HUMAN ANTIBODIES TO HUMAN CD20 AND METHOD OF USING THEREOF

Field of the Invention

[0001] The present invention related to human antibodies and antibody fragments specific for human CD20, pharmaceutical compositions, and therapeutic methods thereof

Statement of Related Art

[0002] CD20 (also known as human B-lymphocyte-restricted differentiation antigen or Bp35; B-lymphocyte surface antigen B1, Leu-16, BM5, and LF5) is a hydrophobic transmembrane protein with a molecular weight of ~35 kD expressed on pre-B and mature B lymphocytes (Valentine et al. (1989) J Biol Chem 264:11282; Einfield et al. (1988) EMBO J 7:711-717). The amino acid sequence of human CD20 is shown in SEQ ID NO:1 (GenBank Accession No. NP_690605). Anti-CD20 antibodies are described in, for example, US 5,736,137, WO 2004/056312, and US 2004/0167319.

[0003] Methods for producing antibodies useful as human therapeutics include generation of chimeric antibodies and humanized antibodies (see, for example, US 6,949,245). See also, for example, WO 94/02602 and US 6,596,541 describing methods of generating genetically modified mice capable of producing antibodies useful for making human therapeutics.

BRIEF SUMMARY OF THE INVENTION

[0004] In a first aspect, the invention provides human antibodies, preferably recombinant human antibodies that specifically bind human CD20. These antibodies are characterized by specifically binding to human CD20 and by mediating the killing of B-cell lymphoma cells expressing CD20. The antibodies can be full-length (for example, an IgG1 or IgG4 antibody) or may comprise only an antigen-binding portion (for example, an Fab, $F(ab')_2$ or scFv fragment), and may be modified to effect functionality, e.g., to eliminate or enhance residual effector functions (Reddy et al. (2000) J. Immunol. 164:1925-1933).

[0005] The antibody or an antigen-binding fragment thereof specifically binds human CD20 and is capable of inducing complement dependent cytotoxicity (CDC) of cells expressing CD20 in the presence of complement, wherein the antibody at a concentration of about 10 nM or less induces 50% lysis of Daudi and RL cells in the presence of 5% normal human serum with complement. In preferred embodiments, the antibody concentration which induces 50% lysis is about 5 nM or less; about 2 nM or less; or about 1 nM or less. In one embodiment, the antibody or fragment thereof exhibiting an EC_{50} 0.2 nM or less as measured in Daudi cells, or an EC_{50} of 0.4 nM or less as measured by RL cells. In various embodiments, the antibody or antibody fragment is capable of increasing symptom free survival time between about 2-fold to about 9-fold or more, relative to control-treated animals in a mouse model of human lymphoma.

[0006] The antibody or fragment thereof specifically binds human CD20 and is capable of

inducing antibody-dependent cellular cytotoxicity (ADCC) of cells expressing CD20 in the presence of peripheral blood mononuclear cells (PBMC), wherein the antibody exhibits an EC_{50} of about 1 nM or less, as measured in Daudi cells. In preferred embodiments, the antibody exhibits an EC_{50} of about 50 pM or less; about 20 pM or less; about 10 pM or less. In a preferred embodiment, antibodies exhibits enhanced ADCC activity may comprise reduced levels of fucosylation, for example, about 5% fucose.

[0007] In one embodiment, the antibody or antigen-binding portion of the antibody of the invention comprises a heavy chain variable region (HCVR) sequence selected from the group consisting of SEQ ID NO:3, 19, 23, 27, 43, 47, 51, 67, 71, 75, 91, 95, 99, 115, 119, 123, 139, 143, 147, 163, 167, 171, 187, 191, 195, 211, 215, 219, 235, 239, 243, 259, 263, 267, 283, 287, 291, 307, 311, 315, 331, 335, 339, 355, 359, 363, 379, 383, 387, 395, and 403, or a substantially similar sequence thereof. In a preferred embodiment, the antibody or fragment comprises a HCVR sequence selected from the group consisting of SEQ ID NO:339, 195 and 243.

[0008] In a more specific embodiment, the antibody or antigen-binding fragment thereof further comprises a light chain variable region (LCVR) sequence selected from the group consisting of SEQ ID NO:11, 21, 25, 35, 45, 49, 59, 69, 73, 83, 93, 97, 107, 117, 121, 131, 141, 145, 155, 165, 169, 179, 189, 193, 203, 213, 217, 227, 237, 241, 251, 261, 265, 275, 285, 289, 299, 309, 313, 323, 333, 337, 347, 357, 361, 371, 381 and 385, or a substantially similar sequence thereof. In a preferred embodiment, the antibody or fragment comprises a LCVR selected from the group consisting of SEQ ID NO:347, 203 and 251.

[0009] In specific embodiments, the antibody or fragment thereof comprises HCVR/LCVR sequence pairs selected from the group consisting of SEQ ID NO:3/11, 19/21, 23/25, 27/35, 43/45, 47/49, 51/59, 67/69, 71/73, 75/83, 91/93, 95/97, 99/107, 115/117, 119/121, 123/131, 139/141, 143/145, 147/155, 163/165, 167/169, 171/179, 187/189, 191/193, 195/203, 211/213, 215/217, 219/227, 235/237, 239/241, 243/251, 259/261, 263/265, 267/275, 283/285, 287/289, 291/299, 307/309, 311/313, 315/323, 331/333, 335/337, 339/347, 355/357, 359/361, 363/371, 379/381 and 383/385. In a preferred embodiment, the antibody or fragment thereof comprises HCVR/LCVR sequence pair selected from the group consisting of SEQ ID NO:339/347, 195/203 and 243/251.

[0010] In a second aspect, the invention provides isolated nucleic acid molecules that encode an antibody or fragment thereof. In specific embodiments, the nucleic acid molecule encodes an HCVR wherein the nucleotide sequence is selected from the group consisting of SEQ ID NO:2, 18, 22, 26, 42, 46, 50, 66, 70, 74, 90, 94, 98, 114, 118, 122, 138, 142, 146, 162, 166, 170, 186, 190, 194, 210, 214, 218, 234, 238, 242, 258, 262, 266, 282, 286, 290, 306, 310, 314, 330, 334, 338, 354, 358, 362, 378, 382, 386, 394 and 402, or a substantially identical sequence thereof. In a related aspect, the invention provides an isolated nucleic acid molecule encoding an LCVR, wherein the nucleotide sequence is selected from the group consisting of SEQ ID NO:

10, 20, 24, 34, 44, 48, 58, 68, 72, 82, 92, 96, 106, 116, 120, 130, 140, 144, 154, 164, 168, 178, 188, 192, 202, 212, 216, 226, 236, 240, 250, 260, 264, 274, 284, 288, 298, 308, 312, 322, 332, 336, 346, 356, 360, 370, 380 and 384, or a substantially identical sequence thereof. In a preferred embodiment, the antibody or antibody fragment comprise an HCVR encoded by a nucleic acid molecule selected from the group consisting of SEQ ID NO:338, 194 and 242, and a LCVR encoded by a nucleic acid molecule selected from the group consisting of SEQ ID NO:346, 202 and 250, respectively.

[0011] In a third aspect, the invention features an antibody or antigen-binding fragment thereof, comprising a heavy chain CDR3 (HCDR3) comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 9, 33, 57, 81, 104, 129, 153, 177, 201, 225, 249, 273, 297, 321, 345, 369, 393, 401 and 409; and a light chain CDR3 (LCDR3) comprising an amino acid sequence selected from the group consisting of SEQ ID NO:17, 41, 65, 89, 113, 137, 161, 185, 209, 233, 257, 281, 305, 329, 353 and 377. In a preferred embodiment, the antibody or fragment thereof comprises a HCDR3 and LCDR3 sequence selected from the HCDR3/LCDR3 sequence pairs SEQ ID NO: 345/353, 201/209 and 249/257.

