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(71) Applicant: **ACADEMIA SINICA**; 128 Academia Road, Section 2, Nankang, Taipei, 11529 (TW).

(72) Inventor; and
(71) Applicant : **WONG, Chi-Huey** [US/US]; P.O. Box 8154, Rancho Santa Fe, California 92067 (US).

(72) Inventors: **WU, Chung-Yi**; No. 78-1, Donshi Street, Xi-Zhi District, New Taipei City, 221 (TW). **TSAI, Ming-Hung**; c/o Academia Sinica, 128 Academia Road, Section 2, Nankang, Taipei, 11529 (TW).

(74) Agent: **NORTON, Vicki**; DUANE MORRIS LLP, Suite 2900, 750 B Street, San Diego, California 92101 (US).

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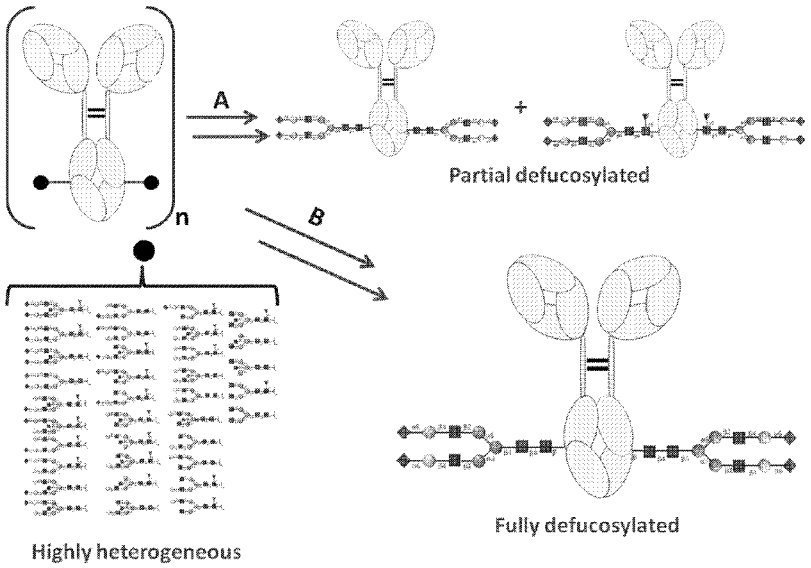

Figure 1

(57) Abstract: The present disclosure relates to a novel class of anti-CD20 monoclonal antibodies comprising a homogeneous population of anti-CD20 IgG molecules having the same N-glycan on each of Fc. The antibodies of the invention can be produced from anti-CD20 monoclonal antibodies by Fc glycoengineering. Importantly, the antibodies of the invention have improved therapeutic values with increased ADCC activity and increased Fc receptor binding affinity compared to the corresponding monoclonal antibodies that have not been glycoengineered.

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ANTI-CD20 GLYCOANTIBODIES AND USES THEREOF

RELATED APPLICATION

[0001] This application claims the benefit of U.S. provisional applications United States Serail Number ("USSN") 62/003,136, May 27, 2014, USSN 62/020,199, July 2, 2014, and USSN 62/110,338, filed January 30, 2015. The contents of each of which is hereby incorporated by reference in its entirety.

FIELD

[0002] Antibodies, antibody variants, antigen binding fragments and conjugates thereof that bind to CD20 are disclosed herein, as well as related compositions and methods of use. Methods of use include, without limitation, cancer therapies and diagnostics.

BACKGROUND OF THE INVENTION

[0003] Immunoglobulins and Fc receptors are critical glycoprotein components of the immune system. Fc receptors bind the Fc (effector) region of antibody molecules and communicate information within the innate and adaptive immune systems. Glycosylation of antibodies, particularly in the Fc region of IgG, plays an important role in the modulation of the activity of the antibody. The N-glycans in the identical heavy chains have been shown to be critical for maintaining structural integrity, communication with the Fc receptor and the downstream immunological response.

[0004] Fc glycosylation has been an important subject in the field of therapeutic monoclonal antibodies. Fc glycosylation can significantly modify Fc effector functions such as Fc receptor binding and complement activation, and thus affect the in vivo safety and efficacy profiles of therapeutic antibodies.

[0005] Several expression systems based on genetically engineering have been reported to produce therapeutic monoclonal antibodies. These include yeasts such as Pichia pastoris, insect cell lines, and even bacteria. However, these expression systems suffer from a number of drawbacks that can negatively affect the effector function of therapeutic antibodies.

