth. Enzymol.* 182:626-646; Rattan *et al.* (1992) *Ann. NY Acad. Sci. 663*:48-62).

The present invention also provides for fusion proteins comprising an anti-CD20 antibody, or antigen-binding fragment, variant, or derivative thereof, and a heterologous polypeptide. The heterologous polypeptide to which the antibody is fused may be useful for function or is useful to target the anti-CD20 polypeptide expressing cells. In one embodiment, a fusion protein of the invention comprises, consists essentially of, or consists of, a polypeptide having the amino acid sequence of

any one or more of the V_H domains of an antibody of the invention or the amino acid sequence of any one or more of the V_L domains of an antibody of the invention or fragments or variants thereof, and a heterologous polypeptide sequence. In another embodiment, a fusion protein for use in the diagnostic and treatment methods disclosed herein comprises, consists essentially of, or consists of a polypeptide having the amino acid sequence of any one, two, three of the CDRs of the V_H domain of an anti-CD20 antibody, or fragments, variants, or derivatives thereof, or the amino acid sequence of any one, two, three of the CDRs of the V_L domain of an anti-CD20 antibody, or fragments, variants, or derivatives thereof, and a heterologous polypeptide sequence. In one embodiment, the fusion protein comprises a polypeptide having the amino acid sequence of a CDR3 of the V_H domain of an anti-CD20-specific antibody of the present invention, or fragment, derivative, or variant thereof, and a heterologous polypeptide sequence, which fusion protein specifically binds to at least one epitope of CD20. In another embodiment, a fusion protein comprises a polypeptide having the amino acid sequence of at least one V_H domain of an anti-CD20 antibody of the invention and the amino acid sequence of at least one V_L domain of an anti-CD20 antibody of the invention or fragments, derivatives or variants thereof, and a heterologous polypeptide sequence. Preferably, the V_H and V_L domains of the fusion protein correspond to a single source antibody (or scFv or Fab fragment) that specifically binds at least one epitope of CD20. In yet another embodiment, a fusion protein for use in the diagnostic and treatment methods disclosed herein comprises a polypeptide having the amino acid sequence of any one, two, three or more of the CDRs of the V_H domain of an anti-CD20 antibody and the amino acid sequence of any one, two, three or more of the CDRs of the V_L domain of an anti-CD20 antibody, or fragments or variants thereof, and a heterologous polypeptide sequence. Preferably, two, three, four, five, six, or more of the CDR(s) of the V_H domain or V_L domain correspond to single source antibody (or scFv or Fab fragment) of the invention. Nucleic acid molecules encoding these fusion proteins are also encompassed by the invention.

Exemplary fusion proteins reported in the literature include fusions of the T cell receptor (Gascoigne *et al.*(1987) *Proc. Natl. Acad. Sci. USA* 84:2936-2940); CD4 (Capon *et al.* (1989) *Nature* 337:525-531; Traunecker *et al.* (1989) *Nature* 339:68-70;

Zettmeissl *et al.* (1990) *DNA Cell Biol. USA* 9:347-353; and Byrn *et al.* (1990) *Nature* 344:667-670); L-selectin (homing receptor) (Watson *et al.* (1990) *J. Cell. Biol.* 110:2221-2229; and Watson *et al.* (1991) *Nature* 349:164-167); CD44 (Aruffo *et al.* (1990) *Cell* 61:1303-1313); CD28 and B7 (Linsley *et al.* (1991) *J. Exp. Med.* 173:721-730); CTLA-4 (Lisley *et al.* (1991) *J. Exp. Med.* 174:561-569); CD22 (Stamenkovic *et al.* (1991) *Cell* 66:1133-1144); TNF receptor (Ashkenazi *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:10535-10539; Lesslauer *et al.* (1991) *Eur. J. Immunol.* 27:2883-2886; and Peppel *et al.* (1991) *J. Exp. Med.* 174:1483-1489); and IgE receptor a (Ridgway and Gorman (1991) *J. Cell. Biol.* Vol. 115, Abstract No. 1448).

As discussed elsewhere herein, anti-CD20 antibodies of the invention, or antigen-binding fragments, variants, or derivatives thereof, may be fused to heterologous polypeptides to increase the *in vivo* half life of the polypeptides or for use in immunoassays using methods known in the art. For example, in one embodiment, PEG can be conjugated to the anti-CD20 antibodies of the invention to increase their half-life *in vivo*. *See* Leong *et al.* (2001) *Cytokine* 16:106; *Adv. in Drug Deliv. Rev.* (2002) 54:531; or Weir *et al.* (2002) *Biochem. Soc. Transactions* 30:512.

Moreover, anti-CD20 antibodies of the invention, or antigen-binding fragments, variants, or derivatives thereof, can be fused to marker sequences, such as a peptide to facilitate their purification or detection. In preferred embodiments, the marker amino acid sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, Calif., 91311), among others, many of which are commercially available. As described in Gentz *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:821-824, for instance, hexa-histidine provides for convenient purification of the fusion protein. Other peptide tags useful for purification include, but are not limited to, the "HA" tag, which corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson *et al.* (1984) *Cell* 37:767) and the "flag" tag.

Fusion proteins can be prepared using methods that are well known in the art (see for example U.S. Patent Nos. 5,116,964 and 5,225,538). The precise site at

which the fusion is made may be selected empirically to optimize the secretion or binding characteristics of the fusion protein. DNA encoding the fusion protein is then transfected into a host cell for expression.

Anti-CD20 antibodies of the present invention, or antigen-binding fragments, variants, or derivatives thereof, may be used in non-conjugated form or may be conjugated to at least one of a variety of molecules, *e.g.*, to improve the therapeutic properties of the molecule, to facilitate target detection, or for imaging or therapy of the patient. Anti-CD20 antibodies of the invention, or antigen-binding fragments, variants, or derivatives thereof, can be labeled or conjugated either before or after purification, when purification is performed.

In particular, anti-CD20 antibodies of the invention, or antigen-binding fragments, variants, or derivatives thereof, may be conjugated to therapeutic agents, prodrugs, peptides, proteins, enzymes, viruses, lipids, biological response modifiers, pharmaceutical agents, or PEG.

Those skilled in the art will appreciate that conjugates may also be assembled using a variety of techniques depending on the selected agent to be conjugated. For example, conjugates with biotin are prepared, *e.g.*, by reacting a binding polypeptide with an activated ester of biotin such as the biotin N-hydroxysuccinimide ester. Similarly, conjugates with a fluorescent marker may be prepared in the presence of a coupling agent, *e.g.* those listed herein, or by reaction with an isothiocyanate, preferably fluorescein-isothiocyanate. Conjugates of the anti-CD20 antibodies of the invention, or antigen-binding fragments, variants, or derivatives thereof, are prepared in an analogous manner.

The present invention further encompasses anti-CD20 antibodies of the invention, or antigen-binding fragments, variants, or derivatives thereof, conjugated to a diagnostic or therapeutic agent. The anti-CD20 antibodies, including antigen-binding fragments, variants, and derivatives thereof, can be used diagnostically to, for example, monitor the development or progression of a disease as part of a clinical testing procedure to, *e.g.*, determine the efficacy of a given treatment and/or prevention regimen. Detection can be facilitated by coupling the anti-CD20 antibody, or antigen-binding fragment, variant, or derivative thereof, to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups,

fluorescent materials, luminescent materials, bioluminescent materials, radioactive materials, positron emitting metals using various positron emission tomographies, and nonradioactive paramagnetic metal ions. See, for example, U.S. Patent No. 4,741,900 for metal ions which can be conjugated to antibodies for use as diagnostics according to the present invention. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin; and examples of suitable radioactive material include ^{125}I, ^{131}I, ^{111}In, ^{90}Y, or ^{99}Tc.

An anti-CD20 antibody, or antigen-binding fragment, variant, or derivative thereof, may be conjugated to a therapeutic moiety such as a cytotoxin, a therapeutic agent or a radioactive metal ion. A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells. Examples include selenium, taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Therapeutic agents include, but are not limited to, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclothosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (e.g., daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (e.g., vincristine and vinblastine). The conjugates of the invention can be used for modifying a given biological response. The drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins

may include, for example, a toxin such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin; a protein such as tumor necrosis factor, alpha-interferon, beta-interferon, nerve growth factor, platelet derived growth factor, tissue plasminogen activator; or, biological response modifiers such as, for example, lymphokines, interleukin-1 ("IL-1"), interleukin-2 ("IL-2"), interleukin-6 ("IL-6"), granulocyte macrophage colony stimulating factor ("GM-CSF"), granulocyte colony stimulating factor ("G-CSF"), or other growth factors.

An anti-CD20 antibody, or antigen-binding fragment, variant, or derivative thereof, also can be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged anti-CD20 antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

One of the ways in which an anti-CD20 antibody, or antigen-binding fragment, variant, or derivative thereof, can be detectably labeled is by linking the same to an enzyme and using the linked product in an enzyme immunoassay (EIA) (Voller, A., "The Enzyme Linked Immunosorbent Assay (ELISA)" *Microbiological Associates Quarterly Publication*, Walkersville, Md.; *Diagnostic Horizons* (1978) 2:1-7; Voller *et al.* (1978) *J. Clin. Pathol. 31*:507-520; Butler (1981) *Meth. Enzymol.* 73:482-523; Maggio, ed. (1980) *Enzyme Immunoassay*, CRC Press, Boca Raton, Fla.; Ishikawa *et al.*, eds. (1981) *Enzyme Immunoassay* (Kgaku Shoin, Tokyo). The enzyme, which is bound to the anti-CD20 antibody will react with an appropriate substrate, preferably a chromogenic substrate, in such a manner as to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorimetric or by visual means. Enzymes which can be used to detectably label the antibody include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate, dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase. Additionally, the detection can be accomplished by colorimetric

methods which employ a chromogenic substrate for the enzyme. Detection may also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

Detection may also be accomplished using any of a variety of other immunoassays. For example, by radioactively labeling the anti-CD20 antibody, or antigen-binding fragment, variant, or derivative thereof, it is possible to detect the antibody through the use of a radioimmunoassay (RIA) (*see*, for example, Weintraub (March, 1986) *Principles of Radioimmunoassays, Seventh Training Course on Radioligand Assay Techniques* (The Endocrine Society), which is incorporated by reference herein). The radioactive isotope can be detected by means including, but not limited to, a gamma counter, a scintillation counter, or autoradiography.

An anti-CD20 antibody, or antigen-binding fragment, variant, or derivative thereof, can also be detectably labeled using fluorescence emitting metals such as 152Eu, or others of the lanthanide series. These metals can be attached to the antibody using such metal chelating groups as diethylenetriaminepentacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

Techniques for conjugating various moieties to an anti-CD20 antibody, or antigen-binding fragment, variant, or derivative thereof, are well known, see, *e.g.*, Arnon *et al.* (1985) "Monoclonal Antibodies for Immunotargeting of Drugs in Cancer Therapy," in *Monoclonal Antibodies and Cancer Therapy*, ed. Reisfeld *et al.* (Alan R. Liss, Inc.), pp. 243-56; Hellstrom *et al.* (1987) "Antibodies for Drug Delivery," in *Controlled Drug Delivery*, ed. Robinson *et al.* (2nd ed.; Marcel Dekker, Inc.), pp. 623-53); Thorpe (1985) "Antibody Carriers of Cytotoxic Agents in Cancer Therapy: A Review," in *Monoclonal Antibodies '84: Biological and Clinical Applications*, ed. Pinchera *et al.*, pp. 475-506; "Analysis, Results, and Future Prospective of the Therapeutic Use of Radiolabeled Antibody in Cancer Therapy," in *Monoclonal Antibodies for Cancer Detection and Therapy*, ed. Baldwin *et al.*, Academic Press, pp. 303-16 (1985); and Thorpe *et al.* (1982) "The Preparation and Cytotoxic Properties of Antibody-Toxin Conjugates," *Immunol. Rev.* 62:119-58.

VI. EXPRESSION OF ANTIBODY POLYPEPTIDES

DNA sequences that encode the light and the heavy chains of the antibody may be made, either simultaneously or separately, using reverse transcriptase and DNA polymerase in accordance with well known methods. PCR may be initiated by consensus constant region primers or by more specific primers based on the published heavy and light chain DNA and amino acid sequences. As discussed above, PCR also may be used to isolate DNA clones encoding the antibody light and heavy chains. In this case the libraries may be screened by consensus primers or larger homologous probes, such as mouse constant region probes.

DNA, typically plasmid DNA, may be isolated from the cells using techniques known in the art, restriction mapped and sequenced in accordance with standard, well known techniques set forth in detail, *e.g.*, in the foregoing references relating to recombinant DNA techniques. Of course, the DNA may be synthetic according to the present invention at any point during the isolation process or subsequent analysis.

Following manipulation of the isolated genetic material to provide anti-CD20 antibodies, or antigen-binding fragments, variants, or derivatives thereof, of the invention, the polynucleotides encoding the anti-CD20 antibodies are typically inserted in an expression vector for introduction into host cells that may be used to produce the desired quantity of anti-CD20 antibody.

Recombinant expression of an antibody, or fragment, derivative or analog thereof, *e.g.*, a heavy or light chain of an antibody that binds to a target molecule described herein, *e.g.*, CD20, requires construction of an expression vector containing a polynucleotide that encodes the antibody. Once a polynucleotide encoding an antibody molecule or a heavy or light chain of an antibody, or portion thereof (preferably containing the heavy or light chain variable domain), of the invention has been obtained, the vector for the production of the antibody molecule may be produced by recombinant DNA technology using techniques well known in the art. Thus, methods for preparing a protein by expressing a polynucleotide containing an antibody encoding nucleotide sequence are described herein. Methods that are well known to those skilled in the art can be used to construct expression vectors containing antibody coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro*

recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. The invention, thus, provides replicable vectors comprising a nucleotide sequence encoding an antibody molecule of the invention, or a heavy or light chain thereof, or a heavy or light chain variable domain, operably linked to a promoter. Such vectors may include the nucleotide sequence encoding the constant region of the antibody molecule (see, *e.g.*, PCT Publication WO 86/05807; PCT Publication WO 89/01036; and U.S. Patent No. 5,122,464) and the variable domain of the antibody may be cloned into such a vector for expression of the entire heavy or light chain.

The term "vector" or "expression vector" is used herein to mean vectors used in accordance with the present invention as a vehicle for introducing into and expressing a desired gene in a host cell. As known to those skilled in the art, such vectors may easily be selected from the group consisting of plasmids, phages, viruses and retroviruses. In general, vectors compatible with the instant invention will comprise a selection marker, appropriate restriction sites to facilitate cloning of the desired gene and the ability to enter and/or replicate in eukaryotic or prokaryotic cells.

For the purposes of this invention, numerous expression vector systems may be employed. For example, one class of vector utilizes DNA elements that are derived from animal viruses such as bovine papilloma virus, polyoma virus, adenovirus, vaccinia virus, baculovirus, retroviruses (RSV, MMTV or MOMLV) or SV40 virus. Others involve the use of polycistronic systems with internal ribosome binding sites. Additionally, cells that have integrated the DNA into their chromosomes may be selected by introducing one or more markers which allow selection of transfected host cells. The marker may provide for prototrophy to an auxotrophic host, biocide resistance (*e.g.*, antibiotics) or resistance to heavy metals such as copper. The selectable marker gene can either be directly linked to the DNA sequences to be expressed, or introduced into the same cell by cotransformation. Additional elements may also be needed for optimal synthesis of mRNA. These elements may include signal sequences, splice signals, as well as transcriptional promoters, enhancers, and termination signals.

In particularly preferred embodiments, the cloned variable region genes are inserted into an expression vector along with the heavy and light chain constant region genes (preferably human) synthesized as discussed above. Of course, any expression vector that is capable of eliciting expression in eukaryotic cells may be used in the present invention. Examples of suitable vectors include, but are not limited to plasmids pcDNA3, pHCMV/Zeo, pCR3.1, pEF1/His, pIND/GS, pRc/HCMV2, pSV40/Zeo2, pTRACER-HCMV, pUB6/V5-His, pVAX1, and pZeoSV2 (available from Invitrogen, San Diego, CA), and plasmid pCI (available from Promega, Madison, WI). In general, screening large numbers of transformed cells for those that express suitably high levels of immunoglobulin heavy and light chains is routine experimentation that can be carried out, for example, by robotic systems.

More generally, once the vector or DNA sequence encoding a monomeric subunit of the anti-CD20 antibody has been prepared, the expression vector may be introduced into an appropriate host cell. Introduction of the plasmid into the host cell can be accomplished by various techniques well known to those of skill in the art. These include, but are not limited to, transfection (including electroporation), protoplast fusion, calcium phosphate precipitation, cell fusion with enveloped DNA, microinjection, and infection with intact virus. *See*, Ridgway (1988) "Mammalian Expression Vectors" in *Vectors*, ed. Rodriguez and Denhardt (Butterworths, Boston, Mass.), Chapter 24.2, pp. 470-472. Typically, plasmid introduction into the host is via electroporation. The host cells harboring the expression construct are grown under conditions appropriate to the production of the light chains and heavy chains, and assayed for heavy and/or light chain protein synthesis. Exemplary assay techniques include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), or fluorescence-activated cell sorter analysis (FACS), immunohistochemistry and the like.

The expression vector is transferred to a host cell by conventional techniques, and the transfected cells are then cultured by conventional techniques to produce an antibody for use in the methods described herein. Thus, the invention includes host cells containing a polynucleotide encoding an antibody of the invention, or a heavy or light chain thereof, operably linked to a heterologous promoter. In preferred

embodiments for the expression of double-chained antibodies, vectors encoding both the heavy and light chains may be co-expressed in the host cell for expression of the entire immunoglobulin molecule, as detailed below.

As used herein, "host cells" refers to cells that harbor vectors constructed using recombinant DNA techniques and encoding at least one heterologous gene. In descriptions of processes for isolation of antibodies from recombinant hosts, the terms "cell" and "cell culture" are used interchangeably to denote the source of antibody unless it is clearly specified otherwise. In other words, recovery of polypeptide from the "cells" may mean either from spun down whole cells, or from the cell culture containing both the medium and the suspended cells.

A variety of host-expression vector systems may be utilized to express antibody molecules for use in the methods described herein. Such host-expression systems represent vehicles by which the coding sequences of interest may be produced and subsequently purified, but also represent cells that may, when transformed or transfected with the appropriate nucleotide coding sequences, express an antibody molecule of the invention *in situ*. These include, but are not limited to, microorganisms such as bacteria (*e.g.*, *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing antibody coding sequences; yeast (*e.g.*, *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors containing antibody coding sequences; insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing antibody coding sequences; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing antibody coding sequences; or mammalian cell systems (*e.g.*, COS, CHO, BLK, 293, 3T3 cells) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the adenovirus late promoter; the vaccinia virus 7.5K promoter). Preferably, bacterial cells such as *Escherichia coli*, and more preferably, eukaryotic cells, especially for the expression of whole recombinant antibody molecule, are used for the expression of a recombinant antibody molecule. For example, mammalian cells such as Chinese

hamster ovary cells (CHO), in conjunction with a vector such as the major intermediate early gene promoter element from human cytomegalovirus is an effective expression system for antibodies (Foecking *et al.* (1986) *Gene* 45:101; Cockett *et al.* (1990) *Bio/Technology* 8:2).

The host cell line used for protein expression is often of mammalian origin; those skilled in the art are credited with ability to preferentially determine particular host cell lines that are best suited for the desired gene product to be expressed therein. Exemplary host cell lines include, but are not limited to, CHO (Chinese Hamster Ovary), DG44 and DUXB11 (Chinese Hamster Ovary lines, DHFR minus), HELA (human cervical carcinoma), CVI (monkey kidney line), COS (a derivative of CVI with SV40 T antigen), VERY, BHK (baby hamster kidney), MDCK, 293, WI38, R1610 (Chinese hamster fibroblast) BALBC/3T3 (mouse fibroblast), HAK (hamster kidney line), SP2/O (mouse myeloma), P3x63-Ag3.653 (mouse myeloma), BFA-1c1BPT (bovine endothelial cells), RAJI (human lymphocyte) and 293 (human kidney). Host cell lines are typically available from commercial services, the American Tissue Culture Collection or from published literature.

In addition, a host cell strain may be chosen that modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells that possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines that stably express the antibody molecule may be engineered. Rather than using expression vectors that contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (*e.g.*, promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker.

Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which stably express the antibody molecule.