[0012] In a more specific embodiment, the antibody or fragment thereof further comprises a heavy chain CDR1 (HCDR1) domain sequence selected from the group consisting of SEQ ID NO:5, 29, 53, 77, 101, 125, 149, 173, 197, 221, 245, 269, 293, 317, 341, 365, 389, 397 and 405; a heavy chain CDR2 (HCDR2) domain sequence selected from the group consisting of SEQ ID NO:7, 31, 55, 79, 103, 127, 151, 175, 199, 223, 247, 271, 295, 319, 343, 367, 391, 399 and 407; a light chain CDR1 (LCDR1) domain sequence selected from the group consisting of SEQ ID NO:13, 37, 61, 85, 109, 133, 157, 181, 205, 229, 253, 277, 301, 325, 349 and 373; and a light chain CDR2 (LCDR2) domain sequence selected from the group consisting of SEQ ID NO:15, 39, 63, 87, 111, 135, 159, 183, 207, 231, 255, 279, 303, 327, 351 and 375. In a preferred embodiment, the antibody or fragment thereof comprises heavy and light chain CDRs sequences selected from the group consisting of SEQ ID NO:341, 343, 345, 349, 351 and 353; 197, 199, 201, 205, 207 and 209; and 245, 247, 249, 253, 255 and 257, respectively.

[0013] In a fourth aspect, the invention features isolated nucleic acid molecules encoding an antibody or antigen-binding fragments of the invention, wherein the nucleic acid molecules encoding a HCDR3 domain and a LCDR3 domain are selected from the group consisting of SEQ ID NO:9 and 16; 33 and 41; 57 and 65; 81 and 89; 104 and 113; 129 and 137; 153 and 161; 177 and 185; 201 and 209; 225 and 233; 249 and 257; 273 and 281; 297 and 305; 321 and 329; 345 and 353; and 369 and 377, respectively.

[0014] In a fifth aspect, the invention features an antibody or antigen-binding fragment, comprising a HCDR3 domain and a LCDR3 domain, wherein the HCDR3 domain comprises an amino acid sequence of the formula $X^1 - X^2 - X^3 - X^4 - X^5 - X^6 - X^7 - X^8 - X^9 - X^{10} - X^{11} - X^{12} - X^{13} - X^{14} - X^{15} - X^{16} - X^{17} - X^{18} - X^{19}$ (SEQ ID NO:412) wherein X^1= A, V or T; X^2=K; X^3=D; X^4= P, F or G; X^5= S or H; X^6=Y; X^7=G; X^8=S or H; X^9= G or F; X^{10}= S or Y; X^{11}= Y, N or S; X^{12}= Y, G or

H; X^{13}= G, L or S; X^{14}= Y, M or D; X^{15}= Y, D or V; X^{16}= G, V or absent; X^{17}= M or absent; X^{18}= D or absent; X^{19}= V or absent; and the LCDR3 domain comprises an amino acid sequence of the formula X^1 - X^2 - X^3 - X^4 - X^5 - X^6 - X^7 - X^8 –X^9 (SEQ ID NO:415), wherein X^1 = Q; X^2 = Q; X^3 = R or S; X^4 = N, Y or F; X^5 = N, D, or Y; X^6 = W; X^7 = P; X^8 = L; X^9 = T.

[0015] In a more specific embodiment, the antibody or antigen-binding fragment further comprises heavy and light chain CDR1 and CDR2 domains, wherein the HCDR1 domain comprises an amino acid sequence of the formula X^1 - X^2 - X^3 - X^4 - X^5 - X^6 - X^7 - X^8 (SEQ ID NO:410) wherein X^1=G; X^2=F or I; X^3=T; X^4=F; X^5= H, R or Y; X^6=D; X^7=Y; X^8=T or A; the HCDR2 domain comprises an amino acid sequence of the formula X^1 - X^2 - X^3 - X^4 - X^5 - X^6 - X^7 - X^8 (SEQ ID NO:411) wherein X^1=I; X^2=S; X^3=W; X^4=N; X^5=S; X^6= G or D; X^7=S, Y or T; X^8=I or L; the LCDR1 domain comprises an amino acid sequence of the formula X^1 - X^2 - X^3 - X^4 - X^5 - X^6 (SEQ ID NO:413) wherein X^1= Q; X^2=S; X^3= V or I; X^4= S; X^5 = S or R; X^6 = Y or N; and the LCDR2 domain comprises an amino acid sequence of the formula X^1 - X^2 - X^3 (SEQ ID NO:414) wherein X^1 = E, G or V; X^2 = A; X^3= S.

[0016] In a sixth aspect, the invention provides recombinant expression vectors carrying the nucleic acid molecules of the invention, and host cells into which such vectors have been introduced, as well as methods of making the antibodies or fragments thereof of the invention obtained by culturing the host cells of the invention. The host cell may be a prokaryotic or eukaryotic cell, preferably the host cell is an *E. coli* cell or a mammalian cell, such as a CHO cell. In a preferred embodiment, an antibody may be produced with varying amounts of fucosylation. For example, a CHO cell line may be selected to produce an antibody or antibody fragment with a range of fucosylation from a minimum of about 5% to a maximum of about 95%.

[0017] In a seventh aspect, the invention features a pharmaceutical composition comprising a anti-human CD20 antibody or fragment thereof and a pharmaceutically acceptable carrier.

[0018] In an eighth aspect, the invention features a fully human antibody or antibody fragment capable of binding to human CD20, with a EC_{50} of less than about 10 nM, as measured by cell binding experiments (described below). In a preferred embodiment, the antibody of the invention exhibits an EC_{50} of about 10^{-8} to about 10^{-12} M or higher, for example, at least 10^{-8} M, at least 10^{-9} M, at least 10^{-10} M, at least 10^{-11} M, or at least 10^{-12} M, when measured by binding to antigen presented on cell surface.

[0019] The invention encompasses anti-CD20 antibodies having a modified glycosylation pattern. In some applications, modification to remove undesirable glycosylation sites may be useful, or an antibody lacking a fucose moiety present on the oligosaccharide chain, for example, to increase antibody dependent cellular cytotoxicity (ADCC) function (see Shield et al. (2002) JBC 277:26733). In other applications, modification of a galactosylation can be made in order to modify complement dependent cytotoxicity (CDC).

[0020] In a ninth aspect, the invention features an antibody or antigen-binding fragment of an antibody as described above for use to attenuate or inhibit a CD20-mediated disease or

condition in a human. In a preferred embodiment the disease or condition treated is non-Hodgkin's lymphoma, rheumatoid arthritis, systemic lupus erythematosus, Crohn's disease, chronic lymphocytic leukemia, and inflammatory diseases. In a related embodiment, the invention encompasses a method of attenuating or inhibiting a CD20-mediated disease or condition in a human, comprising administering a therapeutically effective amount of an antibody or antigen-binding fragment of an antibody as described above. Further the invention encompasses the use of an antibody or antigen-binding fragment of an antibody of the invention in the manufacture of a medicament for use to attenuate or inhibit a CD20-mediated disease or condition in a human.

[0021] Other objects and advantages will become apparent from a review of the ensuing detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0022] Fig. 1. Symptom-free survival curve. Results are shown for human Fc control, control antibodies I and II, and antibodies: 8G6-5, 9D4-7, 10F2-13, and 7E1-13.

DETAILED DESCRIPTION

[0023] Before the present methods are described, it is to be understood that this invention is not limited to particular methods and experimental conditions described, as such methods and conditions may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

[0024] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described.

Definitions

[0025] The term "CD20" includes variants and isoforms of human CD20, which are naturally expressed by cells. Binding of an antibody of the invention to the CD20 antigen mediates the killing of cells expressing CD20 (for example, a tumor cell). The killing of cells expressing CD20 may occur in a number of ways, including complement dependent cytotoxicity (CDC) of cells expressing CD20, apoptosis of cells expressing CD20, effector cell phagocytosis of cells expressing CD20, or effector cell antibody dependent cellular cytotoxicity (ADCC) of cells expressing CD20.