[0006] The majority of biopharmaceuticals are produced in yeast or mammalian cell culture systems to deliver proteins with desired glycosylation patterns and thus ensure reduced immunogenicity and higher in vivo efficacy and stability. Non-human mammalian expression systems such as CHO or NS0 cells have the machinery required to add complex, human-type glycans. However, glycans produced in these systems can differ from glycans produced in humans. Their glycosylation machinery often adds undesired carbohydrate determinants which

may alter protein folding, induce immunogenicity, and reduce circulatory life span of the drug. Notably, sialic acid as N-acetylneuraminic acid is not efficiently added in most mammalian cells and the 6-linkage is missing in these cells. Engineering cells with the various enzymatic activities required for sialic acid transfer has not yet succeeded in providing a human-like pattern of glycoforms to protein drugs. To date, there is a need for engineering animal cells or glycoproteins to highly sialylated products that resemble as closely as possible to human proteins.

[0007] Furthermore, mammalian cell culture delivers a heterogeneous mixture of glycosylation patterns which do not all have the same properties. Properties like safety, efficacy and the serum half-life of therapeutic proteins can be affected by these glycosylation patterns.

SUMMARY OF THE INVENTION

[0008] The present disclosure relates to the development of a novel class of monoclonal antibodies, named "glycoantibodies". Accordingly, one aspect of the present disclosure relates to a composition of anti-CD20 glycoantibodies comprising a homogeneous population of anti-CD20 IgG molecules having the same N-glycan on each of Fc. The anti-CD20 glycoantibodies of the invention can be produced from anti-CD20 monoclonal antibodies by Fc glycoengineering. Importantly, the anti-CD20 glycoantibodies have improved therapeutic values with increased ADCC activity and increased Fc receptor binding affinity compared to the corresponding monoclonal antibodies that have not been glycoengineered. In addition, the disclosure also provides combination pharmaceutical compositions suitable for monotherapy or combination therapy that comprises substantially homogeneous glycoantibodies described herein and other antibodies and/or other therapeutic agents. The pharmaceutical composition can be administered as coformulation or used in co-administration therapeutic regimen.

[0009] In one embodiment, the N-glycan is attached to the Asn-297 of the Fc region (CH2 domain).

[0010] In some embodiments, the anti-CD20 glycoantibody described herein comprises a heavy chain having the amino acid sequence set forth in SEQ ID NO: 1, and a light chain having the amino acid sequence set forth in SEQ ID NO: 2. In a preferred embodiment, the glycoantibody comprises a light chain sequence and a heavy chain sequence of Rituximab (Rituxan®). There exist two different types of anti-CD20 antibodies (Cragg, M.S., et al,, Blood, 103 (2004) 2738-2743; and Cragg, M.S., et al, Blood, 101 (2003) 1045-1052). Type I antibodies, as e.g. rituximab (a non-afocusylated, non-glycoengineered antibody with normal glycosylation pattern, also named "RTX"), are potent in complement mediated cytotoxicity, whereas type II antibodies, as e.g. Tositumomab (Bl), 11B8, AT80 or humanized B-Lyl

antibodies, effectively initiate target cell death via caspase-independent apoptosis with concomitant phosphatidylserine exposure.

[0011] Disclosed herein are a number of functionally active anti-CD20 glycoantibodies constructed by Fc glycoengineering from Rituximab. Importantly, anti-CD20 glycoantibodies with optimized glycoforms exhibit significantly improved ADCC activities as compared to Rituximab. This is the first report that shows homogeneously Fc-glycosylated anti-CD20 antibodies with enhanced ADCC activity have been successfully generated.

[0012] In some embodiments, the anti-CD20 glycoantibodies described herein are characterized in that the glycoantibodies exhibit enhanced binding to FcγRIIIA as compared to Rituximab. In certain embodiments, the resultant ADCC activity of the glycoantibody according to the invention is at least 8 fold increased, preferably at least 15 fold, more preferably at least 35 fold increased ADCC activity, preferably at least 50 fold increased ADCC activity, preferably at least 60 fold increased ADCC activity, most preferred at least 80 fold increased ADCC activity compared to the ADCC activity of the parental antibody.

[0013] In some embodiments, the N-glycan described herein has a biantennary structure. In some embodiments, the N-glycan comprises a bisecting GlcNAc.

[0014] In some embodiments, the N-glycan described herein comprises at least one α2–6 terminal sialic acid. In certain embodiments, the N-glycan comprises one α2–6 terminal sialic acid. In a preferred embodiment, the N-glycan comprises two α2–6 terminal sialic acids.