A number of selection systems may be used, including, but not limited to, the herpes simplex virus thymidine kinase (Wigler *et al.* (1977) *Cell* 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska and Szybalski (1992) *Proc. Natl. Acad. Sci. USA* 48:202), and adenine phosphoribosyltransferase (Lowy *et al.* (1980) *Cell* 22:817) genes can be employed in tk-, hgprt- or aprt-cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler *et al.* (1980) *Natl. Acad. Sci. USA* 77:357; O'Hare *et al.* (1981) *Proc. Natl. Acad. Sci. USA* 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan and Berg (1981) *Proc. Natl. Acad. Sci. USA* 78:2072); neo, which confers resistance to the aminoglycoside G-418 *Clinical Pharmacy* 12:488-505; Wu and Wu (1991) *Biotherapy* 3:87-95; Tolstoshev (1993) *Ann. Rev. Pharmacol. Toxicol.* 32:573-596; Mulligan (1993) *Science* 260:926-932; and Morgan and Anderson (1993) *Ann. Rev. Biochem.* 62:191-217 (1993); *TIB TECH* 11(5):155-215 (May, 1993); and hygro, which confers resistance to hygromycin (Santerre *et al.* (1984) *Gene* 30:147. Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel *et al.* (1993) *Current Protocols in Molecular Biology* (John Wiley & Sons, NY); Kriegler (1990) "Gene Transfer and Expression" in *A Laboratory Manual* (Stockton Press, NY); Dracopoli *et al.* (eds) (1994) *Current Prolocols in Human Genetics* (John Wiley & Sons, NY) Chapters 12 and 13; Colberre-Garapin *et al.* (1981) *J. Mol. Biol.* 150:1, which are incorporated by reference herein in their entireties.

The expression levels of an antibody molecule can be increased by vector amplification (for a review, *see* Bebbington and Hentschel (1987) "The Use of Vectors Based on Gene Amplification for the Expression of Cloned Genes in

Mammalian Cells in DNA Cloning" (Academic Press, NY) Vol. 3. When a marker in the vector system expressing antibody is amplifiable, an increase in the level of inhibitor present in culture of host cell will increase the number of copies of the marker gene. Since the amplified region is associated with the antibody gene, production of the antibody will also increase (Crouse *et al.* (1983) *Mol. Cell. Biol.* 3:257).

In vitro production allows scale-up to give large amounts of the desired polypeptides. Techniques for mammalian cell cultivation under tissue culture conditions are known in the art and include homogeneous suspension culture, *e.g.* in an airlift reactor or in a continuous stirrer reactor, or immobilized or entrapped cell culture, *e.g.* in hollow fibers, microcapsules, on agarose microbeads or ceramic cartridges. If necessary and/or desired, the solutions of polypeptides can be purified by the customary chromatography methods, for example gel filtration, ion-exchange chromatography, chromatography over DEAE-cellulose or (immuno-)affinity chromatography, *e.g.*, after preferential biosynthesis of a synthetic hinge region polypeptide or prior to or subsequent to HIC chromatography.

Genes encoding anti-CD20 antibodies, or antigen-binding fragments, variants, or derivatives thereof of the invention can also be expressed by non-mammalian cells such as bacteria or yeast or plant cells. Bacteria that readily take up nucleic acids include members of the enterobacteriaceae, such as strains of *Escherichia coli* or *Salmonella*; Bacillaceae, such as *Bacillus subtilis*; *Pneumococcus*; *Streptococcus*, and *Haemophilus influenzae*. It will further be appreciated that, when expressed in bacteria, the heterologous polypeptides typically become part of inclusion bodies. The heterologous polypeptides must be isolated, purified and then assembled into functional molecules. Where tetravalent forms of antibodies are desired, the subunits will then self-assemble into tetravalent antibodies (WO 02/096948A2).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the antibody molecule being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions of an antibody molecule, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the *E. coli* expression

vector pUR278 (Ruther *et al.* (1983) *EMBO J.* 2:1791), in which the antibody coding sequence may be ligated individually into the vector in frame with the lacZ coding region so that a fusion protein is produced; pIN vectors (Inouye and Inouye (1985) *Nucleic Acids Res.* 13:3101-3109; Van Heeke and Schuster (1989) *J. Biol. Chem.* 24:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption and binding to a matrix glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

In addition to prokaryotes, eukaryotic microbes may also be used. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among eukaryotic microorganisms although a number of other strains are commonly available, *e.g.*, *Pichia pastoris*.

For expression in *Saccharomyces*, the plasmid YRp7, for example, (Stinchcomb *et al.* (1979) *Nature* 282:39; Kingsman *et al.* (1979) *Gene* 7:141; Tschemper *et al.* (1980) *Gene* 10:157) is commonly used. This plasmid already contains the TRP1 gene, which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076 or PEP4-1 (Jones (1977) *Genetics* 85:12). The presence of the trpl lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

In an insect system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is typically used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The antibody coding sequence may be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter).

Once an antibody molecule of the invention has been recombinantly expressed, it may be purified by any method known in the art for purification of an immunoglobulin molecule, for example, by chromatography (*e.g.*, ion exchange,

affinity, particularly by affinity for the specific antigen after Protein A, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. Alternatively, a preferred method for increasing the affinity of antibodies of the invention is disclosed in U.S. Patent Application Publication No. 2002 0123057 A1.

VII. TREATMENT METHODS USING THERAPEUTIC ANTI-CD20 ANTIBODIES

Methods of the invention are directed to the use of anti-CD20 antibodies, including antigen-binding fragments, variants, and derivatives thereof, to treat patients having a disease associated with CD20-expressing cells. By "CD20-expressing cell" is intended normal and malignant B cells expressing CD20 antigen. Methods for detecting CD20 expression in cells are well known in the art and include, but are not limited to, PCR techniques, immunohistochemistry, flow cytometry, Western blot, ELISA, and the like. 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, 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.

Though the following discussion refers to diagnostic methods and treatment of various diseases and disorders with an anti-CD20 antibody of the invention, the methods described herein are also applicable to the antigen-binding fragments, variants, and derivatives of these anti-CD20 antibodies that retain the desired properties of the anti-CD20 antibodies of the invention, *i.e.*, capable of specifically binding CD20 and having increased CDC activity and/or increased binding affinity for the CD20 antigen.

"Treatment" is herein defined as the application or administration of an anti-CD20 antibody to a patient, or application or administration of an anti-CD20 antibody to an isolated tissue or cell line from a patient, where the patient has a disease, a symptom of a disease, or a predisposition toward a disease, where the purpose is to

cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve, or affect the disease, the symptoms of the disease, or the predisposition toward the disease. By "treatment" is also intended the application or administration of a pharmaceutical composition comprising the anti-CD20 antibody to a patient, or application or administration of a pharmaceutical composition comprising the anti-CD20 antibody to an isolated tissue or cell line from a patient, who has a disease, a symptom of a disease, or a predisposition toward a disease, where the purpose is to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve, or affect the disease, the symptoms of the disease, or the predisposition toward the disease.

By "anti-tumor activity" is intended a reduction in the rate of malignant CD20-expressing cell proliferation or accumulation, and hence a decline in growth rate of an existing tumor or in a tumor that arises during therapy, and/or destruction of existing neoplastic (tumor) cells or newly formed neoplastic cells, and hence a decrease in the overall size of a tumor during therapy. By "anti-inflammatory activity" is intended a reduction or prevention of inflammation. Therapy with at least one anti-CD20 antibody causes a physiological response that is beneficial with respect to treatment of disease states associated with CD20-expressing cells in a human.

In this manner, the methods of the invention find use in the treatment of non-Hodgkin's lymphomas related to abnormal, uncontrollable B cell proliferation or accumulation. For purposes of the present invention, such lymphomas will be referred to according to the *Working Formulation* classification scheme, that is those B cell lymphomas categorized as low grade, intermediate grade, and high grade (see "The Non-Hodgkin's Lymphoma Pathologic Classification Project" in *Cancer* 49:2112-2135 (1982)). Thus, low-grade B cell lymphomas include small lymphocytic, follicular small-cleaved cell, and follicular mixed small-cleaved and large cell lymphomas; intermediate-grade lymphomas include follicular large cell, diffuse small cleaved cell, diffuse mixed small and large cell, and diffuse large cell lymphomas; and high-grade lymphomas include large cell immunoblastic, lymphoblastic, and small non-cleaved cell lymphomas of the Burkitt's and non-Burkitt's type.

It is recognized that the methods of the invention are useful in the therapeutic treatment of B cell lymphomas that are classified according to the Revised European and American Lymphoma Classification (REAL) system. Such B cell lymphomas include, but are not limited to, lymphomas classified as precursor B cell neoplasms, such as B lymphoblastic leukemia/lymphoma; peripheral B cell neoplasms, including B cell chronic lymphocytic leukemia/small lymphocytic lymphoma, lymphoplasmacytoid lymphoma/immunocytoma, mantle cell lymphoma (MCL), follicle center lymphoma (follicular) (including diffuse small cell, diffuse mixed small and large cell, and diffuse large cell lymphomas), marginal zone B cell lymphoma (including extranodal, nodal, and splenic types), hairy cell leukemia, plasmacytoma/ myeloma, diffuse large cell B cell lymphoma of the subtype primary mediastinal (thymic), Burkitt's lymphoma, and Burkitt's like high grade B cell lymphoma; acute leukemias; acute lymphocytic leukemias; myeloblastic leukemias; acute myelocytic leukemias; promyelocytic leukemia; myelomonocytic leukemia; monocytic leukemia; erythroleukemia; granulocytic leukemia (chronic myelocytic leukemia); chronic lymphocytic leukemia; polycythemia vera; multiple myeloma; Waldenstrom's macroglobulinemia; heavy chain disease; and unclassifiable low-grade or high-grade B cell lymphomas.

It is recognized that the methods of the invention may be useful in preventing further tumor outgrowths arising during therapy. The methods of the invention are particularly useful in the treatment of subjects having low-grade B cell lymphomas, particularly those subjects having relapses following standard chemotherapy. Low-grade B cell lymphomas are more indolent than the intermediate- and high-grade B cell lymphomas and are characterized by a relapsing/remitting course. Thus, treatment of these lymphomas is improved using the methods of the invention, as relapse episodes are reduced in number and severity.

In accordance with the methods of the present invention, at least one anti-CD20 antibody as defined elsewhere herein is used to promote a positive therapeutic response with respect to a malignant human B cell. By "positive therapeutic response" with respect to cancer treatment is intended an improvement in the disease in association with the anti-tumor activity of these antibodies or fragments thereof, and/or an improvement in the symptoms associated with the disease. That is, an anti-

proliferative effect, the prevention of further tumor outgrowths, a reduction in tumor size, a reduction in the number of cancer cells, and/or a decrease in one or more symptoms associated with the disease can be observed. Thus, for example, an improvement in the disease may be characterized as a complete response. By "complete response" is intended an absence of clinically detectable disease with normalization of any previously abnormal radiographic studies, bone marrow, and cerebrospinal fluid (CSF). Such a response must persist for at least one month following treatment according to the methods of the invention. Alternatively, an improvement in the disease may be categorized as being a partial response. By "partial response" is intended at least about a 50% decrease in all measurable tumor burden (i.e., the number of tumor cells present in the subject) in the absence of new lesions and persisting for at least one month. Such a response is applicable to measurable tumors only.

Tumor response can be assessed for changes in tumor morphology (i.e., overall tumor burden, tumor size, and the like) using screening techniques such as magnetic resonance imaging (MRI) scan, x-radiographic imaging, computed tomographic (CT) scan, bioluminescent imaging, for example, luciferase imaging, bone scan imaging, and tumor biopsy sampling including bone marrow aspiration (BMA). In addition to these positive therapeutic responses, the subject undergoing therapy with the anti-CD20 antibody may experience the beneficial effect of an improvement in the symptoms associated with the disease. Thus for B cell tumors, the subject may experience a decrease in the so-called B symptoms, i.e., night sweats, fever, weight loss, and/or urticaria.

The anti-CD20 antibodies described herein may also find use in the treatment of inflammatory diseases and deficiencies or disorders of the immune system that are associated with CD-20 expressing cells, including, but not limited to, systemic lupus erythematosus, psoriasis, scleroderma, CREST syndrome, inflammatory myositis, Sjogren's syndrome, mixed connective tissue disease, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, acute respiratory distress syndrome, pulmonary inflammation, idiopathic pulmonary fibrosis, osteoporosis, delayed type hypersensitivity, asthma, primary biliary cirrhosis, and idiopathic thrombocytopenic purpura.

Inflammatory diseases are characterized by inflammation and tissue destruction, or a combination thereof. "Inflammatory disease" includes any inflammatory immune-mediated process where the initiating event or target of the immune response involves non-self antigen(s), including, for example, alloantigens, xenoantigens, viral antigens, bacterial antigens, unknown antigens, or allergens.

Further, for purposes of the present invention, the term "inflammatory disease(s)" includes "autoimmune disease(s)." As used herein, the term "autoimmunity" is generally understood to encompass inflammatory immune-mediated processes involving "self" antigens. In autoimmune diseases, self antigen(s) trigger host immune responses.

Also, the present invention includes treatment of inflammation associated with tissue transplant rejection. "Transplant rejection" or "graft rejection" refers to any host-mounted immune response against a graft including but not limited to HLA antigens, blood group antigens, and the like.

The invention can also be used to treat graft versus host disease, such as that associated with bone marrow transplantation, for example. In such graft versus host disease, the donor bone marrow includes lymphocytes and cells that mature into lymphocytes. The donor's lymphocytes recognize the recipient's antigens as non-self and mount an inflammatory immune response. Hence, as used herein, "graft versus host disease" or "graft versus host reaction" refers to any T cell mediated immune response in which donor lymphocytes react to the host's antigens.

The anti-CD20 described herein can be used in accordance with the methods of the invention to treat autoimmune and/or inflammatory disorders including, but not limited to, systemic lupus erythematosus (SLE), discoid lupus, lupus nephritis, sarcoidosis, inflammatory arthritis, including juvenile arthritis, rheumatoid arthritis, psoriatic arthritis, Reiter's syndrome, ankylosing spondylitis, and gouty arthritis, rejection of an organ or tissue transplant, hyperacute, acute, or chronic rejection and/or graft versus host disease, multiple sclerosis, hyper IgE syndrome, polyarteritis nodosa, primary biliary cirrhosis, inflammatory bowel disease, Crohn's disease, celiac's disease (gluten-sensitive enteropathy), autoimmune hepatitis, pernicious anemia, autoimmune hemolytic anemia, psoriasis, scleroderma, myasthenia gravis,

autoimmune thrombocytopenic purpura, autoimmune thyroiditis, Grave's disease, Hashimoto's thyroiditis, immune complex disease, chronic fatigue immune dysfunction syndrome (CFIDS), polymyositis and dermatomyositis, cryoglobulinemia, thrombolysis, cardiomyopathy, pemphigus vulgaris, pulmonary interstitial fibrosis, Type I and Type II diabetes mellitus, type 1, 2, 3, and 4 delayed-type hypersensitivity, allergy or allergic disorders, unwanted/unintended immune responses to therapeutic proteins (see for example, U.S. Patent Application No. US 2002/0119151 and Koren, *et al.* (2002) *Curr. Pharm. Biotechnol.* 3:349-60), asthma, Churg-Strauss syndrome (allergic granulomatosis), atopic dermatitis, allergic and irritant contact dermatitis, urtecaria, IgE-mediated allergy, atherosclerosis, vasculitis, idiopathic inflammatory myopathies, hemolytic disease, Alzheimer's disease, chronic inflammatory demyelinating polyneuropathy, and the like. In some other embodiments, the anti-CD20 antibodies of the invention are useful in treating pulmonary inflammation including but not limited to lung graft rejection, asthma, sarcoidosis, emphysema, cystic fibrosis, idiopathic pulmonary fibrosis, chronic bronchitis, allergic rhinitis and allergic diseases of the lung such as hypersensitivity pneumonitis, eosinophilic pneumonia, bronchiolitis obliterans due to bone marrow and/or lung transplantation or other causes, graft atherosclerosis/graft phlebosclerosis, as well as pulmonary fibrosis resulting from collagen, vascular, and autoimmune diseases such as rheumatoid arthritis and lupus erythematosus.

In accordance with the methods of the present invention, at least one anti-CD20 antibody as defined elsewhere herein is used to promote a positive therapeutic response with respect to treatment or prevention of an autoimmune disease and/or inflammatory disease. By "positive therapeutic response" with respect to an autoimmune disease and/or inflammatory disease is intended an improvement in the disease in association with the anti-inflammatory activity of these antibodies, and/or an improvement in the symptoms associated with the disease. That is, an anti-proliferative effect, the prevention of further proliferation of the CD20-expressing cell, a reduction in the inflammatory response including but not limited to reduced secretion of inflammatory cytokines, adhesion molecules, proteases, immunoglobulins (in instances where the CD20 bearing cell is a B cell), combinations thereof, and the like, increased production of anti-inflammatory proteins, a reduction in the number of

autoreactive cells, an increase in immune tolerance, inhibition of autoreactive cell survival, and/or a decrease in one or more symptoms mediated by stimulation of CD20-expressing cells can be observed. Such positive therapeutic responses are not limited to the route of administration and may comprise administration to the donor, the donor tissue (such as for example organ perfusion), the host, any combination thereof, and the like.

Clinical response can be assessed using screening techniques such as magnetic resonance imaging (MRI) scan, x-radiographic imaging, computed tomographic (CT) scan, flow cytometry or fluorescence-activated cell sorter (FACS) analysis, histology, gross pathology, and blood chemistry, including but not limited to changes detectable by ELISA, RIA, chromatography, and the like. In addition to these positive therapeutic responses, the subject undergoing therapy with the anti-CD20 antibody or antigen-binding fragment thereof may experience the beneficial effect of an improvement in the symptoms associated with the disease.

By "therapeutically effective dose or amount" or "effective amount" is intended an amount of anti-CD20 that when administered brings about a positive therapeutic response with respect to treatment of a patient with a disease associated with CD20-expressing cells. In some embodiments of the invention, a therapeutically effective dose of the anti-CD20 antibody is in the range from about 0.01 mg/kg to about 40 mg/kg, from about 0.01 mg/kg to about 30 mg/kg, from about 0.1 mg/kg to about 30 mg/kg, from about 1 mg/kg to about 30 mg/kg, from about 3 mg/kg to about 30 mg/kg, from about 3 mg/kg to about 25 mg/kg, from about 3 mg/kg to about 20 mg/kg, from about 5 mg/kg to about 15 mg/kg, or from about 7 mg/kg to about 12 mg/kg. It is recognized that the method of treatment may comprise a single administration of a therapeutically effective dose or multiple administrations of a therapeutically effective dose of the anti-CD20 antibody.

The anti-CD20 antibodies can be used in combination with known chemotherapeutics and cytokines for the treatment of disease states comprising CD20-expressing cells. For example, the anti-CD20 antibodies of the invention can be used in combination with cytokines such as interleukin-2. In another embodiment, the anti-CD20 antibodies of the invention can be used in combination with rituximab (IDEC-C2B8; Rituxan®; IDEC Pharmaceuticals Corp., San Diego, California).