[0026] The term "antibody," as used herein, is intended to refer to immunoglobulin molecules comprising four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain comprises a heavy chain variable region

(abbreviated herein as HCVR or VH) and a heavy chain constant region. The heavy chain constant region comprises three domains, CH1, CH2 and CH3. Each light chain comprises a light chain variable region (abbreviated herein as LCVR or VL) and a light chain constant region. The light chain constant region comprises of one domain (CL1). The VH and VL regions can be further subdivided into regions of hypervariability, termed complementary determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4.

[0027] The term "antigen-binding portion" of an antibody (or simply "antibody portion" or "antibody fragment"), as used herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen (e.g., hCD20). It has been shown that fragments of a full-length antibody can perform the antigen-binding function of an antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) an Fab fragment, a monovalent fragment consisting of the VL, VH, CL1 and CH1 domains; (ii) an $F(ab')_2$ fragment, a bivalent fragment comprising two F(ab)' fragments linked by a disulfide bridge at the hinge region; (iii) an Fd fragment consisting of the VH and CH1 domains; (iv) an Fv fragment consisting of the VL and VH domains of a single arm of an antibody; (v) a dAb fragment (Ward et al. (1989) Nature 241:544-546), which consists of a VH domain; and (vi) an isolated complementary determining region (CDR). Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single contiguous chain in which the VL and VH regions pair to form monovalent molecules (known as single chain Fv (scFv); see e.g., Bird et al. (1988) Science 242:423-426; and Huston et al. (1988) Proc. Natl. Acad. Sci. USA 85:5879-5883). Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. Other forms of single chain antibodies, such as diabodies, are also encompassed (see e.g., Holliger et al. (1993) Proc. Natl. Acad Sci. USA 90:6444-6448).

[0028] A "CDR" or complementary determining region is a region of hypervariability interspersed within regions that are more conserved, termed "framework regions" (FR). In various embodiments of the anti-hCD20 antibody or fragment of the invention, the FRs may be identical to the human germline sequences, or may be naturally or artificially modified.

[0029] The term "surface plasmon resonance," as used herein, refers to an optical phenomenon that allows for the analysis of real-time interactions by detection of alterations in protein concentrations within a biosensor matrix, for example using the BIACORE™ system (Pharmacia Biosensor AB).

[0030] The term "epitope" refers to an antigenic determinant that interacts with a specific antigen binding site in the variable region of an antibody molecule known as a paratope. A single antigen may have more than one epitope. Epitopes may be either conformational or

linear. A conformational epitope is produced by spatially juxtaposed amino acids from different segments of one (or more) linear polypeptide chain(s). A linear epitope is an epitope produced by adjacent amino acid residues in a polypeptide chain. In certain circumstance, an epitope may include other moieties, such as saccharides, phosphoryl groups, or sufonyl groups on the antigen.

[0031] The term "substantial identity" or "substantially identical," when referring to a nucleic acid or fragment thereof, indicates that, when optimally aligned with appropriate nucleotide insertions or deletions with another nucleic acid (or its complementary strand), there is nucleotide sequence identity in at least about 95%, and more preferably at least about 96%, 97%, 98% or 99% of the nucleotide bases, as measured by any well-known algorithm of sequence identity, such as FASTA, BLAST or GAP, as discussed below.

[0032] As applied to polypeptides, the term "substantial similarity" or "substantially similar" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 95% sequence identity, even more preferably at least 98% or 99% sequence identity. Preferably, residue positions, which are not identical, differ by conservative amino acid substitutions. A "conservative amino acid substitution" is one in which an amino acid residue is substituted by another amino acid residue having a side chain (R group) with similar chemical properties (e.g., charge or hydrophobicity). In general, a conservative amino acid substitution will not substantially change the functional properties of a protein. In cases where two or more amino acid sequences differ from each other by conservative substitutions, the percent or degree of similarity may be adjusted upwards to correct for the conservative nature of the substitution. Means for making this adjustment are well-known to those of skill in the art. See, e.g., Pearson (1994) Methods Mol. Biol. 24: 307-331. Examples of groups of amino acids that have side chains with similar chemical properties include 1) aliphatic side chains: glycine, alanine, valine, leucine and isoleucine; 2) aliphatic-hydroxyl side chains: serine and threonine; 3) amide-containing side chains: asparagine and glutamine; 4) aromatic side chains: phenylalanine, tyrosine, and tryptophan; 5) basic side chains: lysine, arginine, and histidine; 6) acidic side chains: aspartate and glutamate, and 7) sulfur-containing side chains: cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, glutamate-aspartate, and asparagine-glutamine. Alternatively, a conservative replacement is any change having a positive value in the PAM250 log-likelihood matrix disclosed in Gonnet et al. (1992) Science 256: 1443 45. A "moderately conservative" replacement is any change having a nonnegative value in the PAM250 log-likelihood matrix.

[0033] Sequence similarity for polypeptides is typically measured using sequence analysis software. Protein analysis software matches similar sequences using measures of similarity assigned to various substitutions, deletions and other modifications, including conservative amino acid substitutions. For instance, GCG software contains programs such as GAP and

BESTFIT which can be used with default parameters to determine sequence homology or sequence identity between closely related polypeptides, such as homologous polypeptides from different species of organisms or between a wild type protein and a mutein thereof. See, e.g., GCG Version 6.1. Polypeptide sequences also can be compared using FASTA with default or recommended parameters; a program in GCG Version 6.1. FASTA (e.g., FASTA2 and FASTA3) provides alignments and percent sequence identity of the regions of the best overlap between the query and search sequences (Pearson (2000) *supra*). Another preferred algorithm when comparing a sequence of the invention to a database containing a large number of sequences from different organisms is the computer program BLAST, especially BLASTP or TBLASTN, using default parameters. See, e.g., Altschul et al. (1990) J. Mol. Biol. 215: 403 410 and Altschul et al. (1997) Nucleic Acids Res. 25:3389 402.

[0034] The term "effective amount" is a concentration or amount of an antibody or antigen-binding fragment of an antibody which results in achieving a particular stated purpose. An "effective amount" of an anti-CD20 antibody or antigen-binding fragment of an antibody thereof may be determined empirically. Furthermore, a "therapeutically effective amount" is a concentration or amount of an anti-CD20 antibody or antigen-binding fragment thereof which is effective for achieving a stated therapeutic effect This amount may also be determined empirically.

Preparation of Human Antibodies

[0035] Methods for generating human antibodies include, for example, VelocImmune™ (Regeneron Pharmaceuticals), XenoMouse™ technology (Green et al. (1994) Nature Genetics 7:13-21; Abgenix), the "minilocus" approach, and phage display (and see, for example, US 5,545,807, US 6,787,637). VelocImmune™ technology (US 6, 596,541) encompasses a method of generating a high specificity fully human antibody to a select antigen. This technology involves generation of a transgenic mouse having a genome comprising human heavy and light chain variable regions operably linked to endogenous mouse constant region loci such that the mouse produces an antibody comprising a human variable region and a mouse constant region in response to antigenic stimulation. The DNA encoding the variable regions of the heavy and light chains of the antibody are isolated and operably linked to DNA encoding the human heavy and light chain constant regions. The DNA is then expressed in a cell capable of expressing the fully human antibody. In specific embodiment, the cell is a CHO cell.

[0036] Antibodies may be therapeutically useful in blocking a ligand-receptor interaction or inhibiting receptor component interaction, rather than by killing cells through fixation of complement (CDC) and participation antibody-dependent cell-mediated cytotoxicity (ADCC). The constant region of an antibody is important in the ability of an antibody to fix complement and mediate cell-dependent cytotoxicity. Thus, the isotype of an antibody may be selected on

the basis of whether it is desirable for the antibody to mediate cytotoxicity.