[0015] In some embodiments, the N-glycan described herein comprises at least one α2–3 terminal sialic acid. In certain embodiments, the N-glycan comprises one α2–3 terminal sialic acid. In a preferred embodiment, the N-glycan comprises two α2–3 terminal sialic acids.

[0016] In some embodiments, the N-glycan described herein comprises at least one galactose. In certain embodiments, the N-glycan comprises one galactose. In a preferred embodiment, the N-glycan comprises two galactoses.

[0017] Preferrably, the N-glycan according to the disclosure is free of core fucose.

[0018] In some embodiments, the N-glycan described herein comprises the sequence selected from the group consisting of $Sia_2(\alpha2\text{-}6)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}6)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}3)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}3)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}3/\alpha2\text{-}6)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}6/\alpha2\text{-}3)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}3/\alpha2\text{-}6)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}6/\alpha2\text{-}3)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia(\alpha2\text{-}6)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia(\alpha2\text{-}3)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia(\alpha2\text{-}6)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia(\alpha2\text{-}$

3)$Gal_2GlcNAc_3Man_3GlcNAc_2$, Sia(α2-6)$GalGlcNAc_2Man_3GlcNAc_2$, Sia(α2-3)$GalGlcNAc_2Man_3GlcNAc_2$, Sia(α2-6)$GalGlcNAc_3Man_3GlcNAc_2$, Sia(α2-3)$GalGlcNAc_3Man_3GlcNAc_2$, $Gal_2GlcNAc_2Man_3GlcNAc_2$, $GalGlcNAcMan_3GlcNAc_2$, $Gal_2GlcNAc_3Man_3GlcNAc_2$, $GalGlcNAc_2Man_3GlcNAc_2$, $GalGlcNAc_3Man_3GlcNAc_2$, $GlcNAc_3Man_3GlcNAc_2$, $GlcNAc_2Man_3GlcNAc_2$, $GlcNAcMan_3GlcNAc_2$ and $Man_3GlcNAc_2$.

[0019] In preferred embodiments, the N-glycan described herein has the sequence selected from the group consisting of $Sia_2(\alpha2\text{-}6)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}6)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}3)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}3)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}3/\alpha2\text{-}6)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}6/\alpha2\text{-}3)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}3/\alpha2\text{-}6)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}6/\alpha2\text{-}3)Gal_2GlcNAc_3Man_3GlcNAc_2$, Sia(α2-6)$Gal_2GlcNAc_2Man_3GlcNAc_2$, Sia(α2-3)$Gal_2GlcNAc_2Man_3GlcNAc_2$, Sia(α2-6)$Gal_2GlcNAc_3Man_3GlcNAc_2$, Sia(α2-3)$Gal_2GlcNAc_3Man_3GlcNAc_2$, Sia(α2-6)$GalGlcNAc_2Man_3GlcNAc_2$, Sia(α2-3)$GalGlcNAc_2Man_3GlcNAc_2$, Sia(α2-6)$GalGlcNAc_3Man_3GlcNAc_2$, Sia(α2-3)$GalGlcNAc_3Man_3GlcNAc_2$, $Gal_2GlcNAc_2Man_3GlcNAc_2$, $GalGlcNAcMan_3GlcNAc_2$ and $Gal_2GlcNAc_3Man_3GlcNAc_2$.

[0020] Another aspect of the present disclosure features a pharmaceutical composition comprising a composition of anti-CD20 glycoantibodies described herein and a pharmaceutically acceptable carrier.

[0021] The pharmaceutical composition according to the disclosure may be used in therapeutics. Disclosed herein include methods for the treatment of cancer in a patient, the method comprising administering to the patient an effective amount of a pharmaceutical composition described herein.

[0022] Examples of cancers include, but not limited to, CD20 expressing cancers, B cell lymphomas, NHL, precursor B cell lymphoblastic leukemia/lymphoma and mature B cell neoplasms, B cell chronic lymphocytic leukemia (CLL)/small lymphocytic lymphoma (SLL), B cell prolymphocytic leukemia, lymphoplasmacytic lymphoma, mantle cell lymphoma (MCL), follicular lymphoma (FL), low-grade, intermediate-grade and high-grade (FL), cutaneous follicle center lymphoma, marginal zone B cell lymphoma, MALT type marginal zone B cell lymphoma, nodal marginal zone B cell lymphoma, splenic type 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 (ALCL).

[0023] In certain embodiments, the cancer is B-cell lymphoma such as non-Hodgkin's lymphoma.