In this manner, the anti-CD20 antibodies described herein are administered in combination with at least one other cancer therapy, including, but not limited to, surgery or surgical procedures (e.g. splenectomy, hepatectomy, lymphadenectomy, leukophoresis, bone marrow transplantation, and the like); radiation therapy; chemotherapy, optionally in combination with autologous bone marrow transplant, where suitable chemotherapeutic agents include, but are not limited to, fludarabine or fludarabine phosphate, chlorambucil, vincristine, pentostatin, 2-chlorodeoxyadenosine (cladribine), cyclophosphamide, doxorubicin, prednisone, and combinations thereof, for example, anthracycline-containing regimens such as CAP (cyclophosphamide, doxorubicin plus prednisone), CHOP (cyclophosphamide, vincristine, prednisone plus doxorubicin), VAD (vincritsine, doxorubicin, plus dexamethasone), MP (melphalan plus prednisone), and other cytotoxic and/or therapeutic agents used in chemotherapy such as mitoxantrone, daunorubicin, idarubicin, asparaginase, and antimetabolites, including, but not limited to, cytarabine, methotrexate, 5-fluorouracil decarbazine, 6-thioguanine, 6-mercaptopurine, and nelarabine; other anti-cancer monoclonal antibody therapy (for example, alemtuzumab (Campath®) or other anti-CD52 antibody targeting the CD52 cell-surface glycoprotein on malignant B cells; rituximab (Rituxan®), the fully human antibody HuMax-CD20, R-1594, IMMU-106, TRU-015, AME-133, tositumomab/I-131 tositumomab (Bexxar®), ibritumomab tiuxetan (Zevalin®), or any other therapeutic anti-CD20 antibody targeting the CD20 antigen on malignant B cells; anti-CD19 antibody (for example, MT103, a bispecific antibody); anti-CD22 antibody (for example, the humanized monoclonal antibody epratuzumab); bevacizumab (Avastin®) or other anti-cancer antibody targeting human vascular endothelial growth factor; anti-CD22 antibody targeting the CD22 antigen on malignant B cells (for example, the monoclonal antibody BL-22, an alphaCD22 toxin); α-M-CSF antibody targeting macrophage colony stimulating factor; antibodies targeting the receptor activator of nuclear factor-kappaB (RANK) and its ligand (RANKL), which are overexpressed in multiple myeloma; anti-CD23 antibody targeting the CD23 antigen on malignant B cells (for example, IDEC-152); anti-CD80 antibody targeting the CD80 antigen (for example, IDEC-114); anti-CD38 antibody targeting the CD38 antigen on malignant B cells; antibodies targeting major histocompatibility complex class II receptors (anti-MHC antibodies) expressed on

malignant B cells; anti-CD40 antibodies (for example, SGN-40) targeting the CD40 antigen on malignant B cells; and antibodies targeting tumor necrosis factor-related apoptosis-inducing ligand receptor 1 (TRAIL-R1) (for example, the agonistic human monoclonal antibody HGS-ETR1) and TRAIL-R2 expressed on a number of solid tumors and tumors of hematopoietic origin); small molecule-based cancer therapy, including, but not limited to, microtubule and/or topoisomerase inhibitors (for example, the mitotic inhibitor dolastatin and dolastatin analogues; the tubulin-binding agent T900607; XL119; and the topoisomerase inhibitor aminocamptothecin), SDX-105 (bendamustine hydrochloride), ixabepilone (an epothilone analog, also referred to as BMS-247550), protein kinase C inhibitors, for example, midostaurin ((PKC-412, CGP 41251, N-benzoylstaurosporine), pixantrone, eloxatin (an antineoplastic agent), ganite (gallium nitrate), Thalomid® (thalidomide), immunomodulatory derivatives of thalidomide (for example, revlimid (formerly revimid)), Affinitak™ (antisense inhibitor of protein kinase C-alpha), SDX-101 (R-etodolac, inducing apoptosis of malignant lymphocytes), second-generation purine nucleoside analogs such as clofarabine, inhibitors of production of the protein Bcl-2 by cancer cells (for example, the antisense agents oblimersen and Genasense®), proteasome inhibitors (for example, Velcade™ (bortezomib)), small molecule kinase inhibitors (for example, CHIR-258), small molecule VEGF inhibitors (for example, ZD-6474), small molecule inhibitors of heat shock protein (HSP) 90 (for example, 17-AAG), small molecule inhibitors of histone deacetylases (for example, hybrid/polar cytodifferentiation HPC) agents such as suberanilohydroxamic acid (SAHA), and FR-901228) and apoptotic agents such as Trisenox® (arsenic trioxide) and Xcytrin® (motexafin gadolinium); vaccine /immunotherapy-based cancer therapies, including, but not limited to, vaccine approaches (for example, Id-KLH, oncophage, vitalethine), personalized immunotherapy or active idiotype immunotherapy (for example, MyVax® Personalized Immunotherapy, formally designated GTOP-99), Promune® (CpG 7909, a synthetic agonist for toll-like receptor 9 (TLR9)), interferon-alpha therapy, interleukin-2 (IL-2) therapy, IL-12 therapy, IL-15 therapy, and IL-21 therapy; steroid therapy; or other cancer therapy; where the additional cancer therapy is administered prior to, during, or subsequent to the anti-CD20 antibody therapy.

Thus, where the combined therapies comprise administration of an anti-CD20 antibody in combination with administration of another therapeutic agent, as with chemotherapy, radiation therapy, other anti-cancer antibody therapy, small molecule-based cancer therapy, or vaccine/immunotherapy-based cancer therapy, the methods of the invention encompass coadministration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order. Where the methods of the present invention comprise combined therapeutic regimens, these therapies can be given simultaneously, i.e., the anti-CD20 antibody is administered concurrently or within the same time frame as the other cancer therapy (i.e., the therapies are going on concurrently, but the anti-CD20 antibody is not administered precisely at the same time as the other cancer therapy). Alternatively, the anti-CD20 antibody of the present invention may also be administered prior to or subsequent to the other cancer therapy. Sequential administration of the different cancer therapies may be performed regardless of whether the treated subject responds to the first course of therapy to decrease the possibility of remission or relapse. Where the combined therapies comprise administration of the anti-CD20 antibody in combination with administration of a cytotoxic agent, preferably the anti-CD20 antibody is administered prior to administering the cytotoxic agent.

In some embodiments of the invention, the anti-CD20 antibodies described herein are administered in combination with chemotherapy, and optionally in combination with autologous bone marrow transplantation, wherein the antibody and the chemotherapeutic agent(s) may be administered sequentially, in either order, or simultaneously (i.e., concurrently or within the same time frame). Examples of suitable chemotherapeutic agents include, but are not limited to, fludarabine or fludarabine phosphate, chlorambucil, vincristine, pentostatin, 2-chlorodeoxyadenosine (cladribine), cyclophosphamide, doxorubicin, prednisone, and combinations thereof, for example, anthracycline-containing regimens such as CAP (cyclophosphamide, doxorubicin plus prednisone), CHOP (cyclophosphamide, vincristine, prednisone plus doxorubicin), VAD (vincritsine, doxorubicin, plus dexamethasone), MP (melphalan plus prednisone), and other cytotoxic and/or therapeutic agents used in chemotherapy such as mitoxantrone, daunorubicin, idarubicin, asparaginase, and antimetabolites, including, but not limited to, cytarabine, methotrexate, 5-fluorouracil decarbazine, 6-

thioguanine, 6-mercaptopurine, and nelarabine. In some embodiments, the anti-CD20 antibody is administered prior to treatment with the chemotherapeutic agent. In alternative embodiments, the anti-CD20 antibody is administered after treatment with the chemotherapeutic agent. In yet other embodiments, the chemotherapeutic agent is administered simultaneously with the anti-CD20 antibody.

Thus, for example, in some embodiments, the anti-CD20 antibody is administered in combination with fludarabine or fludarabine phosphate. In one such embodiment, the anti-CD20 antibody is administered prior to administration of fludarabine or fludarabine phosphate. In alternative embodiments, the anti-CD20 antibody is administered after treatment with fludarabine or fludarabine phosphate. In yet other embodiments, the fludarabine or fludarabine phosphate is administered simultaneously with the anti-CD20 antibody.

In other embodiments of the invention, chlorambucil, an alkylating drug, is administered in combination with an anti-CD20 antibody described herein. In one such embodiment, the anti-CD20 antibody is administered prior to administration of chlorambucil. In alternative embodiments, the anti-CD20 antibody is administered after treatment with chlorambucil. In yet other embodiments, the chlorambucil is administered simultaneously with the anti-CD20 antibody.

In yet other embodiments, anthracycline-containing regimens such as CAP (cyclophosphamide, doxorubicin plus prednisone) and CHOP (cyclophosphamide, vincristine, prednisone plus doxorubicin) may be combined with administration of an anti-CD20 antibody described herein. In one such embodiment, the anti-CD20 antibody is administered prior to administration of anthracycline-containing regimens. In other embodiments, the anti-CD20 antibody is administered after treatment with anthracycline-containing regimens. In yet other embodiments, the anthracycline-containing regimen is administered simultaneously with the anti-CD20 antibody.

In alternative embodiments, an anti-CD20 antibody described herein is administered in combination with alemtuzumab (Campath®; distributed by Berlex Laboratories, Richmond, California). Alemtuzumab is a recombinant humanized monoclonal antibody (Campath-1H) that targets the CD52 antigen expressed on malignant B cells. In one such embodiment, the anti-CD20 antibody is administered prior to administration of alemtuzumab. In other embodiments, the anti-CD20

antibody is administered after treatment with alemtuzumab. In yet other embodiments, the alemtuzumab is administered simultaneously with the anti-CD20 antibody.

In alternative embodiments, an anti-CD20 antibody described herein is administered in combination with another therapeutic anti-CD20 antibody targeting the CD20 antigen on malignant B cells, for example, rituximab (Rituxan®), the fully human antibody HuMax-CD20, R-1594, IMMU-106, TRU-015, AME-133, tositumomab/I-131 tositumomab (Bexxar®), or ibritumomab tiuxetan (Zevalin®). In one such embodiment, the anti-CD20 antibody of the invention is administered prior to administration of the other anti-CD20 antibody. In other embodiments, the anti-CD20 antibody of the invention is administered after treatment with the other anti-CD20 antibody. In yet other embodiments, the anti-CD20 antibody of the invention is administered simultaneously with the other anti-CD20 antibody.

In alternative embodiments, an anti-CD20 antibody described herein is administered in combination with a small molecule-based cancer therapy, including, but not limited to, microtubule and/or topoisomerase inhibitors (for example, the mitotic inhibitor dolastatin and dolastatin analogues; the tubulin-binding agent T900607; XL119; and the topoisomerase inhibitor aminocamptothecin), SDX-105 (bendamustine hydrochloride), ixabepilone (an epothilone analog, also referred to as BMS-247550), protein kinase C inhibitors, for example, midostaurin ((PKC-412, CGP 41251, N-benzoylstaurosporine), pixantrone, eloxatin (an antineoplastic agent), ganite (gallium nitrate), Thalomid® (thalidomide), immunomodulatory derivatives of thalidomide (for example, revlimid (formerly revimid)), Affinitak™ (antisense inhibitor of protein kinase C-alpha), SDX-101 (R-etodolac, inducing apoptosis of malignant lymphocytes), second-generation purine nucleoside analogs such as clofarabine, inhibitors of production of the protein Bcl-2 by cancer cells (for example, the antisense agents oblimersen and Genasense®), proteasome inhibitors (for example, Velcade™ (bortezomib)), small molecule kinase inhibitors (for example, CHIR-258), small molecule VEGF inhibitors (for example, ZD-6474), small molecule inhibitors of heat shock protein (HSP) 90 (for example, 17-AAG), small molecule inhibitors of histone deacetylases (for example, hybrid/polar cytodifferentiation HPC) agents such as suberanilohydroxamic acid (SAHA), and FR-901228) and apoptotic agents such as

Trisenox® (arsenic trioxide) and Xcytrin® (motexafin gadolinium). In one such embodiment, the anti-CD20 antibody is administered prior to administration of the small molecule-based cancer therapy. In other embodiments, the anti-CD20 antibody is administered after treatment with the small molecule-based cancer therapy. In yet other embodiments, the small molecule-based cancer therapy is administered simultaneously with the anti-CD20 antibody.

In yet other embodiments, an anti-CD20 antibody described herein can be used in combination with vaccine/immunotherapy-based cancer therapy, including, but not limited to, vaccine approaches (for example, Id-KLH, oncophage, vitalethine), personalized immunotherapy or active idiotype immunotherapy (for example, MyVax® Personalized Immunotherapy, formally designated GTOP-99), Promune® (CpG 7909, a synthetic agonist for toll-like receptor 9 (TLR9)), interferon-alpha therapy, interleukin-2 (IL-2) therapy, IL-12 therapy, IL-15 therapy, or IL-21 therapy; or steroid therapy. In one such embodiment, the anti-CD20 antibody is administered prior to administration of the vaccine/immunotherapy-based cancer therapy. In other embodiments, the anti-CD20 antibody is administered after treatment with the vaccine/immunotherapy-based cancer therapy. In yet other embodiments, the vaccine/immunotherapy-based cancer therapy is administered simultaneously with the anti-CD20 antibody.

In one such embodiment, an anti-CD20 antibody described herein can be used in combination with IL-2. IL-2, an agent known to expand the number of natural killer (NK) effector cells in treated patients, can be administered prior to, or concomitantly with, the anti-CD20 antibody of the invention. This expanded number of NK effector cells may lead to enhanced ADCC activity of the administered anti-CD20 antibody. In other embodiments, IL-21 serves as the immunotherapeutic agent to stimulate NK cell activity when administered in combination with an anti-CD20 antibody described herein.

The anti-CD20 antibodies of the invention can be used in combination with any known therapies for autoimmune and inflammatory diseases, including any agent or combination of agents that are known to be useful, or which have been used or are currently in use, for treatment of autoimmune and inflammatory diseases. Such therapies and therapeutic agents include, but are not limited to, surgery or surgical

procedures (e.g., splenectomy, lymphadenectomy, thyroidectomy, plasmaphoresis, leukophoresis, cell, tissue, or organ transplantation, intestinal procedures, organ perfusion, and the like), radiation therapy, therapy such as steroid therapy and non-steroidal therapy, hormone therapy, cytokine therapy, therapy with dermatological agents (for example, topical agents used to treat skin conditions such as allergies, contact dermatitis, and psoriasis), immunosuppressive therapy, and other anti-inflammatory monoclonal antibody therapy, and the like. In this manner, the anti-CD20 antibodies described herein are administered in combination with at least one other therapy, including, but not limited to, surgery, organ perfusion, radiation therapy, steroid therapy, non-steroidal therapy, antibiotic therapy, antifungal therapy, hormone therapy, cytokine therapy, therapy with dermatological agents (for example, topical agents used to treat skin conditions such as allergies, contact dermatitis, and psoriasis), immunosuppressive therapy, other anti-inflammatory monoclonal antibody therapy, combinations thereof, and the like. Thus, where the combined therapies comprise administration of an anti-CD20 antibody in combination with administration of another therapeutic agent, as with steroids as one example, the methods of the invention encompass coadministration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order.

Where the methods of the present invention comprise combined therapeutic regimens, these therapies can be given simultaneously, i.e., the anti-CD20 antibody is administered concurrently or within the same time frame as the other therapy (i.e., the therapies are going on concurrently, but the anti-CD20 antibody is not administered precisely at the same time as the other therapy). Alternatively, the anti-CD20 antibody of the present invention may also be administered prior to or subsequent to the other therapy. Sequential administration of the different therapies may be performed regardless of whether the treated subject responds to the first course of therapy to decrease the possibility of remission or relapse.

In some embodiments of the invention, the anti-CD20 antibodies described herein are administered in combination with immunosuppressive drugs or anti-inflammatory drugs, wherein the antibody and the therapeutic agent(s) may be administered sequentially, in either order, or simultaneously (i.e., concurrently or within the same time frame). Examples of suitable immunosuppressive drugs that can

be administered in combination with the anti-CD20 antibodies of the invention include, but are not limited to, methotrexate, cyclophosphamide, mizoribine, chlorambucil, cyclosporine, such as, for example, aerosolized cyclosporine (see, U.S. Patent Application Publication No. US20020006901, herein incorporated by reference in its entirety), tacrolimus (FK506; ProGraf™), mycophenolate mofetil, and azathioprine (6-mercaptopurine), sirolimus (rapamycin), deoxyspergualin, leflunomide and its malononitriloamide analogs; and immunosuppressive proteins, including, for example, anti-CTLA4 antibodies and Ig fusions, anti-B lymphocyte stimulator antibodies (e.g., LYMPHOSTAT-B™) and Ig fusions (BLyS-Ig), anti-CD80 antibodies and etanercept (Enbrel®), as well as anti-T cell antibodies such as anti-CD3 (OKT3), anti-CD4, and the like. Examples of suitable anti-inflammatory agents include, but are not limited to, corticosteroids such as, for example, clobetasol, halobetasol, hydrocortisone, triamcinolone, betamethasone, fluocinole, fluocinonide, prednisone, prednisolone, methylprednisolone; non-steroidal anti-inflammatory drugs (NSAIDs) such as, for example, sulfasalazine, medications containing mesalamine (known as 5-ASA agents), celecoxib, diclofenac, etodolac, fenprofen, flurbiprofen, ibuprofen, ketoprofen, meclofamate, meloxicam , nabumetone, naproxen, oxaprozin, piroxicam, rofecoxib, salicylates, sulindac, and tolmetin; anti-inflammatory antibodies such as adalimumab (HUMIRA®, a TNF-α antagonist) and infliximab (Remicade®, a TNF-α antagonist), and the like.

Transplant rejection and graft versus host disease can be hyperacute (humoral), acute (T cell mediated), or chronic (unknown etiology), or a combination thereof. Thus, the anti-CD20 antibodies of the invention are used in some embodiments to prevent and/or ameliorate rejection and/or symptoms associated with hyperacute, acute, and/or chronic transplant rejection of any tissue, including, but not limited to, liver, kidney, pancreas, pancreatic islet cells, small intestine, lung, heart, corneas, skin, blood vessels, bone, heterologous or autologous bone marrow, and the like. Graft tissues may be obtained from any donor and transplanted into any recipient host, and thus the transplant procedure may comprise transplanting animal tissue to humans (e.g., xenografts), transplanting tissue from one human to another human (e.g., allografts), and/or transplanting tissue from one part of a human's body to another (e.g., autografts). Treatment with the antibodies of the invention may also

reduce transplantation sequelae such as fever, anorexia, hemodynamic abnormalities, leukopenia, white cell infiltration of the transplanted organ/tissue, as well as opportunistic infections.

In some embodiments, the anti-CD20 antibodies of the invention may be used alone or in combination with immunosuppressive drugs to treat and/or prevent transplant rejection such as hyperacute, acute, and/or chronic rejection and/or graft versus host disease. Thus, in some embodiments where the anti-CD20 antibodies of the invention are used to treat graft rejection, the antibodies may used in combination with suitable immunosuppressive drugs, including, but not limited, to methotrexate; cyclophosphamide; mizoribine; chlorambucil; cyclosporine, such as, for example, aerosolized cyclosporine (see, U.S. Patent Application Publication No. US20020006901, herein incorporated by reference in its entirety), tacrolimus (FK506; ProGraf™), mycophenolate mofetil, and azathioprine (6-mercaptopurine), sirolimus (rapamycin), deoxyspergualin, leflunomide and its malononitriloamide analogs; and immunosuppressive proteins, including, for example, anti-CTLA antibodies and Ig fusions, anti-B lymphocyte stimulator antibodies (e.g., LYMPHOSTAT-B™) and Ig fusions (BLyS-Ig), anti-CD80 antibodies and etanercept (Enbrel®), as well as anti-T cell antibodies such as anti-CD3 (OKT3), anti-CD4, and the like.

As such, it is specifically contemplated that the compositions and methods of the invention are used in combination with other drugs to further improve symptoms and outcomes in transplant recipients, such as those receiving lung grafts, for example. Thus, in some embodiments, the anti-CD20 antibodies of the invention are used to treat transplant rejection (such as, for example hyperacute, acute, and/or chronic rejection or graft versus host disease in lung transplant recipients) alone or in combination with parenterally and/or non-parenterally administered cyclosporine, including for example oral cyclosporine, injectable cyclosporine, aerosolized (e.g., inhaled) cyclosporine, and combinations thereof. In some embodiments where at least a component of the therapy is aerosolized cyclosporine, the cyclosporine is delivered to the lung of the recipient by inhalation of cyclosporine in aerosol spray form using, for example, a pressurized delivery device or nebulizer. The cyclosporine may be administered in either dry powder or wet form.

In some other embodiments, the anti-CD20 antibodies of the invention may be used alone or in combination with immunosuppressive drugs to treat and/or prevent rheumatoid arthritis. Thus in some embodiments where the anti-CD20 antibodies of the invention are used to treat rheumatoid arthritis, the antibodies may used in combination with suitable immunosuppressive drugs, including, but not limited to, methotrexate, cyclophosphamide, mizoribine, chlorambucil, cyclosporine, tacrolimus (FK506; PROGRAF™), mycophenolate mofetil, and azathioprine (6-mercaptopurine), sirolimus (rapamycin), deoxyspergualin, leflunomide and its malononitriloamide analogs; and immunosuppressive proteins, including, for example, anti-CTLA antibodies and Ig fusions, anti-B lymphocyte stimulator antibodies (e.g., LYMPHOSTAT-B™) and Ig fusions (BLyS-Ig), other anti-CD20 antibodies (e.g. RITUXAN®); the fully human antibody HuMax-CD20, R-1594, IMMU-106, TRU-015, AME-133, tositumomab/I-131, tositumomab (Bexxar®), ibritumomab tituxetan (Zevalin®); anti-CD80 antibodies, and etanercept (ENBREL®), as well as anti-T cell antibodies such as anti-CD3 (OKT3), anti-CD4, and the like. As discussed above, treatment effectiveness may be assessed using any means and includes, but is not limited to, effectiveness as measured by clinical responses defined by the American College of Rheumatology criteria, the European League of Rheumatism criteria, or any other criteria. See for example, Felson *et al.* (1995) *Arthritis. Rheum.* 38:727-35 and van Gestel *et al.* (1996) *Arthritis Rheum.* 39:34-40.