[0037] Human immunoglobulins can exist in two forms that are associated with hinge heterogeneity. In one form, an immunoglobulin molecule comprises a stable four-chain construct of approximately 150-160 kDa in which the dimers are held together by an interchain heavy chain disulfide bond. In a second form, the dimers are not linked via interchain disulfide bonds and a molecule of about 75-80 kDa is formed composed of a covalently coupled light and heavy chain (half-antibody). These forms have been extremely difficult to separate, even after affinity purification. The frequency of appearance of the second form in various intact IgG isotypes is due to, but not limited to, structural differences associated with the hinge region isotype of the antibody. A single amino acid substitution in the hinge region of the human IgG4 hinge can significantly reduce the appearance of the second form (Angal et al. (1993) Molecular Immunology 30: 105) to levels typically observed using a human IgG1 hinge. The instant invention encompasses antibodies having one or more mutations in the hinge, CH2 or CH3 region, which may be desirable, for example, in production, to improve the yield of the desired antibody, form.

[0038] Antibodies of the invention are preferably prepared with the use of VelocImmune™ technology. A transgenic mouse in which the endogenous immunoglobulin heavy and light chain variable regions are replaced with the corresponding human variable regions is challenged with the antigen of interest, and lymphatic cells (such as B-cells) are recovered from the mice that express antibodies. The lymphatic cells may be fused with a myeloma cell line to prepare immortal hybridoma cell lines, and such hybridoma cell lines are screened and selected to identify hybridoma cell lines that produce antibodies specific to the antigen of interest. DNA encoding the variable regions of the heavy chain and light chain may be isolated and linked to desirable isotypic constant regions of the heavy chain and light chain. Such an antibody protein may be produced in a cell, such as a CHO cell. Alternatively, DNA encoding the antigen-specific chimeric antibodies or the variable domains of the light and heavy chains may be isolated directly from antigen-specific lymphocytes.

[0039] In general, the antibodies of the instant invention possess very high affinities, typically possessing K_D or EC_{50} of from about 10^{-8} through about 10^{-12} M or higher, for example, at least 10^{-8} M, at least 10^{-9} M, at least 10^{-10} M, at least 10^{-11} M, or at least 10^{-12} M, when measured by binding to antigen presented on cell surface.

[0040] Initially, high affinity chimeric antibodies are isolated having a human variable region and a mouse constant region. As described below, the antibodies are characterized and selected for desirable characteristics, including affinity, selectivity, epitope, etc. The mouse constant regions are replaced with a desired human constant region to generate the fully human antibody of the invention, for example wild-type or modified IgG1 or IgG4 (for example, SEQ ID NO:416, 417, 418). While the constant region selected may vary according to specific use, high affinity antigen-binding and target specificity characteristics reside in the variable region.

Epitope Mapping and Related Technologies

[0041] To screen for antibodies which bind to a particular epitope, a routine cross-blocking assay such as that described in "Antibodies: A Laboratory Manual" 1988 Cold Spring Harbor Laboratory, Harlow and Lane, eds., can be performed. Other methods include alanine scanning mutants, peptide blots (Reineke (2004) Methods Mol Biol 248:443-63), or peptide cleavage analysis as described in the examples below. In addition, methods such as epitope excision, epitope extraction and chemical modification of antigens can be employed (Tomer (2000) Protein Science: 9: 487-496).

[0042] To ascertain the binding characteristics of the antibodies, mutant CD20 proteins consisting of selected amino acid substitutions were constructed. The mutant CD20 proteins contained substitutions of certain amino acids occurring in the human protein with corresponding amino acids occurring in the mouse protein. This approach helped ensure that the mutant CD20 proteins maintained their tertiary structure and, presumably, any conformational epitopes. Binding of the test antibodies to these mutant CD20 proteins was compared with binding of control (known) CD20 antibodies, as measured by FACS. None of the inventive antibodies displayed a binding profile that was identical (with respect to each and every mutant) to either of the control antibodies.

Immunoconjugates

[0043] The invention encompasses a human anti-CD20 monoclonal antibody conjugated to a therapeutic moiety ("immunoconjugate"), such as a cytotoxin, a chemotherapeutic drug, an immunosuppressant or a radioisotope. Cytotoxin agents include any agent that is detrimental to cells. Examples of suitable cytotoxin agents and chemotherapeutic agents for forming immunoconjugates are known in the art, see for example, WO 05/103081.

Bispecifics

[0044] The antibodies of the present invention may be monospecific, bispecific, or multispecific. Multispecific antibodies may be specific for different epitopes of one target polypeptide or may contain antigen-binding domains specific for more than one target polypeptide. See, e.g., Tutt et al. (1991) J. Immunol. 147:60-69. The human anti-CD20 antibodies can be linked to or co-expressed with another functional molecule, e.g., another peptide or protein. For example, an antibody or fragment thereof can be functionally linked (e.g., by chemical coupling, genetic fusion, noncovalent association or otherwise) to one or more other molecular entities, such as another antibody or antibody fragment, to produce a bispecific or a multispecific antibody with a second binding specificity. A multispecific antibody of the invention may specifically bind both a CD20 expressing cell and a human effector cell expressing a polypeptide, such as a human Fc receptor, and/or components of the T cell receptor complex. In one embodiment, the multispecific antibody of the invention comprises a CD20-binding portion and a cytokine.

Therapeutic Uses

[0045] The human antibodies, antigen-binding fragments of antibodies, immunoconjugates, and bispecific molecules of the invention are useful in therapeutic methods for treating human diseases which are inhibited or ameliorated by inhibiting growth of cells expressing CD20 and/or killing cells expressing CD20. The mechanism of action by which the therapeutic methods of the invention are achieved include killing of the cell expressing CD20 in the presence of effector cells, for example, by CDC, apoptosis, ADCC, phagocytosis, or by a combination of two or more of these mechanisms. The mechanism for achieving the therapeutic effect of the molecules of the invention may result in direct killing or inhibition of cells expressing CD20, or indirectly, through inhibiting cells which do not express CD20 for, for example, express a structurally related cell-surface antigen (i.e., without cross-reactivity to related but functionally distinct cell surface antigens). Cells expressing CD20 which can be inhibited or killed using the human antibodies of the invention include, for example, tumorigenic B cells.

[0046] Examples of diseases and conditions that can be treated or ameliorated with the anti-CD20 antibodies and fragments thereof include, but are not limited to, tumorigenic diseases, such as B cell lymphoma (NHL, precursor B cell lymphoblastic leukemia/lymphoma, mature B cell neoplasms, B cell chronic lymphocytic leukemia/small lymphocytic lymphoma, B cell prolymphocytic leukemia, lymphoplasmacytic lymphoma, mantle cell lymphoma, follicular lymphoma, cutaneous follicle center lymphoma, marginal zone B cell lymphoma, hairy cell leukemia, diffuse large B cell lymphoma, Burkitt's lymphoma, plasmacytoma, plasma cell myeloma, post-transplant lymphoproliferative disorder, Waldenstrom's macroglobulinemia, and anaplastic large-cell lymphoma); immune diseases, such as autoimmune diseases (psoriasis, psoriatic arthritis, dermatitis, systemic scleroderma and sclerosis, inflammatory bowel disease, Crohn's disease, ulcerative colitis, respiratory distress syndrome, meningitis, encephalitis, uveitis, glomerulonephritis, eczema, asthma, atherosclerosis, leukocyte adhesion deficiency, multiple sclerosis, Raynaud's syndrome, Sjogren's syndrome, juvenile onset diabetes, Reiter's disease, Behcet's disease, immune complex nephritis, IgA nephropathy, IgM polyneuropathies); immune-mediated thrombocytopenias, acute idiopathic thrombocytopenic purpura and chronic idiopathic thrombocytopenic purpura, hemolytic anemia, myasthenia gravis, lupus nephritis, systemic lupus erythematosus, rheumatoid arthritis, atopic dermatitis, pemphigus, Graves' disease, Hashimoto's thyroiditis, Wegener's granulomatosis, Omenn's syndrome, chronic renal failure, acute infectious mononucleosis, HIV, and herpes virus associated diseases; severe acute respiratory distress syndrome and choreoretinitis; diseases and disorders caused by infection of B-cells with virus, such as Epstein-Barr virus.