[0024] Further, the pharmaceutical composition described herein may be used for treating a patient having an autoimmune or inflammatory disease. The method of the treatment comprises administering to the patient an effective amount of a pharmaceutical composition described herein. In certain embodiments, one or more additional other cytotoxic, chemotherapeutic or anti-cancer agents, or compounds or ionizing radiation that enhance the effects of such agents are co-administered.

[0025] Examples of the autoimmune or inflammatory disease include, but are not limited to, rheumatoid arthritis, juvenile rheumatoid arthritis, systemic lupus erythematosus (SLE), Wegener's disease, inflammatory bowel disease, idiopathic thrombocytopenic purpura (ITP), thrombotic thrombocytopenic purpura (TTP), autoimmune thrombocytopenia, multiple sclerosis, psoriasis, IgA nephropathy, IgM polyneuropathies, myasthenia gravis, vasculitis, diabetes mellitus, Reynaud's syndrome, Crohn's diasease, ulcerative colitis, gastritis, Hashimoto's thyroiditis, ankylosing spondylitis, hepatitis C-associated cryoglobulinemic vasculitis, chronic focal encephalitis, bullous pemphigoid, hemophilia A, membranoproliferative glomerulnephritis, adult and juvenile dermatomyositis, adult polymyositis, chronic urticaria, primary biliary cirrhosis, neuromyelitis optica, Graves' dysthyroid disease, bullous pemphigoid, membranoproliferative glonerulonephritis, Churg-Strauss syndrome, asthma, psoriatic arthritis, dermatitis, respiratory distress syndrome, meningitis, encephalitits, uveitis, eczema, atherosclerosis, leukocyte adhesion deficiency, juvenile onset diabetes, Reiter's disease, Behcet's disease, hemolytic anemia, atopic dermatitis, Wegener's granulomatosis, Omenn's syndrome, chronic renal failure, acute infectious mononucleosis, HIV and herpes-associated disease, systemic sclerosis, Sjorgen's syndrome and glomerulonephritis, dermatomyositis, ANCA, aplastic anemia, autoimmune hemolytic anemia (AIHA), factor VIII deficiency, hemophilia A, autoimmune neutropenia, Castleman's syndrome, Goodpasture's syndrome, solid organ transplant rejection, graft versus host disease (GVHD), autoimmune hepatitis, lymphoid interstitial pneumonitis (HIV), bronchiolitis obliterans (non-transplant), Guillain-Barre Syndrome, large vessel vasculitis, giant cell (Takayasu's) arteritis, medium vessel vasculitis, Kawasaki's Disease, and polyarteritis nodosa.

[0026] In certain embodiments, the autoimmune or inflammatory disease is rheumatoid arthritis.

[0027] In these treatment methods described herein, the pharmaceutical composition of anti-CD20 glycoantibodies can be administered alone or in conjunction with a second therapeutic

agent such as a second antibody, or a chemotherapeutic agent or an immunosuppressive agent. The second antibody can be one that binds CD20 or a different B cell antigen, or a NK or T cell antigen.

[0028] The anti-CD20 glycoantibodies described herein may be generated from anti-CD20 monoclonal antibodies approved by FDA or in development. The anti-CD20 monoclonal antibodies may be humanized, human or chimeric.

[0029] The anti-CD20 glycoantibodies described herein may be produced *in vitro*. The anti-CD20 glycoantibodies may be generated by Fc glycoengineering. In certain embodiments, the anti-CD20 glycoantibodies are enzymatically or chemoenzymatically engineered from the anti-CD20 monoclonal antibodies obtained by mammalian cell culturing.

[0030] In yet another aspect, the present disclosure relates to a method of making an anti-CD20 glycoantibody, the method comprising: (a) contacting an anti-CD20 monoclonal antibody with an alpha-fucosidase and at least one endoglycosidase, thereby an anti-CD20 monoclonal antibody bearing a defucosylated monosaccharide GlcNAc on the Fc is produced, and (b) adding an carbohydrate moiety to GlcNAc under suitable conditions.

[0031] In some embodiments, the anti-CD20 monoclonal antibody used for making an anti-CD20 glycoantibody is Rituximab.