In yet other embodiments, the anti-CD20 antibodies of the invention may be used alone or in combination with immunosuppressive drugs to treat and/or prevent multiple sclerosis. Thus in some embodiments where the anti-CD20 antibodies of the invention are used to treat multiple sclerosis, the antibodies may be used in combination with suitable immunosuppressive drugs, including, but not limited to, methotrexate, cyclophosphamide, mizoribine, chlorambucil, cyclosporine, tacrolimus (FK506; PROGRAF™), mycophenolate mofetil, and azathioprine (6-mercaptopurine), sirolimus (rapamycin), deoxyspergualin, leflunomide and its malononitriloamide analogs; and immunosuppressive proteins, including, for example, anti-CTLA antibodies and Ig fusions, anti-B lymphocyte stimulator antibodies (e.g., LYMPHOSTAT-B™) and Ig fusions (BLyS-Ig), other anti-CD20 antibodies (e.g., RITUXAN®); the fully human antibody HuMax-CD20, R-1594,

IMMU-106, TRU-015, AME-133, tositumomab/I-131, tositumomab (Bexxar®), ibritumomab tituxetan (Zevalin®); anti-CD80 antibodies, and etanercept (ENBREL®), as well as anti-T cell antibodies such as anti-CD3 (OKT3), anti-CD4, and the like.

Further, combination therapy with two or more therapeutic agents and an anti-CD20 antibody described herein can also be used for treatment of disease states associated with CD20-expressing cells, for example, B cell-related cancers, and autoimmune and/or inflammatory disorders. Without being limiting, examples include triple combination therapy, where two chemotherapeutic agents are administered in combination with an anti-CD20 antibody described herein, and where a chemotherapeutic agent and another anti-cancer monoclonal antibody (for example, alemtuzumab, rituximab, or anti-CD23 antibody) are administered in combination with an anti-CD20 antibody described herein. Examples of such combinations include, but are not limited to, combinations of fludarabine, cyclophosphamide, and the anti-CD20 antibody; and combinations of fludarabine, another anti-CD20 antibody, for example, rituximab (Rituxan®; IDEC Pharmaceuticals Corp., San Diego, California), and an anti-CD20 antibody of the invention.

A further embodiment of the invention is the use of anti-CD20 antibodies for diagnostic monitoring of protein levels in tissue as part of a clinical testing procedure, e.g., to determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin; and examples of suitable radioactive material include ^{125}I, ^{131}I, ^{35}S, or ^{3}H.

VIII. PHARMACEUTICAL COMPOSITIONS AND ADMINISTRATION METHODS

Methods of preparing and administering the anti-CD20 antibodies, or antigen-binding fragments, variants, or derivatives thereof of the invention to a subject in need thereof are well known to or are readily determined by those skilled in the art. The route of administration of the anti-CD20 antibody, or antigen-binding fragment, variant, or derivative thereof may be, for example, oral, parenteral, by inhalation or topical. The term parenteral as used herein includes, *e.g.*, intravenous, intraarterial, intraperitoneal, intramuscular, subcutaneous, rectal, or vaginal administration. While all these forms of administration are clearly contemplated as being within the scope of the invention, a form for administration would be a solution for injection, in particular for intravenous or intraarterial injection or drip. Usually, a suitable pharmaceutical composition for injection may comprise a buffer (*e.g.* acetate, phosphate or citrate buffer), a surfactant (*e.g.* polysorbate), optionally a stabilizer agent (*e.g.* human albumin), etc. However, in other methods compatible with the teachings herein, anti-CD20 antibodies, or antigen-binding fragments, variants, or derivatives thereof of the invention can be delivered directly to the site of the adverse cellular population thereby increasing the exposure of the diseased tissue to the therapeutic agent.

As previously discussed, anti-CD20 antibodies, or antigen-binding fragments, variants, or derivatives thereof, of the invention may be administered in a pharmaceutically effective amount for the *in vivo* treatment of CD20-expressing cell-mediated diseases such as SLE, PBC, ITP, multiple sclerosis, psoriasis, Crohn's disease, graft rejection, and B-cell lymphoma. In this regard, it will be appreciated that the disclosed antibodies will be formulated so as to facilitate administration and promote stability of the active agent. Preferably, pharmaceutical compositions in accordance with the present invention comprise a pharmaceutically acceptable, non-toxic, sterile carrier such as physiological saline, non-toxic buffers, preservatives and the like. For the purposes of the instant application, a pharmaceutically effective amount of an anti-CD20 antibody, or antigen-binding fragment, variant, or derivative thereof, conjugated or unconjugated, shall be held to mean an amount sufficient to achieve effective binding to a target and to achieve a benefit, *e.g.*, to ameliorate symptoms of a disease or disorder or to detect a substance or a cell.

The pharmaceutical compositions used in this invention comprise pharmaceutically acceptable carriers, including, *e.g.*, ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts or electrolytes, such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, polyethylene glycol, sodium carboxymethylcellulose, polyacrylates, waxes, polyethylene-polyoxypropylene-block polymers, polyethylene glycol, and wool fat.

Preparations for parenteral administration includes sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. In the subject invention, pharmaceutically acceptable carriers include, but are not limited to, 0.01-0.1 M and preferably 0.05 M phosphate buffer or 0.8% saline. Other common parenteral vehicles include sodium phosphate solutions, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers, such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present such as for example, antimicrobials, antioxidants, chelating agents, and inert gases and the like.

More particularly, pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In such cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It should be stable under the conditions of manufacture and storage and will preferably be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures

thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Suitable formulations for use in the therapeutic methods disclosed herein are described in Remington's *Pharmaceutical Sciences* (Mack Publishing Co.) 16th ed. (1980).

Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols, such as mannitol, sorbitol, or sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

In any case, sterile injectable solutions can be prepared by incorporating an active compound (*e.g.*, an anti-CD20 antibody, or antigen-binding fragment, variant, or derivative thereof, by itself or in combination with other active agents) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated herein, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle, which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying, which yields a powder of an active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The preparations for injections are processed, filled into containers such as ampoules, bags, bottles, syringes or vials, and sealed under aseptic conditions according to methods known in the art. Further, the preparations may be packaged and sold in the form of a kit such as those described in co-pending U.S. Patent Application No., published as 09/259,337 US-2002-0102208 A1, which is incorporated herein by reference in its entirety. Such articles of manufacture will preferably have labels or package inserts indicating that the associated compositions are useful for treating a subject suffering from, or predisposed to a disease or disorder.

Parenteral formulations may be a single bolus dose, an infusion or a loading bolus dose followed with a maintenance dose. These compositions may be administered at specific fixed or variable intervals, *e.g.*, once a day, or on an "as needed" basis.

Certain pharmaceutical compositions used in this invention may be orally administered in an acceptable dosage form including, *e.g.*, capsules, tablets, aqueous suspensions or solutions. Certain pharmaceutical compositions also may be administered by nasal aerosol or inhalation. Such compositions may be prepared as solutions in saline, employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, and/or other conventional solubilizing or dispersing agents.

The amount of an anti-CD20 antibody, or fragment, variant, or derivative thereof that may be combined with the carrier materials to produce a single dosage form will vary depending upon the host treated and the particular mode of administration. The composition may be administered as a single dose, multiple doses or over an established period of time in an infusion. Dosage regimens also may be adjusted to provide the optimum desired response (*e.g.*, a therapeutic or prophylactic response).

In keeping with the scope of the present disclosure, anti-CD20 antibodies, or antigen-binding fragments, variants, or derivatives thereof of the invention may be administered to a human or other animal in accordance with the aforementioned methods of treatment in an amount sufficient to produce a therapeutic effect. The anti-CD20 antibodies, or antigen-binding fragments, variants, or derivatives thereof of the invention can be administered to such human or other animal in a conventional dosage form prepared by combining the antibody of the invention with a conventional pharmaceutically acceptable carrier or diluent according to known techniques. It will be recognized by one of skill in the art that the form and character of the pharmaceutically acceptable carrier or diluent is dictated by the amount of active ingredient with which it is to be combined, the route of administration and other well-known variables. Those skilled in the art will further appreciate that a cocktail

comprising one or more species of anti-CD20 antibodies, or antigen-binding fragments, variants, or derivatives thereof of the invention may prove to be particularly effective.

Effective doses of the compositions of the present invention, for treatment of CD20-expressing cell-mediated diseases such as SLE, PBC, ITP, multiple sclerosis, psoriasis, Crohn's disease, graft rejection, and B-cell lymphoma vary depending upon many different factors, including means of administration, target site, physiological state of the patient, whether the patient is human or an animal, other medications administered, and whether treatment is prophylactic or therapeutic. Usually, the patient is a human, but non-human mammals including transgenic mammals can also be treated. Treatment dosages may be titrated using routine methods known to those of skill in the art to optimize safety and efficacy.

The amount of at least one anti-CD20 antibody to be administered is readily determined by one of ordinary skill in the art without undue experimentation given the disclosure of the present invention. Factors influencing the mode of administration and the respective amount of at least one anti-CD20 antibody, antigen-binding fragment, variant or derivative thereof include, but are not limited to, the severity of the disease, the history of the disease, and the age, height, weight, health, and physical condition of the individual undergoing therapy. Similarly, the amount of anti-CD20 antibody, or fragment, variant, or derivative thereof to be administered will be dependent upon the mode of administration and whether the subject will undergo a single dose or multiple doses of this agent. The dose of anti-CD20 antibody, or fragment, or variant, or derivative thereof to be administered is in the range from about 0.0001 to 100 mg/kg, 0.003 mg/kg to about 50 mg/kg, or about 0.01 mg/kg to about 40 mg/kg. Thus, for example, the dose can be 0.01 mg/kg, 0.03 mg/kg, 0.1 mg/kg, 0.3 mg/kg, 0.5 mg/kg, 1 mg/kg, 1.5 mg/kg, 2 mg/kg, 2.5 mg/kg, 3 mg/kg, 5 mg/kg, 7 mg/kg, 10 mg/kg, 15 mg/kg, 20 mg/kg, 25 mg/kg, 30 mg/kg, 35 mg/kg, 40 mg/kg, 45 mg/kg, 50 mg/kg, 60 mg/kg, 70 mg/kg, 80 mg/kg, 90 mg/kg, or 100 mg/kg.

In some embodiments, for treatment of CD20-expressing cell-mediated diseases such as SLE, PBC, ITP, multiple sclerosis, psoriasis, Crohn's disease, graft rejection, and B-cell lymphoma with an anti-CD20 antibody, or antigen-binding fragment, variant, or derivative thereof, the dosage can range, *e.g.*, from about 0.0001

to 100 mg/kg, and more usually 0.01 to 5 mg/kg (*e.g.*, 0.02 mg/kg, 0.25 mg/kg, 0.5 mg/kg, 0.75 mg/kg, 1 mg/kg, 2 mg/kg, etc.), of the host body weight. For example dosages can be 1 mg/kg body weight or 10 mg/kg body weight or within the range of 1-10 mg/kg, preferably at least 1 mg/kg. Doses intermediate in the above ranges are also intended to be within the scope of the invention. Subjects can be administered such doses daily, on alternative days, weekly or according to any other schedule determined by empirical analysis. Exemplary dosage schedules include 1-10 mg/kg or 15 mg/kg on consecutive days, 30 mg/kg on alternate days, or 60 mg/kg weekly. In some methods, two or more monoclonal antibodies with different binding specificities are administered simultaneously, in which case the dosage of each antibody administered falls within the ranges indicated.

Anti-CD20 antibodies, or antigen-binding fragments, variants, or derivatives thereof of the invention can be administered on multiple occasions. Intervals between single dosages can be daily, weekly, monthly or yearly. Intervals can also be irregular as indicated by measuring blood levels of target polypeptide or target molecule in the patient. In some methods, dosage is adjusted to achieve a plasma polypeptide concentration of 1-1000 μg/ml and in some methods 25-300 μg/ml. Alternatively, anti-CD20 antibodies, or antigen-binding fragments, variants, or derivatives thereof of the invention can be administered as a sustained release formulation, in which case less frequent administration is required. Dosage and frequency vary depending on the half-life of the antibody in the patient. The half-life of an anti-CD20 antibody can also be prolonged via fusion to a stable polypeptide or moeity, *e.g.*, albumin or PEG. In general, humanized antibodies show the longest half-life, followed by chimeric antibodies and nonhuman antibodies. In one embodiment, the anti-CD20 antibodies, or antigen-binding fragments, variants, or derivatives thereof of the invention can be administered in unconjugated form, In another embodiment, the anti-CD20 antibodies, or antigen-binding fragments, variants, or derivatives thereof of the invention can be administered multiple times in conjugated form. In still another embodiment, anti-CD20 antibodies, or antigen-binding fragments, variants, or derivatives thereof of the invention can be administered in unconjugated form, then in conjugated form, or vice versa.

In another embodiment of the invention, the method comprises administration of multiple doses of anti-CD20 antibody, or antigen-binding fragment, variant, or derivative thereof. The method may comprise administration of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, or more therapeutically effective doses of a pharmaceutical composition comprising an anti-CD20 antibody, fragment, variant, or derivative thereof. The frequency and duration of administration of multiple doses of the pharmaceutical compositions comprising the antibody molecule can be readily determined by one of skill in the art without undue experimentation given the disclosure herein. Moreover, treatment of a subject with a therapeutically effective amount of an antibody can include a single treatment or, preferably, can include a series of treatments. In a preferred example, a subject is treated with anti-CD20 antibody, or antigen-binding fragment, variant, or derivative thereof in the range of between about 0.1 to 20 mg/kg body weight, once per week for between about 1 to 10 weeks, preferably between about 2 to 8 weeks, more preferably between about 3 to 7 weeks, and even more preferably for about 4, 5, or 6 weeks. Treatment may occur annually to prevent relapse or upon indication of relapse. It will also be appreciated that the effective dosage of antibody molecule used for treatment may increase or decrease over the course of a particular treatment. Changes in dosage may result and become apparent from the results of diagnostic assays as described herein.

Thus, in one embodiment, the dosing regimen includes a first administration of a therapeutically effective dose of at least one anti-CD20 antibody, or antigen-binding fragment, variant, or derivative thereof, on days 1, 7, 14, and 21 of a treatment period. In another embodiment, the dosing regimen includes a first administration of a therapeutically effective dose of at least one anti-CD20 antibody, or antigen-binding fragment, variant, or derivative thereof, on days 1, 2, 3, 4, 5, 6, and 7 of a week in a treatment period. Further embodiments include a dosing regimen having a first administration of a therapeutically effective dose of at least one anti-CD20 antibody, or antigen-binding fragment, variant, or derivative thereof, on days 1, 3, 5, and 7 of a week in a treatment period; a dosing regimen including a first administration of a therapeutically effective dose of at least one anti-CD20 antibody, or antigen-binding fragment, variant, or derivative thereof, on days 1 and 3 of a week in a treatment period; and a preferred dosing regimen including a first administration

of a therapeutically effective dose of at least one anti-CD20 antibody, or antigen-binding fragment, variant, or derivative thereof, on day 1 of a week in a treatment period. The treatment period may comprise 1 week, 2 weeks, 3 weeks, a month, 3 months, 6 months, or a year. Treatment periods may be subsequent or separated from each other by a day, a week, 2 weeks, a month, 3 months, 6 months, or a year.

In some embodiments, the therapeutically effective doses of anti-CD20 antibody, or antigen-binding fragment, variant, or derivative thereof, ranges from about 0.0001 mg/kg to about100 mg/kg, from about 0.003 mg/kg to about 50 mg/kg, from about 0.01 mg/kg to about 40 mg/kg, from about 0.01 mg/kg to about 30 mg/kg, from about 0.1 mg/kg to about 30 mg/kg, from about 0.5 mg/kg to about 30 mg/kg, from about 1 mg/kg to about 30 mg/kg, from about 3 mg/kg to about 30 mg/kg, from about 3 mg/kg to about 25 mg/kg, from about 3 mg/kg to about 20 mg/kg, from about 5 mg/kg to about 15 mg/kg, or from about 7 mg/kg to about 12 mg/kg. Thus, for example, the dose of any one anti-CD20 antibody, or antigen-binding fragment, variant, or derivative thereof, can be 0.003 mg/kg, 0.01 mg/kg, 0.03 mg/kg, 0.1 mg/kg, 0.3 mg/kg, 0.5 mg/kg, 1 mg/kg, 1.5 mg/kg, 2 mg/kg, 2.5 mg/kg, 3 mg/kg, 5 mg/kg, 7 mg/kg, 10 mg/kg, 15 mg/kg, 20 mg/kg, 25 mg/kg, 30 mg/kg, 35 mg/kg, 40 mg/kg, 45 mg/kg, 50 mg/kg, or other such doses falling within the range of about 0.0001 mg/kg to about 100 mg/kg. The same therapeutically effective dose of an anti-CD20 antibody, or antigen-binding fragment, variant, or derivative thereof, can be administered throughout each week of antibody dosing. Alternatively, different therapeutically effective doses of an anti-CD20 antibody, or antigen-binding fragment, variant, or derivative thereof, can be used over the course of a treatment period.

IX. USE OF ANTI-CD20 ANTIBODIES IN THE MANUFACTURE OF MEDICAMENTS

The present invention also provides for the use of an anti-CD20 antibody or antigen-binding fragment, variant, or derivative thereof in the manufacture of a medicament for treating a subject for a cancer characterized by neoplastic B cell growth, wherein the medicament is coordinated with treatment with at least one other cancer therapy. Cancers characterized by neoplastic B cell growth include, but are

not limited to, the B cell-related cancers discussed herein above, for example, non-Hodgkin's lymphoma, chronic lymphocytic leukemia, multiple myeloma, B cell lymphoma, high-grade B cell lymphoma, intermediate-grade B cell lymphoma, low-grade B cell lymphoma, B cell acute lympohoblastic leukemia, myeloblastic leukemia, Hodgkin's disease, plasmacytoma, follicular lymphoma, follicular small cleaved lymphoma, follicular large cell lymphoma, follicular mixed small cleaved lymphoma, diffuse small cleaved cell lymphoma, diffuse small lymphocytic lymphoma, prolymphocytic leukemia, lymphoplasmacytic lymphoma, marginal zone lymphoma, mucosal associated lymphoid tissue lymphoma, monocytoid B cell lymphoma, splenic lymphoma, hairy cell leukemia, diffuse large cell lymphoma, mediastinal large B cell lymphoma, lymphomatoid granulomatosis, intravascular lymphomatosis, diffuse mixed cell lymphoma, diffuse large cell lymphoma, immunoblastic lymphoma, Burkitt's lymphoma, AIDS-related lymphoma, and mantle cell lymphoma.