[0047] In a specific embodiment, the subject being administered the antibody is additionally treated with a chemotherapeutic agent, radiation, or an agent that modulates (enhances or inhibits) the expression or activity of an Fc receptor, such as a cytokine. Typical, cytokines for administration during treatment include granulocyte colony-stimulating factor (G-CSF),

granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-gamma. (IFN-γ), and tumor necrosis factor (TNF). Typical therapeutic agents include, among others, anti-neoplastic agents such as doxorubicin, cisplatin, bleomycin, carmustine, chlorambucil, and cyclophosphamide.

Therapeutic Administration and Formulations

[0048] The invention provides therapeutic compositions comprising the human anti-CD20 antibodies or antigen-binding fragments thereof of the present invention. The therapeutic compositions in accordance with the invention will be administered with suitable carriers, excipients, and other agents that are incorporated into formulations to provide improved transfer, delivery, tolerance, and the like. In general, carriers, excipients, or other agents can include, for example, oils (e.g., canola, cottonseed, peanut, safflower, sesame, soybean), fatty acids and salts and esters thereof (e.g., oleic acid, stearic acid, palmitic acid), alcohols (e.g., ethanol, benzyl alcohol), polyalcohols (e.g., glycerol, propylene glycols and polyethylene glycols, e.g., PEG 3350), polysorbates (e.g., polysorbate 20, polysorbate 80), gelatin, albumin (e.g., human serum albumin), salts (e.g., sodium chloride), succinic acid and salts thereof (e.g., sodium succinate), amino acids and salts thereof (e.g., alanine, histidine, glycine, arginine, lysine), acetic acid or a salt or ester thereof (e.g., sodium acetate, ammonium acetate), citric acid and salts thereof (e.g., sodium citrate), benzoic acid and salts thereof, phosphoric acid and salts thereof (e.g., monobasic sodium phosphate, dibasic sodium phosphate), lactic acid and salts thereof, polylactic acid, glutamic acid and salts thereof (e.g., sodium glutamate), calcium and salts thereof (e.g., calcium chloride, calcium acetate), phenol, sugars (e.g., glucose, sucrose, lactose, maltose, trehalose), erythritol, arabitol, isomalt, lactitol, maltitol, mannitol, sorbitol, xylitol, nonionic surfactants (e.g., TWEEN® 20, TWEEN® 80), ionic surfactants (e.g., sodium dodecyl sulfate), chlorobutanol, DMSO, sodium hydroxide, glycerin, m-cresol, imidazole, protamine, zinc and salts thereof (e.g, zinc sulfate), thimerosal, methylparaben, propylparaben, carboxymethylcellulose, chlorobutanol, and heparin. Other non-therapeutic agents are described in US 7,001,892, in particular in Table A. A multitude of appropriate formulations can be found in the formulary known to all pharmaceutical chemists: Remington's Pharmaceutical Sciences (Mack Publishing Company, Easton, Pa). These formulations include, for example, powders, pastes, ointments, jellies, waxes, oils, lipids, lipid (cationic or anionic) containing vesicles (such as LIPOFECTIN™), DNA conjugates, anhydrous absorption pastes, oil-in-water and water-in-oil emulsions, emulsions carbowax (polyethylene glycols of various molecular weights), semi-solid gels, and semi-solid mixtures containing carbowax. Any of the foregoing mixtures may be appropriate in treatments and therapies in accordance with the present invention, provided that the active ingredient in the formulation is not inactivated by the formulation and the formulation is physiologically compatible and tolerable with the route of administration. See also Powell et al. PDA (1998) J Pharm Sci Technol. 52:238-311 and the

citations therein for additional information related to excipients and carriers well known to pharmaceutical chemists.

[0049] The dose of the therapeutic compositions may vary depending upon the age and the size of a subject to be administered, target disease, conditions, route of administration, and the like. When the antibody of the present invention is used for treating various conditions and diseases associated with CD20 activity, including non-Hodgkin's lymphoma, rheumatoid arthritis, systemic lupus erythematosus, Crohn's disease, chronic lymphocytic leukemia, inflammatory diseases, and the like, in an adult patient, it is advantageous to intravenously administer the antibody of the present invention normally at a single dose of about 0.01 to about 20 mg/kg body weight, preferably about 0.1 to about 10 mg/kg body weight, and more preferably about 0.1 to about 5 mg/kg body weight. Depending on the severity of the condition or disease, the frequency and the duration of the treatment can be adjusted. In other parenteral administration and oral administration, the antibody can be administered in a dose corresponding to the dose given above.

[0050] Various delivery systems are known and can be used to administer the pharmaceutical composition of the invention, e.g., encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the mutant viruses, receptor mediated endocytosis (see, e.g., Wu et al. (1987) J. Biol. Chem. 262:4429-4432). Methods of introduction include, but are not limited to, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The composition may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration may preferably be systemic or local.

[0051] The pharmaceutical composition can be also delivered in a vesicle, in particular a liposome (see Langer (1990) Science 249:1527-1533). In certain situations, the pharmaceutical composition can be delivered in a controlled release system. In one embodiment, a pump may be used (see Langer, supra; Sefton (1987) CRC Crit. Ref. Biomed. Eng. 14:201). In another embodiment, polymeric materials can be used (see Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York (1984). In yet another embodiment, a controlled release system can be placed in proximity of the composition's target, thus requiring only a fraction of the systemic dose (see, *e.g.*, Goodson, in Medical Applications of Controlled Release, supra, vol. 2, pp. 115-138, 1984).

[0052] The injectable preparations may include dosage forms for intravenous, subcutaneous, intracutaneous and intramuscular injections, drip infusions, etc. These injectable preparations may be prepared by methods publicly known. For example, the injectable preparations may be prepared, *e.g.*, by dissolving, suspending or emulsifying the antibody or its salt described above

in a sterile aqueous medium or an oily medium conventionally used for injections. As the aqueous medium for injections, there are, for example, physiological saline, an isotonic solution containing glucose and other auxiliary agents, etc., which may be used in combination with an appropriate solubilizing agent such as an alcohol (e.g., ethanol), a polyalcohol (e.g., propylene glycol, polyethylene glycol), a nonionic surfactant [e.g., polysorbate 80, HCO-50 (polyoxyethylene (50 mol) adduct of hydrogenated castor oil)], etc. As the oily medium, there are employed, e.g., sesame oil, soybean oil, etc., which may be used in combination with a solubilizing agent such as benzyl benzoate, benzyl alcohol, etc. The injection thus prepared is preferably filled in an appropriate ampoule.

[0053] Advantageously, the pharmaceutical compositions for oral or parenteral use described above are prepared into dosage forms in a unit dose suited to fit a dose of the active ingredients. Such dosage forms in a unit dose include, for example, tablets, pills, capsules, injections (ampoules), suppositories, etc. The amount of the aforesaid antibody contained is generally about 5 to 500 mg per dosage form in a unit dose; especially in the form of injection, it is preferred that the aforesaid antibody is contained in about 5 to 100 mg and in about 10 to 250 mg for the other dosage forms.

EXAMPLES

[0054] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the methods and compositions of the invention, and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

Example 1. Generation of Human Antibodies to Human CD20.