[0032] In some embodiments, the carbohydrate moiety is selected from the group consisting of $Sia_2(\alpha2\text{-}6)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}6)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}3)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}3)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}3/\alpha2\text{-}6)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}6/\alpha2\text{-}3)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}3/\alpha2\text{-}6)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}6/\alpha2\text{-}3)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia(\alpha2\text{-}6)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia(\alpha2\text{-}3)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia(\alpha2\text{-}6)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia(\alpha2\text{-}3)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia(\alpha2\text{-}6)GalGlcNAc_2Man_3GlcNAc_2$, $Sia(\alpha2\text{-}3)GalGlcNAc_2Man_3GlcNAc_2$, $Sia(\alpha2\text{-}6)GalGlcNAc_3Man_3GlcNAc_2$, $Sia(\alpha2\text{-}3)GalGlcNAc_3Man_3GlcNAc_2$, $Gal_2GlcNAc_2Man_3GlcNAc_2$, $GalGlcNAcMan_3GlcNAc_2$, $Gal_2GlcNAc_3Man_3GlcNAc_2$, $GalGlcNAc_2Man_3GlcNAc_2$, $GalGlcNAc_3Man_3GlcNAc_2$, $GlcNAc_3Man_3GlcNAc_2$, $GlcNAc_2Man_3GlcNAc_2$, $GlcNAcMan_3GlcNAc_2$ and $Man_3GlcNAc_2$.

[0033] In preferred embodiments, the carbohydrate moiety is selected from the group consisting of $Sia_2(\alpha2\text{-}6)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}6)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}3)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}3)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}3/\alpha2\text{-}6)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}6/\alpha2\text{-}3)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}3/\alpha2\text{-}$

6)$Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}6/\alpha2\text{-}3)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia(\alpha2\text{-}6)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia(\alpha2\text{-}3)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia(\alpha2\text{-}6)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia(\alpha2\text{-}3)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia(\alpha2\text{-}6)GalGlcNAc_2Man_3GlcNAc_2$, $Sia(\alpha2\text{-}3)GalGlcNAc_2Man_3GlcNAc_2$, $Sia(\alpha2\text{-}6)GalGlcNAc_3Man_3GlcNAc_2$, $Sia(\alpha2\text{-}3)GalGlcNAc_3Man_3GlcNAc_2$, $Gal_2GlcNAc_2Man_3GlcNAc_2$, $GalGlcNAcMan_3GlcNAc_2$ and $Gal_2GlcNAc_3Man_3GlcNAc_2$.

[0034] The adding in step (b) can be performed by a transglycosylase. Transglycosylase includes, but are not limited to EndoS, EndoS2, EndoH, EndoA, EndoM, EndoF, EndoF1, EndoF2 and EndoF3.

[0035] Endoglycosidases useful for the method of the invention include, but are not limited to EndoS, EndoS2, EndoH, EndoA, EndoM, EndoF, EndoF1, EndoF2 and EndoF3.

[0036] In some embodiments, the alpha-fucosidase comprises a polypeptide having an amino acid sequence at least about 90%, 91%, 92 %, 93%, 94%, 95 %, 96%, 97%, 98 % or 99% identical to SEQ ID NO: 5.

[0037] In certain embodiments, the alpha-fucosidase is a recombinant *Bacteroides* alpha-L-fucosidase.

[0038] The details of one or more embodiments of the invention are set forth in the description below. Other features or advantages of the present invention will be apparent from the following drawings and detailed description of several embodiments, and also from the appending claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0039] Figure 1. shows a schematic representation of Fc glycoengineering of monoclonal antibodies. Route (A) shows the methods known in the art that lead to a mixture of fucosylated and nonfucosylated antibodies. Route (B) shows the method of the present invention that leads to a homogeneous glycoantibodies.

[0040] Figure 2. shows SDS-PAGE analyses of anti-CD20 GAbs 101, 102, 104, 105, 106, 107, 108, 109, 110, 111, 201 and Rituximab.

[0041] Figure 3. shows N-glycan profiling for anti-CD20 GAbs 101,102, 104, 105, 106, 107, 108, 109, 110 and 111.

[0042] Figure 4. shows binding to (A) Ramos cells and (B) SKW6.4 cells by Rituximab, GAb101 and GAb104, and binding to (C) Ramos, Raji and SU-DHL4 cells by Rituximab, GAb101 and GAb117.

[0043] Figure 5. shows apoptosis to (A) Ramos cells and (B) SKW6.4 cells by Rituximab, GAb101 and GAb104.

[0044] Figure 6. shows CDC to Ramos cells induced by (A) Rituximab, GAb101 and GAb117; (B) GAb101, GAb104 and GAb108.

[0045] Figure 7. shows ADCC to (A) Ramos cells and (B)(C) SKW6.4 cells induced by Rituximab, GAb101, GAb104 and GAb117 using effector PBMC cells.