By "coordinated" is intended the medicament comprising the anti-CD20 antibody or antigen-binding fragment, variant, or derivative thereof is to be used either prior to, during, or after treatment of the subject with at least one other cancer therapy. Examples of other cancer therapies include, but are not limited to, surgery; radiation therapy; chemotherapy, optionally in combination with autologous bone marrow transplant, where suitable chemotherapeutic agents include, but are not limited to, fludarabine or fludarabine phosphate, chlorambucil, vincristine, pentostatin, 2-chlorodeoxyadenosine (cladribine), cyclophosphamide, doxorubicin, prednisone, and combinations thereof, for example, anthracycline-containing regimens such as CAP (cyclophosphamide, doxorubicin plus prednisone), CHOP (cyclophosphamide, vincristine, prednisone plus doxorubicin), VAD (vincritsine, doxorubicin, plus dexamethasone), MP (melphalan plus prednisone), and other cytotoxic and/or therapeutic agents used in chemotherapy such as mitoxantrone, daunorubicin, idarubicin, asparaginase, and antimetabolites, including, but not limited to, cytarabine, methotrexate, 5-fluorouracil decarbazine, 6-thioguanine, 6-mercaptopurine, and nelarabine; other anti-cancer monoclonal antibody therapy (for example, alemtuzumab (Campath®) or other anti-CD52 antibody targeting the CD52 cell-surface glycoprotein on malignant B cells; rituximab (Rituxan®), the fully human

antibody HuMax-CD20, R-1594, IMMU-106, TRU-015, AME-133, tositumomab/I-131 tositumomab (Bexxar®), ibritumomab tiuxetan (Zevalin®), or any other therapeutic anti-CD20 antibody targeting the CD20 antigen on malignant B cells; anti-CD19 antibody (for example, MT103, a bispecific antibody); anti-CD22 antibody (for example, the humanized monoclonal antibody epratuzumab); bevacizumab (Avastin®) or other anti-cancer antibody targeting human vascular endothelial growth factor; anti-CD22 antibody targeting the CD22 antigen on malignant B cells (for example, the monoclonal antibody BL-22, an alphaCD22 toxin); α-M-CSF antibody targeting macrophage colony stimulating factor; antibodies targeting the receptor activator of nuclear factor-kappaB (RANK) and its ligand (RANKL), which are overexpressed in multiple myeloma; anti-CD23 antibody targeting the CD23 antigen on malignant B cells (for example, IDEC-152); anti-CD38 antibody targeting the CD38 antigen on malignant B cells; antibodies targeting major histocompatibility complex class II receptors (anti-MHC antibodies) expressed on malignant B cells; anti-CD40 antibodies (for example, SGN-40) targeting the CD40 antigen on malignant B cells; and antibodies targeting tumor necrosis factor-related apoptosis-inducing ligand receptor 1 (TRAIL-R1) (for example, the agonistic human monoclonal antibody HGS-ETR1) expressed on a number of solid tumors and tumors of hematopoietic origin); small molecule-based cancer therapy, including, but not limited to, microtubule and/or topoisomerase inhibitors (for example, the mitotic inhibitor dolastatin and dolastatin analogues; the tubulin-binding agent T900607; XL119; and the topoisomerase inhibitor aminocamptothecin), SDX-105 (bendamustine hydrochloride), ixabepilone (an epothilone analog, also referred to as BMS-247550), protein kinase C inhibitors, for example, midostaurin ((PKC-412, CGP 41251, N-benzoylstaurosporine), pixantrone, eloxatin (an antineoplastic agent), ganite (gallium nitrate), Thalomid® (thalidomide), immunomodulatory derivatives of thalidomide (for example, revlimid (formerly revimid)), Affinitak™ (antisense inhibitor of protein kinase C-alpha), SDX-101 (R-etodolac, inducing apoptosis of malignant lymphocytes), second-generation purine nucleoside analogs such as clofarabine, inhibitors of production of the protein Bcl-2 by cancer cells (for example, the antisense agents oblimersen and Genasense®), proteasome inhibitors (for example, Velcade™ (bortezomib)), small molecule kinase inhibitors (for example, CHIR-258),

small molecule VEGF inhibitors (for example, ZD-6474), small molecule inhibitors of heat shock protein (HSP) 90 (for example, 17-AAG), small molecule inhibitors of histone deacetylases (for example, hybrid/polar cytodifferentiation HPC) agents such as suberanilohydroxamic acid (SAHA), and FR-901228) and apoptotic agents such as Trisenox® (arsenic trioxide) and Xcytrin® (motexafin gadolinium); vaccine /immunotherapy-based cancer therapies, including, but not limited to, vaccine approaches (for example, Id-KLH, oncophage, vitalethine), personalized immunotherapy or active idiotype immunotherapy (for example, MyVax® Personalized Immunotherapy, formally designated GTOP-99), Promune® (CpG 7909, a synthetic agonist for toll-like receptor 9 (TLR9)), interferon-alpha therapy, interleukin-2 (IL-2) therapy, IL-12 therapy; IL-15 therapy, and IL-21 therapy; steroid therapy; or other cancer therapy; where treatment with the additional cancer therapy, or additional cancer therapies, occurs prior to, during, or subsequent to treatment of the subject with the medicament comprising the anti-CD20 antibody or antigen-binding fragment, variant, or derivative thereof, as noted herein above.

In some embodiments, the present invention provides for the use of the anti-CD20 antibody or antigen-binding fragment, variant, or derivative thereof in the manufacture of a medicament for treating a B cell lymphoma, for example non-Hodgkin's lymphoma, in a subject, wherein the medicament is coordinated with treatment with at least one other cancer therapy selected from the group consisting of chemotherapy, anti-cancer antibody therapy, small molecule-based cancer therapy, and vaccine/immunotherapy-based cancer therapy, wherein the medicament is to be used either prior to, during, or after treatment of the subject with the other cancer therapy or, in the case of multiple combination therapies, either prior to, during, or after treatment of the subject with the other cancer therapies.

Thus, for example, in some embodiments, the invention provides for the use of the monoclonal antibody 1589 or other anti-CD20 antibodies of the invention, or antigen-binding fragment, variant, or derivative thereof, in the manufacture of a medicament for treating a B cell lymphoma, for example, non-Hodgkin's lymphoma, in a subject, wherein the medicament is coordinated with treatment with chemotherapy, where the chemotherapeutic agent is selected from the group consisting of cytoxan, doxorubicin, vincristine, prednisone, and combinations thereof,

for example CHOP. In other embodiments, the invention provides for the use of the monoclonal antibody 1589, or antigen-binding fragment thereof, in the manufacture of a medicament for treating a B cell lymphoma, for example non-Hodgkin's lymphoma, in a subject, wherein the medicament is coordinated with treatment with at least one other anti-cancer antibody selected from the group consisting of alemtuzumab (Campath®) or other anti-CD52 antibody targeting the CD52 cell-surface glycoprotein on malignant B cells; rituximab (Rituxan®), the fully human antibody HuMax-CD20, R-1594, IMMU-106, TRU-015, AME-133, tositumomab/I-131 tositumomab (Bexxar®), ibritumomab tiuxetan (Zevalin®), or any other therapeutic anti-CD20 antibody targeting the CD20 antigen on malignant B cells; anti-CD19 antibody (for example, MT103, a bispecific antibody); anti-CD22 antibody (for example, the humanized monoclonal antibody epratuzumab); bevacizumab (Avastin®) or other anti-cancer antibody targeting human vascular endothelial growth factor; and any combinations thereof; wherein the medicament is to be used either prior to, during, or after treatment of the subject with the other cancer therapy or, in the case of multiple combination therapies, either prior to, during, or after treatment of the subject with the other cancer therapies.

In yet other embodiments, the present invention provides for the use of the monoclonal antibody 1589, or antigen-binding fragment, variant, or derivative thereof, in the manufacture of a medicament for treating a B cell lymphoma, for example non-Hodgkin's lymphoma, in a subject, wherein the medicament is coordinated with treatment with at least one other small molecule-based cancer therapy selected from the group consisting of microtubule and/or topoisomerase inhibitors (for example, the mitotic inhibitor dolastatin and dolastatin analogues; the tubulin-binding agent T900607; XL119; and the topoisomerase inhibitor aminocamptothecin), SDX-105 (bendamustine hydrochloride), ixabepilone (an epothilone analog, also referred to as BMS-247550), protein kinase C inhibitors, for example, midostaurin ((PKC-412, CGP 41251, N-benzoylstaurosporine), pixantrone, eloxatin (an antineoplastic agent), ganite (gallium nitrate), Thalomid® (thalidomide), an apoptotic agent such as Xcytrin® (motexafin gadolinium), inhibitors of production of the protein Bcl-2 by cancer cells (for example, the antisense agents oblimersen and Genasense®), nelarabine, and any combinations thereof; wherein the medicament is to

be used either prior to, during, or after treatment of the subject with the other cancer therapy or, in the case of multiple combination therapies, either prior to, during, or after treatment of the subject with the other cancer therapies.

In still other embodiments, the present invention provides for the use of the monoclonal antibody 1589, or antigen-binding fragment, variant, or derivative thereof, in the manufacture of a medicament for treating a B cell lymphoma, for example non-Hodgkin's lymphoma, in a subject, wherein the medicament is coordinated with treatment with at least one other vaccine/immunotherapy-based cancer therapy selected from the group consisting of vaccine approaches (for example, Id-KLH, oncophage, vitalethine), personalized immunotherapy or active idiotype immunotherapy (for example, MyVax® Personalized Immunotherapy, formally designated GTOP-99), Promune® (CpG 7909, a synthetic agonist for toll-like receptor 9 (TLR9)), interleukin-2 (IL-2) therapy, IL-12 therapy; IL-15 therapy, and IL-21 therapy, and any combinations thereof; wherein the medicament is to be used either prior to, during, or after treatment of the subject with the other cancer therapy or, in the case of multiple combination therapies, either prior to, during, or after treatment of the subject with the other cancer therapies.

In some embodiments, the present invention provides for the use of the anti-CD20 antibody, for example, the monoclonal antibody 1589, or antigen-binding fragment, variant, or derivative thereof, in the manufacture of a medicament for treating a B cell-related leukemia, for example B-cell acute lymphocytic leukemia (B-ALL), in a subject, wherein the medicament is coordinated with treatment with at least one other cancer therapy selected from the group consisting of chemotherapy and anti-metabolite therapy, wherein the medicament is to be used either prior to, during, or after treatment of the subject with the other cancer therapy or, in the case of multiple combination therapies, either prior to, during, or after treatment of the subject with the other cancer therapies. Examples of such embodiments include, but are not limited to, those instances where the medicament comprising the anti-CD20 antibody, for example, the monoclonal antibody 1589, or antigen-binding fragment, variant, or derivative thereof, is coordinated with treatment with a chemotherapeutic agent or anti-metabolite selected from the group consisting of cytoxan, doxorubicin, vincristine, prednisone, cytarabine, mitoxantrone, idarubicin, asparaginase,

methotrexate, 6-thioguanine, 6-mercaptopurine, and combinations thereof; wherein the medicament is to be used either prior to, during, or after treatment of the subject with the other cancer therapy or, in the case of multiple combination therapies, either prior to, during, or after treatment of the subject with the other cancer therapies. In one such example, the medicament is coordinated with treatment with cytarabine plus daunorubicin, cytarabine plus mitoxantrone, and/or cytarabine plus idarubicin; wherein the medicament is to be used either prior to, during, or after treatment of the B-ALL subject with the other cancer therapy or, in the case of multiple combination therapies, either prior to, during, or after treatment of the subject with the other cancer therapies.

The invention also provides for the use of an anti-CD20 antibody, for example, the monoclonal antibody 1589 disclosed herein, or antigen-binding fragment, variant, or derivative thereof, in the manufacture of a medicament for treating a subject for a cancer characterized by neoplastic B cell growth, including the B cell-related cancers described herein above, wherein the medicament is used in a subject that has been pretreated with at least one other cancer therapy. By "pretreated" or "pretreatment" is intended the subject has received one or more other cancer therapies (i.e., been treated with at least one other cancer therapy) prior to receiving the medicament comprising the anti-CD20 antibody or antigen-binding fragment, variant, or derivative thereof. "Pretreated" or "pretreatment" includes subjects that have been treated with at least one other cancer therapy within 2 years, within 18 months, within 1 year, within 6 months, within 2 months, within 6 weeks, within 1 month, within 4 weeks, within 3 weeks, within 2 weeks, within 1 week, within 6 days, within 5 days, within 4 days, within 3 days, within 2 days, or even within 1 day prior to initiation of treatment with the medicament comprising the anti-CD20 antibody, for example, the monoclonal antibody 1589 disclosed herein, or antigen-binding fragment, variant, or derivative thereof. It is not necessary that the subject was a responder to pretreatment with the prior cancer therapy, or prior cancer therapies. Thus, the subject that receives the medicament comprising the anti-CD20 antibody or antigen-binding fragment, variant, or derivative thereof could have responded, or could have failed to respond (i.e., the cancer was refractory), to pretreatment with the prior cancer therapy, or to one or more of the prior cancer

therapies where pretreatment comprised multiple cancer therapies. Examples of other cancer therapies for which a subject can have received pretreatment prior to receiving the medicament comprising the anti-CD20 antibody or antigen-binding fragment, variant, or derivative thereof include, but are not limited to, surgery; radiation therapy; chemotherapy, optionally in combination with autologous bone marrow transplant, where suitable chemotherapeutic agents include, but are not limited to, those listed herein above; other anti-cancer monoclonal antibody therapy, including, but not limited to, those anti-cancer antibodies listed herein above; small molecule-based cancer therapy, including, but not limited to, the small molecules listed herein above; vaccine/immunotherapy-based cancer therapies, including, but limited to, those listed herein above; steroid therapy; other cancer therapy; or any combination thereof.

"Treatment" in the context of coordinated use of a medicament described herein with one or more other cancer therapies is herein defined as the application or administration of the medicament or of the other cancer therapy to a subject, or application or administration of the medicament or other cancer therapy to an isolated tissue or cell line from a subject, where the subject has a cancer characterized by neoplastic B cell growth, a symptom associated with such a cancer, or a predisposition toward development of such a cancer, where the purpose is to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve, or affect the cancer, any associated symptoms of the cancer, or the predisposition toward the development of the cancer.

The present invention also provides for the use of an anti-CD20 antibody or antigen-binding fragment, variant, or derivative thereof in the manufacture of a medicament for treating an autoimmune disease and/or inflammatory disease in a subject, wherein the medicament is coordinated with treatment with at least one other therapy. By "coordinated" is intended the medicament is to be used either prior to, during, or after treatment of the subject with at least one other therapy for the autoimmune disease and/or inflammatory disease. Examples of other therapies include, but are not limited to, those described herein above, i.e., surgery or surgical procedures (e.g. splenectomy, lymphadenectomy, thyroidectomy, plasmaphoresis, leukophoresis, cell, tissue, or organ transplantation, organ perfusion, intestinal

procedures, and the like), radiation therapy, therapy such as steroid therapy and non-steroidal therapy, hormone therapy, cytokine therapy, therapy with dermatological agents (for example, topical agents used to treat skin conditions such as allergies, contact dermatitis, and psoriasis), immunosuppressive therapy, and other anti-inflammatory monoclonal antibody therapy, and the like, where treatment with the additional therapy, or additional therapies, occurs prior to, during, or subsequent to treatment of the subject with the medicament comprising the anti-CD20 antibody or antigen-binding fragment, variant, or derivative thereof, as noted herein above. In one such embodiment, the present invention provides for the use of the monoclonal antibody 1589, or antigen-binding fragment, variant, or derivative thereof, in the manufacture of a medicament for treating an autoimmune disease and/or inflammatory disease in a subject, wherein the medicament is coordinated with treatment with at least one other therapy as noted herein above.

In some embodiments, the medicament comprising the anti-CD20 antibody, for example, the monoclonal antibody 1589 disclosed herein, or antigen-binding fragment, variant, or derivative thereof, is coordinated with treatment with two other therapies. Where the medicament comprising the anti-CD20 antibody is coordinated with two other therapies, use of the medicament can be prior to, during, or after treatment of the subject with either or both of the other therapies.

The invention also provides for the use of an anti-CD20 antibody, for example, the monoclonal antibody 1589 disclosed herein, or antigen-binding fragment, variant, or derivative thereof, in the manufacture of a medicament for treating an autoimmune disease and/or inflammatory disease in a subject, wherein the medicament is used in a subject that has been pretreated with at least one other therapy. By "pretreated" or "pretreatment" is intended the subject has been treated with one or more other therapies prior to receiving the medicament comprising the anti-CD20 antibody or antigen-binding fragment, variant, or derivative thereof. "Pretreated" or "pretreatment" includes subjects that have been treated with the other therapy, or other therapies, within 2 years, within 18 months, within 1 year, within 6 months, within 2 months, within 6 weeks, within 1 month, within 4 weeks, within 3 weeks, within 2 weeks, within 1 week, within 6 days, within 5 days, within 4 days, within 3 days, within 2 days, or even within 1 day prior to initiation of treatment with

the medicament comprising the anti-CD20 antibody, for example, the monoclonal antibody 1589 disclosed herein, or antigen-binding fragment, variant, or derivative thereof. It is not necessary that the subject was a responder to pretreatment with the prior therapy, or prior therapies. Thus, the subject that receives the medicament comprising the anti-CD20 antibody or antigen-binding fragment, variant, or derivative thereof could have responded, or could have failed to respond, to pretreatment with the prior therapy, or to one or more of the prior therapies where pretreatment comprised multiple therapies.

"Treatment" in the context of coordinated use of a medicament described herein with one or more other autoimmune disease and/or inflammatory disease therapies is herein defined as the application or administration of the medicament or of the other therapy to a subject, or application or administration of the medicament or other therapy to an isolated tissue or cell line from a subject, where the subject has an autoimmune disease and/or inflammatory disease associated with CD20-expressing cells, a symptom associated with such a disease, or a predisposition toward development of such a disease, where the purpose is to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve, or affect the disease, any associated symptoms of the disease, or the predisposition toward the development of the disease.

X. DIAGNOSTICS

The invention further provides a diagnostic method useful during diagnosis of CD20-expressing cell-mediated diseases such as SLE, PBC, ITP, multiple sclerosis, psoriasis, Crohn's disease, graft rejection, and B-cell lymphoma, which involves measuring the expression level of CD20 protein or transcript in tissue or other cells or body fluid from an individual and comparing the measured expression level with a standard CD20 expression level in normal tissue or body fluid, whereby an increase in the expression level compared to the standard is indicative of a disorder.

The anti-CD20 antibodies of the invention and antigen-binding fragments, variants, and derivatives thereof, can be used to assay CD20 protein levels in a biological sample using classical immunohistological methods known to those of skill in the art (*e.g.*, see Jalkanen, *et al.* (1985) *J. Cell. Biol.* 101:976-985; Jalkanen *et al.*

(1987) *J. Cell Biol.* 105:3087-3096). Other antibody-based methods useful for detecting CD20 protein expression include immunoassays, such as the enzyme linked immunosorbent assay (ELISA), immunoprecipitation, or western blotting. Suitable assays are described in more detail elsewhere herein.

By "assaying the expression level of CD20 polypeptide" is intended qualitatively or quantitatively measuring or estimating the level of CD20 polypeptide in a first biological sample either directly (*e.g.*, by determining or estimating absolute protein level) or relatively (*e.g.*, by comparing to the disease associated polypeptide level in a second biological sample). Preferably, CD20 polypeptide expression level in the first biological sample is measured or estimated and compared to a standard CD20 polypeptide level, the standard being taken from a second biological sample obtained from an individual not having the disorder or being determined by averaging levels from a population of individuals not having the disorder. As will be appreciated in the art, once the "standard" CD20 polypeptide level is known, it can be used repeatedly as a standard for comparison.

By "biological sample" is intended any biological sample obtained from an individual, cell line, tissue culture, or other source of cells potentially expressing CD20. Methods for obtaining tissue biopsies and body fluids from mammals are well known in the art.

XI. IMMUNOASSAYS

Anti-CD20 antibodies, or antigen-binding fragments, variants, or derivatives thereof of the invention may be assayed for immunospecific binding by any method known in the art. The immunoassays that can be used include but are not limited to competitive and non-competitive assay systems using techniques such as western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, to name but a few. Such assays are routine and well known in the art (*see, e.g.*, Ausubel *et al.*, eds, (1994) *Current Protocols in Molecular Biology* (John

Wiley & Sons, Inc., NY) Vol. 1, which is incorporated by reference herein in its entirety). Exemplary immunoassays are described briefly below (but are not intended by way of limitation).

Immunoprecipitation protocols generally comprise lysing a population of cells in a lysis buffer such as RIPA buffer (1% NP-40 or Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate at pH 7.2, 1% Trasylol) supplemented with protein phosphatase and/or protease inhibitors (*e.g.*, EDTA, PMSF, aprotinin, sodium vanadate), adding the antibody of interest to the cell lysate, incubating for a period of time (*e.g.*, 1-4 hours) at 4°C., adding protein A and/or protein G sepharose beads to the cell lysate, incubating for about an hour or more at 4°C., washing the beads in lysis buffer and resuspending the beads in SDS/sample buffer. The ability of the antibody of interest to immunoprecipitate a particular antigen can be assessed by, *e.g.*, western blot analysis. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the binding of the antibody to an antigen and decrease the background (*e.g.*, pre-clearing the cell lysate with sepharose beads). For further discussion regarding immunoprecipitation protocols see, *e.g.*, Ausubel *et al.*, eds, (1994) *Current Protocols in Molecular Biology* (John Wiley & Sons, Inc., NY) Vol. 1 at 10.16.1.