[0055] Immunization of rodents can be done by any method known in the art (see, for example, Harlow & Lane, eds. (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Press, New York; Malik and Lillehoj, Antibody techniques: Academic Press, 1994, San Diego). In one embodiment, cells expressing CD20 are administered directly to mice which have DNA loci encoding both human Ig heavy chain variable regions and Kappa light chain variable regions (VelocImmune™, Regeneron Pharmaceuticals, Inc.; US 6,596,541), with an adjuvant to stimulate the immune response. Such an adjuvant can include complete and incomplete Freund's adjuvant, MPL+TDM adjuvant system (Sigma), or RIBI (muramyl dipeptides) (see O'Hagan, Vaccine Adjuvant, by Human Press, 2000, Totawa, NJ). To achieve high expression levels of human CD20 on a cell surface, the murine cell lines, MG87 and/or NS/0 cells, are transfected with a plasmid encoding for human CD20, and cells expressing high levels of CD20 are enriched using FACS technology. In one embodiment, CD20 is administered indirectly as a

DNA plasmid that contains a CD20 gene, and CD20 is expressed using the host's protein expression system to produce antigen protein *in vivo*. In both approaches, to attain optimal antibody immune response, mice are given booster injections every 3~4 weeks. The immune response is monitored by a cell-based immunoassay as described below in which serum samples in 1- to 3-fold serial dilutions are immunoassayed. Serum titer is defined as the dilution of serum sample that yielded an assay signal two-fold over background. When animals reach their maximum immune response, antibody-expressing B cells are harvested and fused with mouse myeloma cells to form hybridomas.

Example 2. Screening for Antigen Specific Hybridoma

[0056] In primary screening, NS/0 cells (ATCC) were transfected with the human CD20 gene and high-expressing cells (NS/0-hCD20 cells) were pooled and maintained in culture for use in screening hybridoma-conditioned media, generally about 11 to 14 days following fusion. NS/0-hCD20 cells in RPMI 1640 with 10% fetal calf serum were plated at a density of 50,000 cells per well in 96-well poly-D-lysine plates. Hybridoma-conditioned medium was diluted 5-fold and allowed to bind to the cells for 30 minutes. The cells were then fixed onto the plates with the addition of an equal volume of 8% formaldehyde for 20 min, followed by four successive PBST washes. The plates were incubated with 5% BSA for 2 hrs at room temperature (RT). After washing, plate-bound antibodies were incubated with HRP-conjugated goat anti-mouse IgG Fcγ-specific polyclonal antibodies for 30 min, and the plates developed using 3.3', 5.5'-tetramethyl-benzidine (TMB) substrate (BD Pharmigen) following the final washes. The HRP reaction was stopped with an equal volume of 1 M phosphoric acid. Antibody binding signals were measured by optical density at 450nm. NS/0 parental cells, which have no detectable CD20 expression, were used as a background control to exclude hybridoma supernatants with non-specific cell surface binding. Wells positive for both NS/0 parental cells and CD20-expressing cells were excluded.

Example 3. Sequencing of Human Antibodies Against CD20

[0057] Prior to sequencing, antigen-specific hybridoma cells were single-cell sub-cloned using a MOFLO™ flow cytometer. Sequencing of the variable light and heavy chain regions was performed by standard methods (see for example, US 2004/0167319A1). Total RNA was prepared from each hybridoma cell line with an RNEASY™ kit (Qiagen). cDNA was prepared using the SMART RACE™ cDNA Amplification kit (Clonetech). DNA sequences of HCVRs and LCVR were sequenced and the predicted amino acid sequences for HCVRs and LCVRs provided for selected antibodies (HCVR/LCVR SEQ ID NO): 3B9-10N (3/11); 3B9-10GSP (19/21); 3B9-10FGL (23/25); 9C11-14N (27/35); 9C11-14GSP (43/45); 9C11-14FGL (47/49); 2B7-7N (51/59); 2B7-7GSP (67/69); 2B7-7FGL (71/73); 2C11-4N (75/83); 2C11-4GSP (91/93); 2C11-4FGL (95/97); 3H7-6N (99/107); 3H7-6GSP (115/117); 3H7-6FGL (119/121); 5H2-17N

(123/131); 5H2-17GSP (139/141); 5H2-17FGL (143/145); 6B9-4N (147/155); 6B9-4GSP (163/165); 6B9-4FGL (167/169); 6F6-1N (171/179); 6F6-1GSP (187/189); 6F6-1FGL (191/193); 8G6-5N ("8G6-5") (195/203); 8G6-5GSP (211/213); 8G6-5FGL (215/217); 9C3-8N (219/227); 9C3-8GSP (235/237); 9C3-8FGL (239/241); 9D4-7N ("9D4-7") (243/251); 9D4-7GSP (259/261); 9D4-7FGL (263/265); 9E4-20N (267/275); 9E4-20GSP (283/285); 9E4-20FGL (287/289); 9H4-12N (291/299); 9H4-12GSP (307/309); 9H4-12FGL (311/313); 10E3-17N (315/323); 10E3-17GSP (331/333); 10E3-17FGL (335/337); 10F2-13N ("10F2-13") (339/347); 10F2-13GSP (355/357); 10F2-13FGL (359/361); 7E1-13N (363/371); 7E1-13GSP (379/381); 7E1-13FGL (383/385).

Example 4. Antigen Binding Specificity of the Anti-CD20 Antibodies

[0058] After chimeric antibodies had been converted to fully human IgGs, specific antigen binding properties were determined with an ELISA protocol similar to the protocol described above, except that an HRP-conjugated goat anti-hIgG Fcγ-specific polyclonal was used as the detection antibody and a Daudi cell line (which expresses endogenous CD20) was used as an antigen source. All of the tested antibodies bound specifically to Daudi cells with EC_{50} values ranging from about 0.4 nM to about 20 nM.

[0059] Antigen binding specificity of the fully human anti-CD20 antibodies was verified using flow cytometry as described below, with human CD20-transfected MG87 cells. Briefly, parental MG87 and human CD20-transfected MG87 cells were incubated for 30 min at 4°C with each of the 15 human antibodies and the two control antibodies, followed by incubation with PE-conjugated anti-human IgG antibody. Binding was assessed by flow cytometry. Fluorescence intensities were compared with binding to the parental cell line and control isotype-matched sample. Results are summarized on Table 1. All antibodies bound to human CD20-transfected MG87 cells, whereas no binding was observed to parental MG87 cells, indicating that the antibodies are CD20-specific. Control I: chimeric (murine/human) anti-CD20 mAb, rituximab, (RITUXAN®, IDEC Pharmaceuticals Corp.); control II: human anti-CD20 mAb, 2F2, described in WO 2005/103081).

Table 1

Antibody	Total Mean Fluorescent Intensity	
	Untransfected	Human CD20-Transfected
Unstained	5.78	5.86
Control I	6.15	2955.71
Control II	6.08	3315.94
7E1-13	6.11	3076.88

2B7-7	6.08	3483.32
10F2-13	6.13	3396.69
9H4-12	6.06	2043.95
10E3-17	6.03	3071.01
9D4-7	6.66	3156.91
9C3-8	6.03	2913.87
3B9-10	6.07	2986.32
9E4-20	6.03	2908.67
3H7-6	6.1	3302.01
6B9-4	6.09	2933.36
6F6-1	6.05	3385.59
8G6-5	6.04	3407.87
2C11-4	6.02	2009.86
9C11-14	6.05	2751.56

Example 5. Human Anti-CD20 Antibody Binding to Mutant Human CD20

[0060] Mutant human CD20s were generated by substituting human CD20 amino acid sequences with corresponding mouse amino acids using a Strategene Mutagenesis kit (Table 2). A plasmid vector comprising a mutant human CD20, a CMV promoter, and a hygromycin resistant gene-IRES-GFP marker was then transfected into MG87 cells. For each mutant human CD20, a pool of hygromycin-resistant cells that displayed high GFP expression were collected and a stable line was created for antibody binding assay.