[0046] Figure 8. shows depletion of human B cells in PBMC from three donors in the absence of autologous plasma by Rituximab and anti-CD20 GAbs 101, 102, 105, 106, 107, 108, 109, 110 and 111.

[0047] Figure 9. shows suppression of Ramos tumor xenograft in SCID mice by GAb101.

DETAILED DESCRIPTION OF THE INVENTION

[0048] Rituximab (Rituxan®) is a chimeric anti-CD20 antibody targeting the CD20 protein which is expressed on over 95% of B cell lymphomas. Monoclonal antibody therapy with the anti-CD20 mAb Rituximab represents one of the most important advances in the treatment of lymphoproliferative disorders in the last 30 years. Rituximab is produced in Chinese hamster ovary (CHO) cells. The mammalian cell culture system delivers heterogeneous mixtures of glycosylation patterns which do not all have the same properties. Diversity in Fc glycosylation within an antibody will correspond to diversity in Fc effector functions. Thus, this heterogeneity in Fc glycans has a functional consequence as it influences binding of IgG molecules to Fc receptors and C1q and thereby impacts antibody effector functions, and may trigger undesired effects in patients thus deeming them a safety concern. For example, a proportion of patients with CD20 positive malignancies fail to respond to, or more commonly relapse, after receiving Rituximab-containing immunochemotherapy.

[0049] A need remains for improving monoclonal antibody therapy with improved anti-CD20 antibodies. A few specific glycoforms in the heterogeneous mixtures of glycosylation patterns are known to confer desired biological functions. Thus, it is of great interest to generate therapeutic antibodies containg a well-defined glycan structure and sequence as desired glycoforms for therapeutic purposes.

Definitions

[0050] The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular 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, Molecular Cloning A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press, 1989); DNA Cloning,

Volumes I and II (D. N. Glover ed., 1985); Culture Of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984); the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Methods In Enzymology, Vols. 154 and 155 (Wu et al. eds.), Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Antibodies: A Laboratory Manual, by Harlow and Lane s (Cold Spring Harbor Laboratory Press, 1988); and Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986).

[0051] The term "glycoantibodies" was coined by the inventor, Dr. Chi-Huey Wong, to refer to a homogeneous population of monoclonal antibodies (preferably, therapeutic monoclonal antibodies) having a single, uniformed glycoform bound to the Fc region. The individual glycoantibodies comprising the essentially homogeneous population are identical, bind to the same epitope, and contain the same Fc glycan with a well-defined glycan structure and sequence.

[0052] As used herein, the term "anti-CD20 glycoantibodies" ("anti-CD20 GAbs") refers to a homogeneous population of anti-CD20 IgG molecules having the same glycoform on Fc.

[0053] The term "anti-CD20 glycoantibody" ("anti-CD20 GAb") refers to an individual IgG molecule in the anti-CD20 glycoantibodies. In certain embodiments, anti-CD20 "molecule" can also include antigen binding fragments.

[0054] As used herein, the term "glycan" refers to a polysaccharide, oligosaccharide or monosaccharide. Glycans can be monomers or polymers of sugar residues and can be linear or branched. A glycan may include natural sugar residues (e.g., glucose, N-acetylglucosamine, N-acetyl neuraminic acid, galactose, mannose, fucose, hexose, arabinose, ribose, xylose, etc.) and/or modified sugars (e.g., 2′-fluororibose, 2′-deoxyribose, phosphomannose, 6′ sulfo N-acetylglucosamine, etc). Glycan is also used herein to refer to the carbohydrate portion of a glycoconjugate, such as a glycoprotein, glycolipid, glycopeptide, glycoproteome, peptidoglycan, lipopolysaccharide or a proteoglycan. Glycans usually consist solely of O-glycosidic linkages between monosaccharides. For example, cellulose is a glycan (or more specifically a glucan) composed of ß-1,4-linked D-glucose, and chitin is a glycan composed of ß-1,4-linked N-acetyl-D-glucosamine. Glycans can be homo or heteropolymers of monosaccharide residues, and can be linear or branched. Glycans can be found attached to proteins as in glycoproteins and proteoglycans. They are generally found on the exterior surface of cells. O- and N-linked

glycans are very common in eukaryotes but may also be found, although less commonly, in prokaryotes. N-Linked glycans are found attached to the R-group nitrogen (N) of asparagine in the sequon. The sequon is a Asn-X-Ser or Asn-X-Thr sequence, where X is any amino acid except praline.

[0055] As used herein, the terms "fucose", "core fucose" and " core fucose residue" are used interchangeably and refer to a fucose in α1,6-position linked to the N-acetylglucosamine .