Western blot analysis generally comprises preparing protein samples, electrophoresis of the protein samples in a polyacrylamide gel (*e.g.*, 8%-20% SDS-PAGE depending on the molecular weight of the antigen), transferring the protein sample from the polyacrylamide gel to a membrane such as nitrocellulose, PVDF or nylon, blocking the membrane in blocking solution (*e.g.*, PBS with 3% BSA or non-fat milk), washing the membrane in washing buffer (*e.g.*, PBS-Tween 20), incubating the membrane with primary antibody (the antibody of interest) diluted in blocking buffer, washing the membrane in washing buffer, incubating the membrane with a secondary antibody (which recognizes the primary antibody, *e.g.*, an anti-human antibody) conjugated to an enzymatic substrate (*e.g.*, horseradish peroxidase or alkaline phosphatase) or radioactive molecule (*e.g.*, 32p or 1251) diluted in blocking buffer, washing the membrane in wash buffer, and detecting the presence of the antigen. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the signal detected and to reduce the background noise. For

further discussion regarding western blot protocols see, *e.g.*, Ausubel *et al.*, eds, (1994) *Current Protocols in Molecular Biology* (John Wiley & Sons, Inc., NY) Vol. 1 at 10.8.1.

ELISAs comprise preparing antigen, coating the well of a 96-well microtiter plate with the antigen, adding the antibody of interest conjugated to a detectable compound such as an enzymatic substrate (*e.g.*, horseradish peroxidase or alkaline phosphatase) to the well and incubating for a period of time, and detecting the presence of the antigen. In ELISAs the antibody of interest does not have to be conjugated to a detectable compound; instead, a second antibody (which recognizes the antibody of interest) conjugated to a detectable compound may be added to the well. Further, instead of coating the well with the antigen, the antibody may be coated to the well. In this case, a second antibody conjugated to a detectable compound may be added following the addition of the antigen of interest to the coated well. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the signal detected as well as other variations of ELISAs known in the art. For further discussion regarding ELISAs *see, e.g.*, Ausubel *et al.*, eds, (1994) *Current Protocols in Molecular Biology* (John Wiley & Sons, Inc., NY) Vol. 1 at 11.2.1.

The binding affinity of an antibody to an antigen and the off-rate of an antibody-antigen interaction can be determined by competitive binding assays. One example of a competitive binding assay is a radioimmunoassay comprising the incubation of labeled antigen (*e.g.*, ^{3}H or ^{125}I) with the antibody of interest in the presence of increasing amounts of unlabeled antigen, and the detection of the antibody bound to the labeled antigen. The affinity of the antibody of interest for a particular antigen and the binding off-rates can be determined from the data by scatchard plot analysis. Competition with a second antibody can also be determined using radioimmunoassays. In this case, the antigen is incubated with the antibody of interest conjugated to a labeled compound (*e.g.*, ^{3}H or ^{125}I) in the presence of increasing amounts of an unlabeled second antibody.

Anti-CD20 antibodies, or antigen-binding fragments, variants, or derivatives thereof of the invention, additionally, can be employed histologically, as in immunofluorescence, immunoelectron microscopy or non-immunological assays, for

in situ detection of cancer antigen gene products or conserved variants or peptide fragments thereof. *In situ* detection may be accomplished by removing a histological specimen from a patient, and applying thereto a labeled anti-CD20 antibody, or antigen-binding fragment, variant, or derivative thereof, preferably applied by overlaying the labeled antibody (or fragment) onto a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of CD20 protein, or conserved variants or peptide fragments, but also its distribution in the examined tissue. Using the present invention, those of ordinary skill will readily perceive that any of a wide variety of histological methods (such as staining procedures) can be modified in order to achieve such *in situ* detection.

Immunoassays and non-immunoassays for CD20 gene products or conserved variants or peptide fragments thereof will typically comprise incubating a sample, such as a biological fluid, a tissue extract, freshly harvested cells, or lysates of cells which have been incubated in cell culture, in the presence of a detectably labeled antibody capable of binding to CD20 or conserved variants or peptide fragments thereof, and detecting the bound antibody by any of a number of techniques well known in the art.

The biological sample may be brought in contact with and immobilized onto a solid phase support or carrier such as nitrocellulose, or other solid support which is capable of immobilizing cells, cell particles or soluble proteins. The support may then be washed with suitable buffers followed by treatment with the detectably labeled anti-CD20 antibody, or antigen-binding fragment, variant, or derivative thereof. The solid phase support may then be washed with the buffer a second time to remove unbound antibody. Optionally the antibody is subsequently labeled. The amount of bound label on solid support may then be detected by conventional means.

By "solid phase support or carrier" is intended any support capable of binding an antigen or an antibody. Well-known supports or carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, gabbros, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to an antigen or

antibody. Thus, the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. Preferred supports include polystyrene beads. Those skilled in the art will know many other suitable carriers for binding antibody or antigen, or will be able to ascertain the same by use of routine experimentation.

The binding activity of a given lot of anti-CD20 antibody, or antigen-binding fragment, variant, or derivative thereof may be determined according to well known methods. Those skilled in the art will be able to determine operative and optimal assay conditions for each determination by employing routine experimentation.

There are a variety of methods available for measuring the affinity of an antibody-antigen interaction, but relatively few for determining rate constants. Most of the methods rely on either labeling antibody or antigen, which inevitably complicates routine measurements and introduces uncertainties in the measured quantities.

Surface plasmon reasonance (SPR) as performed on BIAcore offers a number of advantages over conventional methods of measuring the affinity of antibody-antigen interactions including: (i) no requirement to label either antibody or antigen; (ii) antibodies do not need to be purified in advance, cell culture supernatant can be used directly; (iii) real-time measurements, allowing rapid semi-quantitative comparison of different monoclonal antibody interactions, are enabled and are sufficient for many evaluation purposes; (iv) biospecific surface can be regenerated so that a series of different monoclonal antibodies can easily be compared under identical conditions; (v) analytical procedures are fully automated, and extensive series of measurements can be performed without user intervention. BIAapplications Handbook, version AB (reprinted 1998), BIACORE code No. BR-1001-86; BIAtechnology Handbook, version AB (reprinted 1998), BIACORE code No. BR-1001-84.

SPR based binding studies require that one member of a binding pair be immobilized on a sensor surface. The binding partner immobilized is referred to as the ligand. The binding partner in solution is referred to as the analyte. In some cases, the ligand is attached indirectly to the surface through binding to another

immobilized molecule, which is referred as the capturing molecule. SPR response reflects a change in mass concentration at the detector surface as analytes bind or dissociate.

Based on SPR, real-time BIAcore measurements monitor interactions directly as they happen. The technique is well suited to determination of kinetic parameters. Comparative affinity ranking is extremely simple to perform, and both kinetic and affinity constants can be derived from the sensorgram data.

When analyte is injected in a discrete pulse across a ligand surface, the resulting sensorgram can be divided into three essential phases: (i) Association of analyte with ligand during sample injection; (ii) Equilibrium or steady state during sample injection, where the rate of analyte binding is balanced by dissociation from the complex; (iii) Dissociation of analyte from the surface during buffer flow.

The association and dissociation phases provide information on the kinetics of analyte-ligand interaction (k_a and k_d, the rates of complex formation and dissociation, $k_d/k_a = K_D$). The equilibrium phase provides information on the affinity of the analyte-ligand interaction (K_D).

BIAevaluation software provides comprehensive facilities for curve fitting using both numerical integration and global fitting algorithms. With suitable analysis of the data, separate rate and affinity constants for interaction can be obtained from simple BIAcore investigations. The range of affinities measurable by this technique is very broad, ranging from mM to pM.

Epitope specificity is an important characteristic of a monoclonal antibody. Epitope mapping with BIAcore, in contrast to conventional techniques using radioimmunoassay, ELISA or other surface adsorption methods, does not require labeling or purified antibodies, and allows multi-site specificity tests using a sequence of several monoclonal antibodies. Additionally, large numbers of analyses can be processed automatically.

Pair-wise binding experiments test the ability of two MAbs to bind simultaneously to the same antigen. MAbs directed against separate epitopes will bind independently, whereas MAbs directed against identical or closely related epitopes will interfere with each other's binding. These binding experiments with BIAcore are straightforward to carry out.

For example, one can use a capture molecule to bind the first Mab, followed by addition of antigen and second MAb sequentially. The sensorgrams will reveal: 1. how much of the antigen binds to first Mab, 2. to what extent the second MAb binds to the surface-attached antigen, 3. if the second MAb does not bind, whether reversing the order of the pair-wise test alters the results.

Peptide inhibition is another technique used for epitope mapping. This method can complement pair-wise antibody binding studies, and can relate functional epitopes to structural features when the primary sequence of the antigen is known. Peptides or antigen fragments are tested for inhibition of binding of different MAbs to immobilized antigen. Peptides which interfere with binding of a given MAb are assumed to be structurally related to the epitope defined by that MAb.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, Sambrook *et al.*, ed. (1989) *Molecular Cloning A Laboratory Manual* (2nd ed.; Cold Spring Harbor Laboratory Press); Sambrook *et al.*, ed. (1992) *Molecular Cloning: A Laboratory Manual,* (Cold Springs Harbor Laboratory, NY); D. N. Glover ed., (1985) *DNA Cloning*, Volumes I and II; Gait, ed. (1984) *Oligonucleotide Synthesis*; Mullis *et al.* U.S. Patent No. 4,683,195; Hames and Higgins, eds. (1984) *Nucleic Acid Hybridization*; Hames and Higgins, eds. (1984) *Transcription And Translation*; Freshney (1987) *Culture Of Animal Cells* (Alan R. Liss, Inc.); *Immobilized Cells And Enzymes* (IRL Press) (1986); Perbal (1984) *A Practical Guide To Molecular Cloning*; the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); Miller and Calos eds. (1987) *Gene Transfer Vectors For Mammalian Cells*, (Cold Spring Harbor Laboratory); Wu *et al.*, eds., *Methods In Enzymology*, Vols. 154 and 155; Mayer and Walker, eds. (1987) *Immunochemical Methods In Cell And Molecular Biology* (Academic Press, London); Weir and Blackwell, eds., (1986) *Handbook Of Experimental Immunology*, Volumes I-IV; *Manipulating the Mouse Embryo*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., (1986); and in Ausubel *et al.* (1989) *Current Protocols in Molecular Biology* (John Wiley and Sons, Baltimore, Maryland).

General principles of antibody engineering are set forth in Borrebaeck, ed. (1995) *Antibody Engineering* (2nd ed.; Oxford Univ. Press). General principles of protein engineering are set forth in Rickwood *et al.*, eds. (1995) *Protein Engineering, A Practical Approach* (IRL Press at Oxford Univ. Press, Oxford, Eng.). General principles of antibodies and antibody-hapten binding are set forth in: Nisonoff (1984) *Molecular Immunology* (2nd ed.; Sinauer Associates, Sunderland, MA); and Steward (1984) *Antibodies, Their Structure and Function* (Chapman and Hall, New York, NY). Additionally, standard methods in immunology known in the art and not specifically described are generally followed as in *Current Protocols in Immunology*, John Wiley & Sons, New York; Stites *et al.*, eds. (1994) *Basic and Clinical Immunology* (8th ed; Appleton & Lange, Norwalk, CT) and Mishell and Shiigi (eds) (1980) *Selected Methods in Cellular Immunology* (W.H. Freeman and Co., NY).

Standard reference works setting forth general principles of immunology include *Current Protocols in Immunology*, John Wiley & Sons, New York; Klein (1982) *J., Immunology: The Science of Self-Nonself Discrimination* (John Wiley & Sons, NY); Kennett *et al.*, eds. (1980) *Monoclonal Antibodies, Hybridoma: A New Dimension in Biological Analyses* (Plenum Press, NY); Campbell (1984) "Monoclonal Antibody Technology" in *Laboratory Techniques in Biochemistry and Molecular Biology*, ed. Burden *et al.*, (Elsevere, Amsterdam); Goldsby *et al.*, eds. (2000) *Kuby Immunnology* (4th ed.; H. Freemand & Co.); Roitt *et al.* (2001) *Immunology* (6th ed.; London: Mosby); Abbas *et al.* (2005) *Cellular and Molecular Immunology* (5th ed.; Elsevier Health Sciences Division); Kontermann and Dubel (2001) *Antibody Engineering* (Springer Verlan); Sambrook and Russell (2001) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Press); Lewin (2003) *Genes VIII* (Prentice Hall2003); Harlow and Lane (1988) *Antibodies: A Laboratory Manual* (Cold Spring Harbor Press); Dieffenbach and Dveksler (2003) *PCR Primer* (Cold Spring Harbor Press).

All of the references cited above, as well as all references cited herein, are incorporated herein by reference in their entireties.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Example 1: Optimization of Humanized Murine Anti-CD20 Antibody

A humanized murine anti-CD20 monoclonal antibody, mAb 1097, was engineered within one or more of the complementarity determining regions (CDRs) of its variable heavy (V_H; see SEQ ID NO:29) and variable light (V_K; see SEQ ID NO:10) domains to yield optimized mAbs having increased CDC function. The initial humanized murine anti-CD20 mAb is a humanized form of the chimeric murine/human anti-CD20 antibody known as rituximab (i.e., mAb C2B8; see U.S. Patent No. 5,736,137). The three CDRs of the original V_H domain (designated H1286) and original V_K domain (designated L373) within the humanized murine anti-CD20 mAb are shown in Figure 1. The CDR2 of L373 (shown in SEQ ID NO:9) was derived from a human V_K and not a murine V_K.

Initial optimization steps included modifications to the starting CDR3 (designated as "271" in Figure 1; SEQ ID NO:30) of the H1286 V_H domain (see Figure 2). The 271 CDR3 sequence is equivalent to that for the CDR3 of the variable domain within the heavy chain of mAb C2B8. The initial modifications to 271 CDR3 included:

1) a tyrosine to asparagine (Y→N) substitution at position 9 of the starting CDR3 and a valine to asparagine (V→N) substitution at position 12 of the starting CDR3 to yield the "1236" optimized CDR3 (SEQ ID NO:1);

2) a glycine to alanine (G→A) substitution at position 5 of the initial CDR3 in addition to the Y→N and V→N substitutions at positions 9 and 12, respectively, to yield the "1237" optimized CDR3 (SEQ ID NO:2); or

3) a valine to aspartic acid (V→D) substitution at position 12 of the initial CDR3 in addition to the G→A and Y→N substitutions at positions 5 and 9, respectively, to yield the "1238" optimized CDR3 (SEQ ID NO:3).

The individual coding sequences for the optimized CDR3s are set forth in SEQ ID NO:24 (encoding 1236 optimized CDR3), SEQ ID NO:25 (encoding 1237 optimized CDR3), and SEQ ID NO:26 (encoding 1238 optimized CDR3).

These optimized CDR3s were then engineered into the original humanized V_H domain framework of H1286 to yield the following optimized V_H domains: H1569 (SEQ ID NO:12), comprising the 1236 optimized CDR3 of SEQ ID NO:1; H1570 (SEQ ID NO:13), comprising the 1237 optimized CDR3 of SEQ ID NO:2; and H1571 (SEQ ID NO:14), comprising the 1238 optimized CDR3 of SEQ ID NO:3) (see Figure 3). The coding sequences for these optimized V_H domains are shown in Figure 4, and set forth in SEQ ID NO:19 (encoding H1569), SEQ ID NO:20 (encoding H1570), and SEQ ID NO:21 (encoding H1571). Each of these optimized V_H domains was then paired with the original (L373) V_K domain (see Figure 5; SEQ ID NO:10; coding sequence set forth in SEQ ID NO:23) to yield the following optimized humanized mABs: mAb 1236, mAb 1237, and mAb 1238.

Example 2: Binding and Functional Characteristics of Optimized Humanized Murine Anti-CD20 Monoclonal Antibodies

The optimized humanized mAb 1236, mAb 1237, and mAb 1238 described in Example 1 were assessed for their respective binding and functional characteristics. For each assay, mAb 271, having the identical sequence to rituximab (IDEC-C2B8; Rituxan®; IDEC Pharmaceuticals Corp., San Diego, California), served as the positive control.

Binding specificity was assessed by Fluorometric Microvolume Assay Technology (FMAT, Applied Biosystems 8200 Cellular Detection System) and Flow Cytometry, which tabulates Mean Fluorescence Intensities (MFI) of staining on CD20 positive ($CD20^+$) and CD20 negative cell lines. FMAT analysis demonstrated binding specificity for CD20 (Figures 6A-6D). Flow cytometry analysis demonstrated binding of these optimized humanized anti-CD20 mAbs to CD20+ Daudi and EB1 cells (Table 2).

Table 2. Binding to CD20 membrane positive and negative cell lines Flow Cytometry Results (Mean Fluorescence Intensities).

	Daudi	EB1	K562	U266
MAb	CD20 Positive	CD20 Positive	CD20 Negative	CD20 Negative
271	145	429	10	9
1236	145	409	12	11
1237	132	395	14	11
1238	114	389	11	10
11 (Negative control)	8	8	6	5

In order to assess the epitope recognized by mAb1236, mAb 1237, and mAb 1238, cells and CD20-specific mouse 2H7 antibody (or mouse ISO) @ 3 μg/ml were added to the FMAT plate followed by incubation at room temperature for 2 hours. The test mAb (30 ng/ml) and an anti-human IgFc-Alexa 647 were then added, and fluorescence signal detected by FMAT. A decrease in signal with mouse 2H7 (m2H7) indicates epitope blocking.

As can be seen in Figure 7, and as expected, mAb 1236, mAb 1237, and mAb 1238 all recognize the same epitope as mAb 271 (rituximab-sequence antibody).

Complement dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) were assessed. For CDC, an antibody off-rate assay was used. In this manner, target $CD20^+$ Daudi cells were loaded with ^{51}Cr, 200 μCi for 2 hours, and then washed. The washed Daudi cells were incubated with 10 μg/ml of respective mAb for 15 minutes at 25°C. Target Daudi cells were then washed thoroughly and incubated at 37°C. Following 0, 1, 2, 4, and 6 hour incubations at 37°C, the cells were incubated with 6% human serum (complement source) for 45 minutes. Controls included spontaneous and maximum ^{51}Cr release, mAb with Daudi cells alone, and serum with Daudi cells alone at all concentrations. Supernatents were then processed and released ^{51}Cr counted via gamma counter. Antibody-specific CDC was determined by subtracting out mAb and serum alone contributions to lysis.

As can be seen in Figure 8, mAb 1236, mAb 1237, and mAb 1238 have significantly better CDC functional activity than mAb 271 (rituximab-sequence antibody).

ADCC activity was assayed by loading target Daudi cells with ^{51}Cr, 200 μCi for 2 hours, and following with a wash. The washed Daudi cells were incubated with titrated concentrations of respective mAbs and freshly prepared PBMCs (NK source) at 50:1 for 4 hours. Controls included spontaneous and maximum ^{51}Cr release, mAb with Daudi cells alone, and PBMC with Daudi cells alone at all concentrations. Supernatents were then processed and released ^{51}Cr counted via gamma counter. Antibody-specific ADCC was determined by subtracting out PBMC contribution to lysis.

As can be seen in Figure 9, ADCC activity increased with increasing mAb concentration for all mAbs tested. At all mAb concentrations, the mAb 1236, mAb 1237, and mAb1238 have ADCC activity that is equivalent to that observed for the control mAb 271 (rituximab-sequence antibody).

Apoptosis activity was also assessed using a direct apoptosis assay that is CDC and ADCC independent. In this manner, Ramos cells (NHL) were incubated with 10, 2, 0.4, or 0.08 μg/ml of the respective mAb in the presence of cross-linking antibody (5 μg/ml goat anti-human IgG Fc) for 18 hours at 37°C. mAb 11 served as the isotype control. Cells were harvested and washed after 18 hours and incubated with Annexin V-APC and Propidium Iodide for 15 minutes at 25°C. Cells were analyzed by flow cytometry for the presence of Annexin V positive/PI negative cells.

As can be seen in Figure 10, the optimized humanized mAbs 1236, 1237, and 1238 are as effective at inducing apoptosis as mAb 271 (rituximab-sequence antibody).