Table 2

Mutant	Mutation(s)		
#1	Y77F		
#2	N163D		
#3	A170S	P172S	
#4	N166D		
#5	P172S		
#6	Y77F	N166D	
#7	Y77F	N163D	
#8	Y77F	N163D	N166D

#9	N163D	N166D	
#10	A157V		

[0061] Briefly, approximately 1 x 10^6 cells from each stably transfected cell line expressing a mutant human CD20 were collected and incubated with each anti-human antibody, at 10 μg/ml, on ice for 1 hr, followed by incubation with APC-conjugated goat anti-human IgG (Jackson Immunolabs), at 10 μg/ml, on ice for 45 min. For each antibody, binding to each mutant human CD20 was assessed by flow cytometry. Mean fluorescence intensity levels were assessed while gating on a small (approximately 20%) population of cells that displayed a median level of GFP expression to minimize effects due to variable mutant CD20 expression levels within each cell line. For each mutant CD20, the antibody that displayed the highest mean fluorescence intensity was designated as 100% binding. Table 3 shows the percent binding of each anti-CD20 antibody to each mutant human CD20.

Table 3

Antibody	Percent of Binding to Mutant Human CD20 (%)									
	#1	**#2**	**#3**	**#4**	**#5**	**#6**	**#7**	**#8**	**#9**	**#10**
Control II	60	30	70	51	84	1	1	0	4	79
3B9-10	56	6	67	40	72	1	1	0	4	79
9C11-14	89	83	0	87	29	87	96	100	100	83
7E1-13	94	49	95	84	85	1	1	0	4	92
6F6-1	100	74	100	85	100	16	2	0	4	98
8G6-5	86	45	65	70	76	5	1	0	4	96
10F2-13	79	54	55	60	69	1	2	0	4	87
2B7-7	100	40	87	100	88	12	2	0	4	83
10E3-17	61	1	6	1	49	1	1	0	4	87
2C11-4	35	0	11	31	30	1	1	0	3	68
9D4-7	67	2	70	5	66	0	2	0	4	75
6B9-4	74	3	64	3	67	1	2	0	3	75
3H7-6	28	1	43	4	45	0	1	0	3	80
9C3-8	36	0	84	22	76	0	1	0	3	77
9H4-12	19	0	0	0	2	1	2	0	4	52
9E4-20	14	0	57	8	57	1	1	0	3	76
Control I	96	100	0	96	2	100	100	93	97	100

Example 6. Potency in Complement Dependent Cytotoxicity (CDC)

[0062] The anti-human CD20 human antibodies were tested for their ability to promote complement dependent cytotoxicity (CDC) using the human lymphoma cell lines Daudi and RL as target cell lines. The antibodies were serially diluted (final concentration range of 50 nM to 0.85 pM plus buffer control) into media and added to target cells seeded in a 96 well plate format. Human serum with complement components (Quidel) was added to each well to give a final serum concentration of 5%. The cells were incubated at 37°C for 2 hrs with the test antibodies and human serum with complement components and then assayed for cell survival as detected by ALAMARBLUE™. Fluorescence was measured using an excitation wavelength of 560 nm and an emission wavelength of 590 nm (Table 4).

Table 4

Antibody	Daudi EC_{50} (nM)	n	RL EC_{50} (nM)	N
10F2-13	0.17 ± 0.08	3	0.36 ± 0.10	4
8G6-5	0.21 ± 0.08	3	1.06 ± 0.43	4
9D4-7	0.22 ±0.21	4	0.83 ± 0.60	5
2B7-7	0.24 ± 0.09	4	1.03 ± 0.40	5
Control II	0.28 ± 0.11	5	0.77 ± 0.41	6
6B9-4	0.34 ± 0.25	3	0.97 ± 0.32	4
3H7-6	0.44 ± 0.27	2	3.66 ± 3.85	2
6F6-1	0.56 ± 0.35	3	1.20 ± 0.43	4
10E3-17	0.59 ± 0.24	2	7.80 ± 8.64	3
Control I	0.84 ± 0.60	6	>50	4
9E4-20	1.53 ± 0.87	3	1.70 ± 1.80	4
7E1-13	1.59 ± 0.71	3	5.81 ± 3.77	4
3B9-10	1.86± 0.96	3	8.84 ± 6.94	4
9C3-8	2.22 ± 1.62	2	11.13 ± 9.29	2
9C11-14	7.14 ± 6.63	3	12.01 ± 6.61	4
9H4-12	51.10 ± 38.4	2	29.60 ± 23.76	2
2C11-4	>50	2	5.19 ± 3.10	3

Example 7. Functional Off-Rate of Human Anti-CD20 Antibodies

[0063] The off-rates of the anti-CD20 mAbs were analyzed in a CDC assay. The experiments were performed in 3 separate sets. Within each set, the percentage of cell lysis was determined for 5 antibodies at a time relative to controls I and II at 0, 1, and 6 hrs. Antibody was bound to the cells by incubating 2 μg of each antibody with 10^6 Daudi cells for 45 min (RT). For the zero time point, the cells were washed and immediately resuspended in 100 μl of medium containing 20% normal human serum complement, then incubated for 45 min at 37°C, 5% CO_2. For the 1

and 6 hr time points, 10^6 cells were washed following antibody binding, re-suspended in 12 ml fresh media in a 15 ml Falcon tube, and incubated at on a mechanical inverter for 1 and 6 hrs, respectively. Cells were washed at the completion of the selected time points and incubated in medium containing 20% normal human serum complement, and incubated for 45 min. Following serum incubation, 7-amino-actinomycin D (7AAD) was added to each sample and incubated for 15 min at RT to assess cell viability. Percent cytotoxicity was determined at each time point by setting regions as a forward scatter versus 7AAD two-dimensional scatter plot that represented 7AAD positive and negative cells, with debris excluded from both regions. Percent cytotoxicity was plotted for each time point as 100 minus percentage of 7AAD-negative cells (Table 5-7).

Table 5

Antibody	% Cytotoxicity		
	0 hour	1 hour	6 hour
Control I	98.5	86.9	16
Control II	99.6	99.1	98.5
10F2-13	99.6	99.2	98.5
8G6-5	99.6	99.2	97.7
9D4-7	99.4	99.0	96.0
2B7-7	99.4	99.4	98.3
9C11-14	55.8	22.3	12.9

Table 6

Antibody	% Cytotoxicity		
	0 hour	1 hour	6 hour
Control I	91.9	65.3	52.1
Control II	98.3	98.6	97.7
6B9-4	98.1	98.5	97.5
3H7-6	97.5	94.0	67.6
6F6-1	97.1	97.1	76.4
10E3-17	97	96.2	79.2
9E4-20	67.4	31.2	49.9

Table 7

Antibody	% Cytotoxicity		
	0 hour	1 hour	6 hour
Control I	98.3	81.1	20.5
Control II	99.1	99.2	98.7

7E1-13	98.1	98.5	89.3
3B9-10	98.4	97.9	82.2
9C3-8	98.7	98.5	76.9
9H4-12	43.2	17.4	23.1
2C11-4	29.1	14	22.4

Example 8. Biochemical Off-Rate of the Human Anti-CD20 Antibodies

[0064] Biochemical off-rates for selected test anti-CD20 antibodies were determined and compared with control antibodies I and II. Two selected human antibodies, control I or II (each 2 μg/ml) were incubated with CD20-expressing Raji cells, at 10^6/ml, for 2 hrs at RT. The cells were then washed, excess antibody was removed, re-suspended in 1% serum-containing medium, and incubated at 37°C. At time 0, 15, 30, 45, 60, 90, 120, and 180 min, an aliquot of 1 ml of cells was removed, washed, stained with PE-labeled anti-hFc antibody, and FACS analysis conducted. Mean fluorescent intensity (MFI) was used as an indicator of the amount of antibody bound to the cell surface. Biochemical off rates were calculated by setting the percentage binding at time zero as 100%. The experiment was repeated 5 additional times, and biochemical off rate for 12 the test antibodies determined and compared to control I and II (Tables 8-13).