[0056] As used herein, the terms "N-glycan", "N-linked glycan", "N-linked glycosylation", "Fc glycan" and "Fc glycosylation" are used interchangeably and refer to an N-linked oligosaccharide attached by an N-acetylglucosamine (GlcNAc) linked to the amide nitrogen of an asparagine residue in a Fc-containing polypeptide. The term "Fc-containing polypeptide" refers to a polypeptide, such as an antibody, which comprises an Fc region.

[0057] As used herein, the term "glycosylation pattern" and "glycosylation profile" are used interchangeably and refer to the characteristic "fingerprint" of the N-glycan species that have been released from a glycoprotein or antibody, either enzymatically or chemically, and then analyzed for their carbohydrate structure, for example, using LC-HPLC, or MALDI-TOF MS, and the like. See, for example, the review in Current Analytical Chemistry, Vol. 1, No. 1 (2005), pp. 28-57; herein incorporated by reference in its entirety.

[0058] As used herein, the term "glycoengineered Fc" when used herein refers to N-glycan on the Fc region has been altered or engineered either enzymatically or chemically. The term "Fc glycoengineering" as used herein refers to the enzymatic or chemical process used to make the glycoengineered Fc. Exemplary methods of engineering are described in, for example, Wong et al USSN12/959,351, the contents of which is hereby incorporated by reference.

[0059] The terms "homogeneous", "uniform", "uniformly" and "homogeneity" in the context of a glycosylation profile of Fc region are used interchangeably and are intended to mean a single glycosylation pattern represented by one desired N-glycan species, with little or no trace amount of precursor N-glycan. In certain embodiments, the trace amount of the precursor N-glycan is less than about 2%.

[0060] "Essentially pure" protein means a composition comprising at least about 90% by weight of the protein, based on total weight of the composition, including, for example, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% by weight.

[0061] "Essentially homogeneous" protein means a composition comprising at least about 98% by weight of protein, including for example, at least about 98.5 %, at least about 99% based

on total weight of the composition. In certain embodiments, the protein is an antibody, structural variants, and/or antigen binding fragment thereof.

[0062] As used herein, the terms " IgG", "IgG molecule", "monoclonal antibody", "immunoglobulin", and "immunoglobulin molecule" are used interchangeably. In certain embodiments, anti-CD20 "molecule" can also include antigen binding fragments.

[0063] As used herein, the term "Fc receptor" or "FcR" describes a receptor that binds to the Fc region of an antibody. The preferred FcR is a native sequence human FcR. Moreover, a preferred FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) subclasses, including allelic variants and alternatively spliced forms of these receptors. FcγRII receptors include FcγRIIA (an "activating receptor") and FcγRIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor FcγRIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor FcγRIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain. (see review M. in Daëron, *Annu. Rev. Immunol.* 15:203-234 (1997)). FcRs are reviewed in Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991); Capel et al., *Immunomethods* 4:25-34 (1994); and de Haas et al., *J. Lab. Clin. Med.* 126:330-41 (1995). Other FcRs, including those to be identified in the future, are encompassed by the term "FcR" herein. The term also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer et al., *J. Immunol.* 117:587 (1976) and Kim et al., *J. Immunol.* 24:249 (1994)).

[0064] The term "effector function" as used herein refers to a biochemical event that results from the interaction of an antibody Fc region with an Fc receptor or ligand. Exemplary "effector functions" include C1q binding; complement dependent cytotoxicity; Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (e.g. B cell receptor; BCR), etc. Such effector functions can be assessed using various assays known in the art.

[0065] As used herein, the term "Antibody-dependent cell-mediated cytotoxicity" or "ADCC" refers to a form of cytotoxicity in which secreted Ig bound onto Fc receptors (FcRs) present on certain cytotoxic cells (e.g. Natural Killer (NK) cells, neutrophils, and macrophages) enable these cytotoxic effector cells to bind specifically to an antigen-bearing target cell and subsequently kill the target cell with cytotoxins. The antibodies "arm" the cytotoxic cells and are absolutely required for such killing. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on

hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991). To assess ADCC activity of a molecule of interest, an in vitro ADCC assay, such as that described in U.S. Pat. No. 5,500,362 or U.S. Pat. No. 5,821,337 may be performed. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed in vivo, e.g., in a animal model such as that disclosed in Clynes et al. *PNAS (USA)* 95:652-656 (1998).