Example 3: Further Optimization of mAb 1237

B-cell Chronic Lymphocytic Leukemia (B-CLL) cells express lower levels of CD20 than NHL (Non-Hodgkins Lymphoma) cells. Testing showed that optimized mAb 1236, mAb 1237, and mAb 1238 demonstrate weak CDC of B-CLL cells. As a next step the mAb 1237 was selected and subjected to mutagenesis to screen for improvements in affinity and thus improvements in the lysis of B-CLL cells. The

affinity improvement strategy comprised randomizing specific positions in the V_H and the V_K domains, selecting mutant V_H domains with L373, selecting mutant V_K domains with H1570, combining separate beneficial mutations in either V_H or V_K, and combining mutant V_H with mutant V_K domains. The new mutants identified are shown in Table 3.

Table 3. New mutants identified. Residue positions within the V_H and V_K domains are with reference to the Kabat numbering system.

mAb	V_H	V_K
1588	**H1638 (H1570 S31F)**	**L373**
1589	**H1639 (H1570 D56A)**	**L373**
1590	**H1640 (H1570 D56L)**	**L373**
1652	**H1639 (H1570 D56A)**	**L419 (L373 T92Q)**
1692	**H1670 (H1570 N101G)**	**L373**

The optimized mAb 1588 comprises the H1638 V_H domain (SEQ ID NO:15) (which is the H1570 V_H domain with an S→F substitution at Kabat-numbering position 31 of H1570, which corresponds to residue 31 of the H1570 amino acid sequence set forth in SEQ ID NO:13) and the L373 V_L domain (SEQ ID NO:10). This S→F substitution results in an optimized CDR1 (see SEQ ID NO:7) within the H1638 V_H domain.

The optimized mAb 1589 comprises the H1639 V_H domain (SEQ ID NO:16) (which is the H1570 V_H domain with a D→A substitution at Kabat-numbering position 56 of H1570, which corresponds to residue 57 of the H1570 amino acid sequence set forth in SEQ ID NO:13) and the L373 V_L domain (SEQ ID NO:10). This D→A substitution results in an optimized CDR2 (see SEQ ID NO:5; encoded by SEQ ID NO:27) within the H1639 V_H domain.

The optimized mAb1590 comprises the H1640 V_H domain (SEQ ID NO:17) (which is the H1570 V_H domain with a D→L substitution at Kabat-numbering position 56 of H1570, which corresponds to residue 57 of the H1570 amino acid sequence set forth in SEQ ID NO:13) and the L373 V_L domain (SEQ ID NO:10). This D→L substitution results in an optimized CDR2 (see SEQ ID NO:6) within the H1640 V_H domain.

The optimized mAb 1652 comprises the H1639 V_H domain (SEQ ID NO:16) and the L419 V_L domain (SEQ ID NO:11) (which is the L373 V_L domain with a T→Q substitution at Kabat-numbering position 92 of L373, which corresponds to residue 91 of the L373 amino acid sequence set forth in SEQ ID NO:10). This T→Q substitution results in an optimized CDR3 (see SEQ ID NO:8) within the L419 V_L domain.

The optimized mAb 1692 comprises the H1670 V_H domain (SEQ ID NO:18) (which is the H1570 V_H domain with an N→G substitution at Kabat-numbering position 101 of H1570, which corresponds to residue 109 of the H1570 amino acid sequence set forth in SEQ ID NO:13). This N→G substitution results in an optimized CDR3 (see SEQ ID NO:4) within the H1670 V_H domain.

The amino acid sequences for the H1639 V_H domain (SEQ ID NO:16) and L373 V_L domain (SEQ ID NO:10) of the mAb 1589 are shown in Figure 11, and the respective coding sequences are shown in Figure 12; see also SEQ ID NO:22 (coding sequence for H1639) and SEQ ID NO:23 (coding sequence for L373).

Example 4: Binding and Functional Characteristics of Additional Optimized Humanized Murine Anti-CD20 Monoclonal Antibodies

The optimized humanized mAb 1237 of Example 1, and further optimized humanized mAbs 1588, 1589, 1590, 1652, and 1692 described in Example 3 were assessed for their respective binding and functional characteristics. For each assay, mAb 271, having the identical sequence to rituximab (IDEC-C2B8; Rituxan®; IDEC Pharmaceuticals Corp., San Diego, California), served as the positive control.

Binding specificity was assessed by Flow Cytometry, which tabulates Mean Fluorescence Intensities (MFI) of staining on CD20 positive ($CD20^+$) and CD20 negative cell lines. Flow cytometry analysis demonstrated binding of these optimized humanized anti-CD20 mAbs to CD20, as can be seen in Table 4.

Table 4. Binding to CD20 membrane positive and negative cell lines
Flow Cytometry Results (Mean Fluorescence Intensities).

	Daudi	EHEB	CHO.CD20	HL60	K562	CHO Vector
mAb	CD20 Positive	CD20 Positive	CD20 Positive	CD20 Negative	CD20 Negative	CD20 Negative
271	941	130	81	6	3	4
1237	1373	161	106	8	9	6
1588	2068	338	140	9	6	4
1589	1029	345	140	8	6	5
1590	1627	429	135	8	7	6
1652	1422	529	135	6	6	5
1692	1671	450	137	7	4	5
11 (Negative Control)	6	8	5	7	4	6

Epitope conservation was assessed by examining the ability of the mAb 2H7 to block binding. In this manner, mouse 2H7 (or Isotype Ig as control) was added to EB1 cells, then candidate human mAbs and anti-human Ig Alexa 647 were added. Binding was detected by FMAT. A decrease in the signal indicates blocking and common epitope usage.

As can be seen in Figure 13, all tested antibodies recognized identical or overlapping epitopes.

Complement dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) were assessed. For CDC, several assays were used. The first radioactive assay comprised loading target cells with ^{51}Cr, 200μCi for 2 hours. Washed target cells (Daudi (NHL) and B-CLL lines) were incubated with titrated concentrations of mAb and human serum (complement source) for 4 hours. Controls included spontaneous and maximum ^{51}Cr release, mAb with cells alone, and serum with cells alone at all concentrations. Supernatants were processed and released ^{51}Cr counted with a gamma counter. Antibody specific CDC was determined by subtracting out mAb and serum alone contributions to lysis. Results for Daudi and two B-CLL cell lines are shown in Figure 14 and Figures 15A and 15B, respectively. For Daudi cells, the optimized mAbs generally mediated greater CDC function than

mAb 271 (rituximab-sequence antibody), particularly at lower concentrations. For B-CLL cells, the optimized mAbs showed increased CDC function over the mAb 271 (rituximab-sequence antibody).

CDC function was also assessed with an antibody off-rate assay. In this manner, target $CD20^+$ Daudi cells were loaded with ^{51}Cr, 200 μCi for 2 hours, and then washed. The washed Daudi cells were incubated with 10 μg/ml of respective mAb for 15 minutes at 25°C. Target Daudi cells were then washed thoroughly and incubated with 6% human serum (complement source) for 45' following 0, 1, 2, 4, and 6 hour incubations at 37°C. Controls included spontaneous and maximum ^{51}Cr release, mAb with Daudi cells alone, and serum with Daudi cells alone at all concentrations. Supernatents were then processed and released ^{51}Cr counted via gamma counter. Antibody-specific CDC was determined by subtracting out mAb and serum alone contributions to lysis.

As can be seen in Figure 16, all optimized mAbs have significantly better CDC functional activity than mAb 271 (rituximab-sequence antibody).

CDC function was also assessed with a non-radioactive (Alamar Blue™) CDC assay. In this manner, target cells (two each of NHL, B-CLL, and normal B cell lines) were washed with titrated concentrations of mAb and human serum (complement source). Controls included target cells and antibody without human serum (spontaneous cell death), target cells and serum without antibody (background lysis), and wells of target cells with 10% SDS (or 5% Triton X100 in water) replacing the antibody and serum (for maximum cell death). Alamar Blue was added to each well 2 hours later, and fluorescence read after an overnight incubation. In this assay, more fluorescence indicates more live cells and less cytotoxicity due to CDC.

AlamarBlue™ is an oxidation-reduction indicator that fluoresces in response to metabolic activities in proliferating cells. AlamarBlue™ is similar to tetrazolium salts (MTT), in that both can detect changes in cell's metabolism, but AlamarBlue™ is less toxic and more sensitive than MTT. Results obtained in the measurement of cell mediated cytotoxicity comparing the use of AlamarBlue™ with ^{51}Cr release assays indicate that the AamarBlue™ method is as specific as determination of ^{51}Cr release. Results are shown in Figures 17A (Daudi NHL cell line) and 17B (WIL2-S

NHL cell line), Figures 18A (EHEB B-CLL cell line) and 18B (Mec-1 B-CLL cell line), and Figures 19A (SS BLCL "normal" B cells) and 19B (Mel K BLCL "normal" B cells).

The radioactive assay is a 4 hour assay while the non-radioactive assay is an 18-20 hour assay. The optimized mAb candidates usually gave greater lysis in non-radioactive assays, as compared to controls. The non-radioactive assays may represent a more similar situation to the *in-vivo* setting, as the drug would be present at high concentrations for much longer than 4 hours in an *in-vivo* setting.

To summarize the results, the CDC activity of the optimized mAbs is at least equal to mAb 271 (rituximab-sequence antibody) on Daudi NHL cells. The optimized mAbs are better than mAb 271 in the off-rate CDC assay. The lower off-rate indicates that the affinity of the optimized MAbs is higher than the affinity of mAb 271. Finally, the optimized mAbs are better than mAb 271 in lysing B-CLL cell lines and normal B cell targets.

ADCC activity was assayed by loading target cells (Daudi and B-CLL) with ^{51}Cr, 200 μCi for 2 hours, and following with a wash. The washed target cells were incubated with titrated concentrations of respective mAbs and freshly prepared PBMCs (NK source) at 50:1 for 4 hours. Controls included spontaneous and maximum ^{51}Cr release, mAb with target cells alone, and PBMC with target cells alone at all concentrations. Supernatents were then processed and released ^{51}Cr counted via gamma counter. Antibody-specific ADCC was determined by subtracting out PBMC contribution to lysis.

As can be seen in Figure 20 (Daudi NHL cells) and Figure 21 (MEC-1 B-CLL cells), ADCC activity increased with increasing mAb concentration for all mAbs tested. The optimized mAbs had ADCC activity that is similar to that observed for the control mAb 271 (rituximab-sequence antibody).

Apoptosis activity was also assessed using a direct apoptosis assay that is CDC and ADCC independent. In this manner, Ramos cells (NHL) were incubated with 2, 0.4, or 0.08 μg/ml of the respective optimized mAb or control mAb 271 in the presence of cross-linking antibody (5 μg/ml goat anti-human IgG Fc) for 18 hours at

37°C. Cells were harvested and washed after 18 hours and incubated with Annexin V-APC and Propidium Iodide for 15 minutes at 25°C. Cells were analyzed by flow cytometry for the presence of Annexin V positive/PI negative cells.

As can be seen in Figure 22, the optimized mAbs are as effective at inducing apoptosis as mAb 271 (rituximab-sequence antibody).

Cell killing activity was also assessed using a whole blood assay. The protocol was similar to CDC or ADCC assays, except whole blood is used instead of serum (CDC) or purified PBMCs (ADCC). In this assay, the lysis of target cells is due to cumulative activity of CDC, ADCC, and apoptosis.

Results of this whole blood assay are shown in Figure 23 (Daudi NHL cells), Figures 24A (EHEB B-CLL cells) and 24B (MEC-1 B-CLL cells), and Figure 25 (Mel K "normal" B cells). As can be seen in these figures, the optimized mAbs have equivalent or better lysis against these respective target cells as mAb 271 (rituximab-sequence antibody).

Example 5: Administration of the Optimized Humanized Murine Anti-CD20 Monoclonal Antibody 1589 Prolongs Survival in a Daudi Cell Xenograft Mouse Model

Daudi NHL cells are a human B cell lymphoma cell line that grow systematically in SCID mice. Growth of Daudi cells results in paralysis in the mice (Ghetie *et al.* (1990) *Int J Cancer* 45:481-485). Studies were performed to determine if the anti-CD20 antibodies of the invention effect the survival of mice with Daudi cell xenografts.

SCID mice were bred and maintained under pathogen-free conditions. Daudi NHL cells (8×10^6) were injected intravenously into the tail vein of three groups of five SCID mice (15 mice total). On days 7 and 9 post-tumor injection, the mice were injected by the same route with PBS, 50 μg human IgG1 or 50 μg mAb 1589. Animals were observed three times per week and sacrificed at the first signs of hind limb paralysis. As shown in Figure 26, the monoclonal antibody 1589 prolonged the survival of mice with the human Daudi cell xenograft.

Example 6: Outline of Sequences and Antibody Design

The sequence identifiers for the various optimized CDRs, variable heavy and light domains comprising these optimized CDRs, coding sequences for these elements, and variable heavy domain for the original humanized anti-CD20 antibody are summarized below. Table 5 below outlines the anti-CD20 antibody design.

SEQUENCE IDENTIFIERS:

1. CDR3 of H1569 – a.a.
2. CDR3 of H1570 – a.a.
3. CDR3 of H1571 – a.a.
4. CDR3 of H1670 – a.a.
5. CDR2 of H1639 – a.a.
6. CDR2 of H1640 – a.a.
7. CDR1 of H1638 – a.a.
8. CDR3 of L419 – a.a.
9. CDR2 of L373 – a.a.
10. L373 – a.a.
11. L419 – a.a.
12. H1569 – a.a.
13. H1570 – a.a.
14. H1571 – a.a.
15. H1638 – a.a.
16. H1639 – a.a.
17. H1640 – a.a.
18. H1670 – a.a.
19. H1569 – nt seq
20. H1570 – nt seq
21. H1571 – nt seq
22. H1639 – nt seq
23. L373 – nt seq
24. CDR3 of H1569 – nt seq
25. CDR3 of H1570 – nt seq
26. CDR3 of H1571 – nt seq
27. CDR2 of H1639 – nt seq
28. CDR2 of L373 – nt seq
29. H1286 – a.a.
30. CDR3 of H1286 – a.a.

Table 5. Antibody design.

ANTIBODY NAME	V_H DOMAIN	V_L DOMAIN
Parent humanized murine anti-CD20	H1286 (SEQ ID NO:29)	L373 (SEQ ID NO:10)
1236	H1569 (SEQ ID NO:12)	L373 (SEQ ID NO:10)
1237	H1570 (SEQ ID NO:13)	L373 (SEQ ID NO:10)
1238	H1571 (SEQ ID NO:14)	L373 (SEQ ID NO:10)
1588	H1638 (SEQ ID NO:15)	L373 (SEQ ID NO:10)
1589	H1639 (SEQ ID NO:16)	L373 (SEQ ID NO:10)
1590	H1640 (SEQ ID NO:17)	L373 (SEQ ID NO:10)
1593	H1570 (SEQ ID NO:13)	L419 (SEQ ID NO:11)
1652	H1639 (SEQ ID NO:16)	L419 (SEQ ID NO:11)
1692	H1670 (SEQ ID NO:18)	L373 (SEQ ID NO:10)

Many modifications and other embodiments of the inventions set forth herein will come to mind to one skilled in the art to which these inventions pertain having the benefit of the teachings presented in the foregoing descriptions and the associated drawings. Therefore, it is to be understood that the inventions are not to be limited to the specific embodiments disclosed and that modifications and other embodiments are intended to be included within the scope of the appended claims and list of embodiments disclosed herein. Although specific terms are employed herein, they are used in a generic and descriptive sense only and not for purposes of limitation.

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

WHAT IS CLAIMED IS:

1. An immunoglobulin that specifically binds CD20 and exhibits increased complement-dependent cytotoxicity (CDC) as compared to rituximab, wherein said immunoglobulin comprises:

a) a variable heavy (V_H) domain comprising:

i) a complementarity-determining region 1 (CDR1) comprising the amino acid sequence set forth in SEQ ID NO:7 or the amino acid residues 25 through 35 of SEQ ID NO: 12;

ii) a complementarity-determining region 2 (CDR2) comprising the amino acid sequence set forth in SEQ ID NO:5 or 6 or the amino acid residues 50 through 66 of SEQ ID NO:12; and

iii) a complementarity-determining region 3 (CDR3) comprising an amino acid sequence selected from the group consisting of SEQ ID NOS:1-4; and

b) a variable light (V_L) domain comprising:

i) a CDR1 comprising the amino acid sequence set forth in amino acid residues 24 through 33 of SEQ ID NO:10;

ii) a CDR2 comprising the amino acid sequence set forth in SEQ ID NO:9; and

iii) a CDR3 comprising the amino acid sequence set forth in SEQ ID NO:8 or the amino acid residues 88 through 96 of SEQ ID NO:10.

2. The immunoglobulin of claim 1, wherein said immunoglobulin is an IgG1 kappa immunoglobulin.

3. The immunoglobulin of claim 2, wherein said immunoglobulin comprises a human IgG1 constant region within a heavy chain of said immunoglobulin and a human kappa constant region within a light chain of said immunoglobulin.

4. The immunoglobulin of claim 3, wherein said immunoglobulin comprises fully or partially human framework regions within the variable domain of said heavy chain and within the variable domain of said light chain.

5. The immunoglobulin of claim 3, wherein said immunoglobulin comprises murine framework regions within the variable domain of said heavy chain and within the variable domain of said light chain.

6. The immunoglobulin of claim 1, wherein the immunoglobulin comprises an immunoglobulin light chain comprising the amino acid sequence set forth in SEQ ID NO:10 or SEQ ID NO:11.

7. The immunoglobulin of claim 1, wherein said immunoglobulin comprises an immunoglobulin heavy chain comprising an amino acid sequence selected from the group consisting of SEQ ID NOS:12-18.

8. The immunoglobulin of claim 1, wherein said immunoglobulin comprises a heavy chain comprising an amino acid sequence selected from the group consisting of sequences set forth in SEQ ID NO:12-18 and a light chain comprising the amino acid sequence set forth in SEQ ID NO:10 or SEQ ID NO:11.

9. The immunoglobulin of claim 8, wherein said heavy chain comprises the sequence set forth in SEQ ID NO:12 and said light chain comprises the sequence set forth in SEQ ID NO:10.

10. The immunoglobulin of claim 8, wherein said heavy chain comprises the sequence set forth in SEQ ID NO:13 and said light chain comprises the sequence set forth in SEQ ID NO:10.

11. The immunoglobulin of claim 8, wherein said heavy chain comprises the sequence set forth in SEQ ID NO:14 and said light chain comprises the sequence set forth in SEQ ID NO:10.

12. The immunoglobulin of claim 8, wherein said heavy chain comprises the sequence set forth in SEQ ID NO:15 and said light chain comprises the sequence set forth in SEQ ID NO:10.

13. The immunoglobulin of claim 8, wherein said heavy chain comprises the sequence set forth in SEQ ID NO:16 and said light chain comprises the sequence set forth in SEQ ID NO:10.

14. The immunoglobulin of claim 8, wherein said heavy chain comprises the sequence set forth in SEQ ID NO:17 and said light chain comprises the sequence set forth in SEQ ID NO:10.

15. The immunoglobulin of claim 8, wherein said heavy chain comprises the sequence set forth in SEQ ID NO:18 and said light chain comprises the sequence set forth in SEQ ID NO:10.

16. The immunoglobulin of claim 8, wherein said heavy chain comprises the sequence set forth in SEQ ID NO:13 and said light chain comprises the sequence set forth in SEQ ID NO:11.

17. The immunoglobulin of claim 8, wherein said heavy chain comprises the sequence set forth in SEQ ID NO:16 and said light chain comprises the sequence set forth in SEQ ID NO:11.