Table 8

Time (min)	% Binding			
	Control I	**Control II**	**9C11-14**	**10F2-13**
0	100.00	100.00	100.00	100.00
15	58.36	69.04	50.86	74.85
30	47.04	72.03	42.22	73.99
45	33.77	74.00	28.77	70.77
60	22.96	61.38	17.49	54.30
90	11.82	54.43	9.66	51.12
120	6.89	51.33	5.11	47.40
180	2.73	52.73	2.06	51.65

Table 9

Time (min)	% Binding			
	Control I	**Control II**	**8G6-5**	**9D4-7**

0	100.00	100.00	100.00	100.00
15	67.11	80.54	86.48	81.00
30	51.18	81.20	73.09	82.76
45	41.97	85.86	86.95	80.73
60	31.17	85.44	83.93	74.50
90	15.53	81.30	73.26	62.59
120	13.08	73.68	67.93	45.99
180	2.42	51.57	47.96	22.34

Table 10

Time (min)	% Binding			
	Control I	**Control II**	**3H7-6**	**6F6-1**
0	100.00	100.00	100.00	100.00
15	68.02	90.93	69.38	87.04
30	55.97	84.05	56.58	80.86
45	29.49	64.85	33.12	56.45
60	33.24	86.17	36.98	68.75
90	15.42	80.84	19.45	60.57
120	9.40	82.08	12.25	54.56
180	3.40	69.25	3.97	34.60

Table 11

Time (min)	% Binding			
	Control I	**Control II**	**2B7-7**	**6B9-4**
0	100.00	100.00	100.00	100.00
15	59.47	82.97	88.14	91.05
30	58.96	69.72	90.53	95.32
45	49.57	78.71	90.30	96.45
60	30.98	64.19	76.95	79.77

90	18.41	67.06	69.17	64.66
120	8.46	58.03	66.95	60.04
180	2.70	51.73	64.01	49.03

Table 12

Time (min)	% Binding			
	Control I	Control II	7E1-13	10E3-17
0	100.00	100.00	100.00	100.00
15	73.07	81.04	89.23	88.88
30	51.70	86.27	83.34	78.82
45	34.75	87.98	79.89	69.99
60	22.53	76.71	73.89	66.64
90	14.01	87.36	96.29	65.20
120	8.54	93.79	94.46	50.86
180	3.44	84.95	89.58	29.76

Table 13

Time (min)	% Binding			
	Control I	Control II	3B9-10	9E4-20
0	100.00	100.00	100.00	100.00
15	73.84	88.42	77.88	40.59
30	58.06	83.57	76.63	15.90
45	39.64	85.97	70.32	6.76
60	26.86	75.25	62.64	3.31
90	12.89	67.46	55.16	1.60
120	6.83	61.69	47.74	0.97
180	3.27	68.62	48.25	0.81

Example 9. Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) Assay

[0065] ADCC induced by selected human anti-CD20 antibodies was assessed using Daudi cells

(cells from a human lymphoma cell line that endogenously expresses CD20). Briefly, Daudi cells (10,000 cells/well in 50 μl) were first mixed with an equal volume of serially diluted human anti-CD20 antibody, resulting in a final antibody concentration ranging from 0.169 pM to 10 nM, and incubated for 10 min at RT in a 96-well plate (control = wells without ab). Separately, human peripheral blood mononuclear cells (PBMCs, effector cells) were prepared following a conventional Ficoll-Hypaque gradient centrifugation enrichment procedure. Enriched PBMCs were collected, washed, and plated in RPMI 1640 containing 10% heat inactivated FBS, 2 mM glutamine and 50 nM beta-mercaptoethanol. The cells were then stimulated with 5 ng/ml human IL-2 for three days, washed once in media, then used directly in the ADCC assay. Approximately 300,000 PBMCs were added to each mixture of antibody and target cells to give a final ratio of effector to target cells of approximately 30:1. The 96-well plates were then incubated for 4 hr and centrifuged at 250 x *g*. Supernatants were harvested and assayed for lactate dehydrogenase (LDH) activity using the CYTOTOX 96® Non-Radioactive Cytotoxicity Assay system (Promega) (Table 14).

Table 14

Antibody	EC_{50} (pM)	N
9C11-14	10.22	4
9E4-20	2.37	3
3B9-10	6.77	2
8G6-5	14.83	5
10F2-13	6.68	7
6F6-1	5.15	4
7E1-13	2.14	3
9D4-7	1.53	3
2C11-4	1.45	1
10E3-17	1.20	3
2B7-7	1.99	3
6B9-4	4.27	3
9C3-8	11.02	3
3H7-6	11.11	3
9H4-12	33.82	1

Example 10. Therapeutic Activities of Anti-CD20 Antibodies with a Human Lymphoma Xenograft Mouse Model.

[0066] *In vivo* efficacy studies for selected anti-CD20 antibodies were carried out using a human non-Hodgkin's B-cell lymphoma xenograft mouse model. Female severe combined immune deficient (SCID) mice were purchased at 6 weeks of age. After one week of acclimation, 2.5 million freshly harvested Raji cells (cells from a human non-Hodgkin's B-cell lymphoma cell line) were injected intravenously into each mouse. Each Raji cell-engrafted mouse was then treated with human FC (hFC), control I, control II, 8G6-5, 9D4-7, 10F2-13, or 7E1-13, each at 10 mg/kg, via intravenous injection through the lateral tail 3, 6, and 9 days after the engraftment. Mice were monitored for a period up to 180 days. Mice exhibiting signs of disease including hind-limb paralysis, cachexia, and occasional large local tumor mass were euthanized by CO_2 asphyxiation. Symptom-free survival curves were constructed using the Kaplan-Meier method (Fig. 1). The results are expressed as percent survival as a function of symptom free survival time. These results show that ab 10F2-13 increased survival times significantly in the animal model, from about 20 days (hFc control treated animals) to about 180 days (more than a 9-fold increase in survival rate) (50% of treated animals survived about 20 days (hFc control), about 40 days (control I), about 85 days (control II), and more than 180 days (10F2-13). These increased survival times are at least 2-fold greater (relative to control II), about 4.5-fold greater (relative to control I), or at least about 9-fold or greater relative to hFc-treated animals.

Claims:

1. A human antibody or antigen-binding fragment of an antibody that specifically binds human CD20 and is capable of inducing complement dependent cytotoxicity (CDC), wherein the antibody or antibody fragment comprises:

(i) a heavy chain complementarity determining region 1 (HCDR1) having the amino acid sequence of SEQ ID NO:341;
(ii) a heavy chain complementarity determining region 2 (HCDR2) having the amino acid sequence of SEQ ID NO:343;
(iii) a heavy chain complementarity determining region 3 (HCDR3) having the amino acid sequence of SEQ ID NO:345;
(iv) a light chain complementarity determining region 1 (LCDR1) having the amino acid sequence of SEQ ID NO:349;
(v) a light chain complementarity determining region 2 (LCDR2) having the amino acid sequence of SEQ ID NO:351; and
(vi) a light chain complementarity determining region 3 (LCDR3) having the amino acid sequence of SEQ ID NO:353.

2. The antibody or fragment thereof of claim 1, wherein the antibody or fragment thereof exhibits an EC_{50} 0.2 nM or less as measured in Daudi cells, or an EC_{50} of 0.4 nM or less as measured by RL cells.

3. The antibody or fragment thereof of claim 1, wherein the antibody or fragment thereof is capable of increasing symptom free survival time between about 2-fold to about 9-fold or more, relative to control-treated animals in a mouse model of human lymphoma.

4. The antibody or antigen-binding fragment thereof of claim 1, wherein the antibody or fragment thereof comprises a HCVR having the amino acid sequence of SEQ ID NO:339, and a LCVR having the amino acid sequence of SEQ ID NO:347.