18. An immunoglobulin that specifically binds CD20 and exhibits increased complement-dependent cytotoxicity (CDC) as compared to rituximab, wherein said immunoglobulin comprises a variable heavy (V_H) domain, wherein said V_H domain is selected from the group consisting of:

a) a V_H domain comprising ~~an~~ the amino acid sequence selected from the group consisting of SEQ ID NOS:12-18;

b) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:12, wherein said V_H domain comprises the following residues:

i) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:12; and

ii) the asparagine (Asn) residue at the position corresponding to residue 110 of SEQ ID NO:12;

c) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:13, wherein said V_H domain comprises the following residues:

i) the alanine (Ala) residue at the position corresponding to residue 103 of SEQ ID NO:13;

ii) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:13; and

iii) the asparagine (Asn) residue at the position corresponding to residue 110 of SEQ ID NO:13;

d) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:14, wherein said V_H domain comprises the following residues:

i) the alanine (Ala) residue at the position corresponding to residue 103 of SEQ ID NO:14;

ii) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:14; and

iii) the aspartic acid (Asp) residue at the position corresponding to residue 110 of SEQ ID NO:14;

e) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:15, wherein said V_H domain comprises the following residues:

i) the phenylalanine (Phe) residue at the position corresponding to residue 31 of SEQ ID NO:15;

ii) the alanine (Ala) residue at the position corresponding to residue 103 of SEQ ID NO:15;

iii) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:15; and

iv) the asparagine (Asn) residue at the position corresponding to residue 110 of SEQ ID NO:15;

f) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:16, wherein said V_H domain comprises the following residues:

i) the alanine (Ala) residue at the position corresponding to residue 57 of SEQ ID NO:16;

ii) the alanine (Ala) residue at the position corresponding to residue 103 of SEQ ID NO:16;

iii) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:16; and

iv) the asparagine (Asn) residue at the position corresponding to residue 110 of SEQ ID NO:16;

g) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:17, wherein said V_H domain comprises the following residues:

i) the leucine (Leu) residue at the position corresponding to residue 57 of SEQ ID NO:17;

ii) the alanine (Ala) residue at the position corresponding to residue 103 of SEQ ID NO:17;

iii) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:17; and

iv) the asparagine (Asn) residue at the position corresponding to residue 110 of SEQ ID NO:17; and

h) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:18, wherein said V_H domain comprises the following residues:

i) the alanine (Ala) residue at the position corresponding to residue 103 of SEQ ID NO:18;

ii) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:18;

iii) the glycine (Gly) residue at the position corresponding to residue 109 of SEQ ID NO:18; and

iv) the asparagine (Asn) residue at the position corresponding to residue 110 of SEQ ID NO:18.

19. The immunoglobulin of claim 18, wherein said immunoglobulin comprises a variable light (V_L) domain, wherein said V_L domain is selected from the group consisting of:

a) a V_L domain comprising the sequence set forth in SEQ ID NO:10 or SEQ ID NO:11;

b) a V_L domain comprising an amino acid sequence having at least 90% sequence identity to the sequence set forth in SEQ ID NO:10 or SEQ ID NO:11;

c) a V_L domain comprising an amino acid sequence having at least 85% sequence identity to the sequence set forth in SEQ ID NO:10, wherein said V_L domain comprises the complementarity-determining region 2 (CDR2) set forth in SEQ ID NO:9; and

d) a V_L domain comprising an amino acid sequence having at least 85% sequence identity to the sequence set forth in SEQ ID NO:11, wherein said V_L domain comprises at least one of:

i) the complementarity-determining region 2 (CDR2) set forth in SEQ ID NO:9; and

ii) the glutamine (Gln) residue at the position corresponding to residue 91 of SEQ ID NO:11.

20. The immunoglobulin of claim 18 or 19, wherein said immunoglobulin is an IgG1 kappa immunoglobulin.

21. The immunoglobulin of claim 21, wherein said immunoglobulin comprises a human IgG1 constant region within a heavy chain of said immunoglobulin and a human kappa constant region within a light chain of said immunoglobulin.

22. An immunoglobulin comprising a variable heavy (V_H) domain and a variable light (V_L) domain, wherein said V_H domain comprises a polypeptide having an amino acid sequence selected from the group consisting of SEQ NOS:12-18, and wherein said V_L domain comprises a polypeptide having an amino acid sequence set forth in SEQ ID NO:10 or SEQ ID NO:11, wherein said immunoglobulin exhibits increased complement-dependent cytotoxicity (CDC) as compared to rituximab.

23. The immunoglobulin of any one of claims 1 through 22, further comprising a heterologous polypeptide fused thereto.

24. The immunoglobulin of any one of claims 1 through 22, wherein said immunoglobulin is conjugated to an agent selected from the group consisting of a therapeutic agent, a prodrug, a peptide, a protein, an enzyme, a virus, a lipid, a biological response modifier, a pharmaceutical agent, and PEG.

25. A composition comprising the immunoglobulin of any one of claims 1 through 24, and a carrier.

26. An isolated polynucleotide comprising a nucleic acid encoding a variable heavy (V_H) domain of an immunoglobulin heavy chain, wherein said V_H domain is selected from the group consisting of:

a) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:12, wherein said V_H domain comprises the following residues:

i) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:12; and

ii) the asparagine (Asn) residue at the position corresponding to residue 110 of SEQ ID NO:12;

b) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:13, wherein said V_H domain comprises the following residues:

i) the alanine (Ala) residue at the position corresponding to residue 103 of SEQ ID NO:13;

ii) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:13; and

iii) the asparagine (Asn) residue at the position corresponding to residue 110 of SEQ ID NO:13;

c) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:14, wherein said V_H domain comprises the following residues:

i) the alanine (Ala) residue at the position corresponding to residue 103 of SEQ ID NO:14;

ii) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:14; and

iii) the aspartic acid (Asp) residue at the position corresponding to residue 110 of SEQ ID NO:14;

d) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:15, wherein said V_H domain comprises the following residues:

i) the phenylalanine (Phe) residue at the position corresponding to residue 31 of SEQ ID NO:15;

ii) the alanine (Ala) residue at the position corresponding to residue 103 of SEQ ID NO:15;

iii) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:15; and

iv) the asparagine (Asn) residue at the position corresponding to residue 110 of SEQ ID NO:15;

e) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:16, wherein said V_H domain comprises the following residues:

i) the alanine (Ala) residue at the position corresponding to residue 57 of SEQ ID NO:16;

ii) the alanine (Ala) residue at the position corresponding to residue 103 of SEQ ID NO:16;

iii) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:16; and

iv) the asparagine (Asn) residue at the position corresponding to residue 110 of SEQ ID NO:16;

f) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:17, wherein said V_H domain comprises the following residues:

i) the leucine (Leu) residue at the position corresponding to residue 57 of SEQ ID NO:17;

ii) the alanine (Ala) residue at the position corresponding to residue 103 of SEQ ID NO:17;

iii) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:17; and

iv) the asparagine (Asn) residue at the position corresponding to residue 110 of SEQ ID NO:17;

g) a V_H domain comprising an amino acid sequence having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:18, wherein said V_H domain comprises the following residues:

i) the alanine (Ala) residue at the position corresponding to residue 103 of SEQ ID NO:18;

ii) the asparagine (Asn) residue at the position corresponding to residue 107 of SEQ ID NO:18; and

iii) the glycine (Gly) residue at the position corresponding to residue 109 of SEQ ID NO:18; and

iv) the asparagine (Asn) residue at the position corresponding to residue 110 of SEQ ID NO:18; and

h) a V_H domain comprising an amino acid sequence selected from the group consisting of SEQ ID NOs:12-18;

wherein an immunoglobulin comprising said V_H domain specifically binds to CD20 and exhibits increased complement-dependent cytotoxicity (CDC) as compared to rituximab.

27. The isolated polynucleotide of claim 26, wherein said V_H domain is encoded by a nucleic acid comprising a sequence selected from the group consisting of SEQ ID NOS:19-22.

28. A composition comprising the polynucleotide of claim 26 or 27.

29. A vector comprising the polynucleotide of any one of claims 26 through 28.

30. The vector of claim 29, wherein said polynucleotide is operably associated with a promoter.

31. A vector comprising the polynucleotide of claim 26 or 27, and a polynucleotide comprising a nucleic acid encoding a variable light (V_L) domain of an immunoglobulin light chain.

32. A vector comprising a polynucleotide encoding a variable heavy (V_H) domain of an immunoglobulin heavy chain and a polynucleotide encoding a variable light (V_L) domain of an immunoglobulin light chain, wherein said V_H domain comprises:

a) a complementarity-determining region 1 (CDR1) comprising the amino acid sequence set forth in SEQ ID NO:7or the amino acid residues 25 through 35 of SEQ ID NO: 12;

b) a complementarity-determining region 2 (CDR2) comprising the amino acid sequence set forth in SEQ ID NO:5 or SEQ ID NO:6 or the amino acid residues 50 through 66 of SEQ ID NO:12; and

c) a complementarity-determining region 3 (CDR3) comprising the amino acid sequence set forth in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4;

wherein said V_L domain comprises:

a) a CDR1 comprising the amino acid sequence set forth in amino acid residues 24 through 33 of SEQ ID NO:10;

b) a CDR2 comprising the amino acid sequence set forth in SEQ ID NO:9; and

c) a CDR3 comprising the amino acid sequence set forth in SEQ ID NO:8 or the amino acid residues 88 through 96 of SEQ ID NO:10

wherein an immunoglobulin comprising said V_H domain and said V_L domain specifically binds to CD20 and exhibits increased complement-dependent cytotoxicity (CDC) as compared to rituximab.

33. The vector of claim 31 or 32, wherein said polynucleotide encoding said V_H domain and said polynucleotide encoding said V_L domain are fused in frame, are co-transcribed from a single promoter operably associated therewith, and are cotranslated into a single-chain antibody or antigen-binding fragment thereof.

34. The vector of claim 31 or 32, wherein said polynucleotide encoding said V_H domain and said polynucleotide encoding said V_L domain are co-transcribed from a single promoter operably associated therewith, but are separately translated.

35. The vector of claim 34, further comprising an IRES sequence disposed between said polynucleotide encoding said V_H domain and said polynucleotide encoding said V_L domain.

36. The vector of claim 31 or 32, wherein said polynucleotide encoding said V_H domain and said polynucleotide encoding said V_L domain are separately transcribed, each being operably associated with a separate promoter.

37. The vector of claim 36, wherein said separate promoters are copies of the same promoter.

38. The vector of claim 36, wherein said separate promoters are non-identical.

39. A composition comprising the vector of any one of claims 29 through 38.

40. A composition comprising the immunoglobulin of claim 1.

41. A host cell comprising the polynucleotide of claim 26 or 27

42. A host cell comprising the vector of any one of claims 29 through 38.

43. A method of producing an immunoglobulin that specifically binds to CD20, comprising culturing the host cell of claim 41 or 42, and recovering said immunoglobulin.

44. An immunoglobulin produced by the method of claim 43.

45. The immunoglobulin of claim 44, wherein said immunoglobulin is an IgG1 kappa immunoglobulin.

46. The immunoglobulin of claim 45, wherein said immunoglobulin comprises a human IgG1 constant region within a heavy chain of said immunoglobulin and a human kappa constant region within a light chain of said immunoglobulin.

47. The immunoglobulin of claim 46, wherein said immunoglobulin comprises fully or partially human framework regions within the V_H domain of said heavy chain and within the V_L domain of said light chain.

48. The immunoglobulin of claim 46, wherein said immunoglobulin comprises murine framework regions within the V_H domain of said heavy chain and within the V_L domain of said light chain.

49. The use of an effective amount of a composition comprising the immunoglobulin according to any one of claims 1 through 24 in the manufacture of a medicament for treating a cancer in a subject, wherein said cancer is associated with CD20-expressing cells in said subject.

50. The use of claim 49, wherein said cancer is selected from the group consisting of a non-Hodgkins lymphoma, chronic lymphocytic leukemia, multiple myeloma, B cell lymphoma, high-grade B cell lymphoma, intermediate-grade B cell lymphoma, low-grade B cell lymphoma, B cell acute lympohoblastic leukemia, myeloblastic leukemia, and Hodgkin's disease.

51. The use of an effective amount of the immunoglobulin according to any one of claims 1 through 24 in the manufacture of a medicament for inhibiting growth or differentiation of a normal human B cell, wherein said medicament is to be contacted with said B cell.

52. The use of an effective amount of the immunoglobulin according to any one of claims 1 through 24 in the manufacture of a medicament for inhibiting growth of cancer cells of B cell lineage, wherein said medicament is to be contacted with said cancer cells.

53. The use of claim 52, wherein the cancer cells are from a cancer selected from the group consisting of non-Hodgkins lymphoma, chronic lymphocytic leukemia, multiple myeloma, B cell lymphoma, high-grade B cell lymphoma, intermediate-grade B cell lymphoma, low-grade B cell lymphoma, B cell acute lympohoblastic leukemia, myeloblastic leukemia, and Hodgkin's disease.

54. The use of an effective amount of the immunoglobulin according to any one of claims 1 through 24 in the manufacture of a medicament for treating an autoimmune disease or inflammatory disease in a subject, wherein said disease is associated with CD20-expressing cells in said subject.

55. The use of claim 54, wherein said autoimmune disease or inflammatory disease is selected from the group consisting of systemic lupus erythematosus (SLE), discoid lupus, lupus nephritis, sarcoidosis, juvenile arthritis, rheumatoid arthritis, psoriatic arthritis, Reiter's syndrome, ankylosing spondylitis, gouty arthritis, rejection of an organ or tissue transplant, graft versus host disease, multiple sclerosis, hyper IgE syndrome, polyarteritis nodosa, primary biliary cirrhosis, inflammatory bowel disease, Crohn's disease, celiac's disease (gluten-sensitive enteropathy), autoimmune hepatitis, pernicious anemia, autoimmune hemolytic anemia, psoriasis, scleroderma, myasthenia gravis, autoimmune thrombocytopenic purpura, autoimmune thyroiditis, Grave's disease, Hashimoto's thyroiditis, immune complex disease, chronic fatigue immune dysfunction syndrome (CFIDS), polymyositis and dermatomyositis, cryoglobulinemia, thrombolysis, cardiomyopathy, pemphigus vulgaris, pulmonary interstitial fibrosis, sarcoidosis, Type I and Type II diabetes mellitus, type 1, 2, 3, and 4 delayed-type hypersensitivity, allergy or allergic disorders, asthma, Churg-Strauss syndrome (allergic granulomatosis), atopic dermatitis, allergic and irritant contact dermatitis, urtecaria, IgE-mediated allergy, atherosclerosis, vasculitis, idiopathic inflammatory myopathies, hemolytic disease, Alzheimer's disease, and chronic inflammatory demyelinating polyneuropathy.

56. A pharmaceutical composition comprising the immunoglobulin according to any one of claims 1 through 24.

SEQUENCE LISTING

<110> Ernest S. Smith
Terrence L. Fisher, Jr.

<120> Anti-CD20 Antibodies and Methods of Use

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

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

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

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            20   25   30

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        35                  40                  45
Gly Ala Ile Tyr Pro Gly Asn Gly Leu Thr Ser Tyr Asn Gln Lys Phe
    50                  55                  60
Lys Gly Lys Ala Thr Ile Thr Ala Asp Lys Ser Thr Ser Thr Ala Tyr
65                  70                  75                  80
Met Glu Leu Ser Ser Leu Arg Ser Asp Asp Thr Ala Val Tyr Tyr Cys
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            100                 105                 110
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<220>
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            20                  25                  30
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        35                  40                  45
Gly Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr Asn Gln Lys Phe
    50                  55                  60
Lys Gly Lys Ala Thr Ile Thr Ala Asp Lys Ser Thr Ser Thr Ala Tyr
65                  70                  75                  80
Met Glu Leu Ser Ser Leu Arg Ser Asp Asp Thr Ala Val Tyr Tyr Cys
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Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

aat atg cac tgg gtg cga cag gcc cct gga caa ggg cta gag tgg att   144
Asn Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Ile
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 65                  70                  75                  80

atg gag ctc agc agc ctg aga tct gac gac acg gcc gtg tat tac tgt   288
Met Glu Leu Ser Ser Leu Arg Ser Asp Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

gca aga tcg act tac tac ggc ggt gac tgg aac ttc aat aac tgg ggc   336
Ala Arg Ser Thr Tyr Tyr Gly Gly Asp Trp Asn Phe Asn Asn Trp Gly
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 1               5                  10                  15

tca gtg aag gtt tcc tgc aag gca tct ggg tac acc ttc acc agt tac   96
Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

aat atg cac tgg gtg cga cag gcc cct gga caa ggg cta gag tgg att   144
Asn Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Ile
        35                  40                  45

gga gct att tat ccc gga aat ggt gat act tcc tac aat cag aag ttc   192
Gly Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr Asn Gln Lys Phe
    50                  55                  60

aaa ggc aag gcc aca ata act gca gac aaa tcc acg agc aca gcc tac   240
Lys Gly Lys Ala Thr Ile Thr Ala Asp Lys Ser Thr Ser Thr Ala Tyr
 65                  70                  75                  80

atg gag ctc agc agc ctg aga tct gac gac acg gcc gtg tat tac tgt   288
Met Glu Leu Ser Ser Leu Arg Ser Asp Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

gca aga tcg act tac tac gcc ggt gac tgg aac ttc aat aac tgg ggc   336
Ala Arg Ser Thr Tyr Tyr Ala Gly Asp Trp Asn Phe Asn Asn Trp Gly
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tca gtg aag gtt tcc tgc aag gca tct ggg tac acc ttc acc agt tac   96
Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

aat atg cac tgg gtg cga cag gcc cct gga caa ggg cta gag tgg att   144
Asn Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Ile
        35                  40                  45

gga gct att tat ccc gga aat ggt gat act tcc tac aat cag aag ttc   192
Gly Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr Asn Gln Lys Phe
    50                  55                  60

aaa ggc aag gcc aca ata act gca gac aaa tcc acg agc aca gcc tac   240
Lys Gly Lys Ala Thr Ile Thr Ala Asp Lys Ser Thr Ser Thr Ala Tyr
65                  70                  75                  80

atg gag ctc agc agc ctg aga tct gac gac acg gcc gtg tat tac tgt   288
Met Glu Leu Ser Ser Leu Arg Ser Asp Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

gca aga tcg act tac tac gcc ggt gac tgg aac ttc aat gac tgg ggc   336
Ala Arg Ser Thr Tyr Tyr Ala Gly Asp Trp Asn Phe Asn Asp Trp Gly
            100                 105                 110

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tca gtg aag gtt tcc tgc aag gca tct ggg tac acc ttc acc agt tac   96
Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

aat atg cac tgg gtg cga cag gcc cct gga caa ggg cta gag tgg att   144
Asn Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Ile
        35                  40                  45

gga gct att tat ccc gga aat ggt gct act tcc tac aat cag aag ttc   192
Gly Ala Ile Tyr Pro Gly Asn Gly Ala Thr Ser Tyr Asn Gln Lys Phe
    50                  55                  60
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Lys Gly Lys Ala Thr Ile Thr Ala Asp Lys Ser Thr Ser Thr Ala Tyr
 65                  70                  75                  80

atg gag ctc agc agc ctg aga tct gac gac acg gcc gtg tat tac tgt   288
Met Glu Leu Ser Ser Leu Arg Ser Asp Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

gca aga tcg act tac tac gcc ggt gac tgg aac ttc aat aac tgg ggc   336
Ala Arg Ser Thr Tyr Tyr Ala Gly Asp Trp Asn Phe Asn Asn Trp Gly
            100                 105                 110

cag gga acc ctg gtc acc gtc tcc tca                              363
Gln Gly Thr Leu Val Thr Val Ser Ser
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gac aga gtc acc atc act tgc cgg gca agt tcg agc gtt agt tat ata   96
Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Ser Ser Val Ser Tyr Ile
            20                  25                  30

cat tgg ttt cag cag aaa cca ggg aaa gcc cct aaa ccc ctg atc tat   144
His Trp Phe Gln Gln Lys Pro Gly Lys Ala Pro Lys Pro Leu Ile Tyr
        35                  40                  45

gct gca tcc agt ttg caa agt ggg gtc cca tca agg ttc agt ggc agt   192
Ala Ala Ser Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly Ser
    50                  55                  60

gga tct ggg aca gat tac act ctc acc atc agc agt ctg caa cct gag   240
Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu
 65                  70                  75                  80

gat ttc gca act tac tac tgt caa cag tgg act tcc aac ccg ccc act   288
Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Trp Thr Ser Asn Pro Pro Thr
                85                  90                  95

ttc ggc gga ggg acc aag ctc gag atc aaa                          318
Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
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			20					25					30		
Asn	Met	His	Trp	Val	Arg	Gln	Ala	Pro	Gly	Gln	Gly	Leu	Glu	Trp	Ile
		35					40					45			
Gly	Ala	Ile	Tyr	Pro	Gly	Asn	Gly	Asp	Thr	Ser	Tyr	Asn	Gln	Lys	Phe
	50					55					60				
Lys	Gly	Lys	Ala	Thr	Ile	Thr	Ala	Asp	Lys	Ser	Thr	Ser	Thr	Ala	Tyr
65					70					75					80
Met	Glu	Leu	Ser	Ser	Leu	Arg	Ser	Asp	Asp	Thr	Ala	Val	Tyr	Tyr	Cys
				85					90					95	
Ala	Arg	Ser	Thr	Tyr	Tyr	Gly	Gly	Asp	Trp	Tyr	Phe	Asn	Val	Trp	Gly
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