[0066] The term "Complement dependent cytotoxicity" or "CDC" as used herein refers to the lysis of a target cell in the presence of complement. Activation of the classical complement pathway is initiated by the binding of the first component of the complement system (C1q) to antibodies (of the appropriate subclass) which are bound to their cognate antigen. To assess complement activation, a CDC assay, e.g. as described in Gazzano-Santoro et al., *J. Immunol. Methods* 202:163 (1996), may be performed.

[0067] "Chimeric" antibodies (immunoglobulins) have a portion of the heavy and/or light chain identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)). Humanized antibody as used herein is a subset of chimeric antibodies.

[0068] "Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient or acceptor antibody) in which hypervariable region residues of the recipient are replaced by hypervariable region residues from a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance such as binding affinity. Generally, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence although the

FR regions may include one or more amino acid substitutions that improve binding affinity. The number of these amino acid substitutions in the FR is typically no more than 6 in the H chain, and in the L chain, no more than 3. 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., *Nature* 321:522-525 (1986); Reichmann et al., *Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992). See also the following review articles and references cited therein: Vaswani and Hamilton, Ann. Allergy, Asthma & Immunol. 1:105-115 (1998); Harris, Biochem. Soc. Transactions 23:1035-1038 (1995); Hurle and Gross, Curr. Op. Biotech. 5:428-433 (1994).

[0069] As used herein, the term "antigen" is defined as any substance capable of eliciting an immune response. As used herein, the term "antigen specific" refers to a property of a cell population such that supply of a particular antigen, or a fragment of the antigen, results in specific cell proliferation.

[0070] As used herein, the term "immunogenicity" refers to the ability of an immunogen, antigen, or vaccine to stimulate an immune response.

[0071] As used herein, the term "epitope" is defined as the parts of an antigen molecule which contact the antigen binding site of an antibody or a T cell receptor.

[0072] As used herein, the term "specifically binding," refers to the interaction between binding pairs (e.g., an antibody and an antigen). In various instances, specifically binding can be embodied by an affinity constant of about 10-6 moles/liter, about 10-7 moles/liter, or about 10-8 moles/liter, or less.

[0073] An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with research, diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes.

[0074] The phrase "substantially similar," "substantially the same", "equivalent", or "substantially equivalent", as used herein, denotes a sufficiently high degree of similarity between two numeric values (for example, one associated with a molecule and the other associated with a reference/comparator molecule) such that one of skill in the art would consider the difference between the two values to be of little or no biological and/or statistical significance within the context of the biological characteristic measured by said values (e.g., Kd

values, anti-viral effects, etc.). The difference between said two values is, for example, less than about 50%, less than about 40%, less than about 30%, less than about 20%, and/or less than about 10% as a function of the value for the reference/comparator molecule.

[0075] The phrase "substantially reduced," or "substantially different", as used herein, denotes a sufficiently high degree of difference between two numeric values (generally one associated with a molecule and the other associated with a reference/comparator molecule) such that one of skill in the art would consider the difference between the two values to be of statistical significance within the context of the biological characteristic measured by said values (e.g., Kd values). The difference between said two values is, for example, greater than about 10%, greater than about 20%, greater than about 30%, greater than about 40%, and/or greater than about 50% as a function of the value for the reference/comparator molecule.

[0076] "Binding affinity" generally refers to the strength of the sum total of noncovalent interactions between a single binding site of a molecule (e.g., an antibody) and its binding partner (e.g., an antigen). Unless indicated otherwise, as used herein, "binding affinity" refers to intrinsic binding affinity which reflects a 1:1 interaction between members of a binding pair (e.g., antibody and antigen). The affinity of a molecule X for its partner Y can generally be represented by the dissociation constant (Kd). Affinity can be measured by common methods known in the art, including those described herein. Low-affinity antibodies generally bind antigen slowly and tend to dissociate readily, whereas high-affinity antibodies generally bind antigen faster and tend to remain bound longer. A variety of methods of measuring binding affinity are known in the art, any of which can be used for purposes of the present invention. Specific illustrative embodiments are described in the following.

[0077] The "variable region" or "variable domain" of an antibody refers to the amino-terminal domains of heavy or light chain of the antibody. These domains are generally the most variable parts of an antibody and contain the antigen-binding sites.

[0078] The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity-determining regions (CDRs) or hypervariable regions both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a beta-sheet configuration, connected by three

CDRs, which form loops connecting, and in some cases forming part of, the beta-sheet structure. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., Sequences of Proteins of Immunological Interest, Fifth Edition, National Institute of Health, Bethesda, Md. (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

[0079] Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an F(ab′)2 fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

[0080] "Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. In a two-chain Fv species, this region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. In a single-chain Fv species, one heavy- and one light-chain variable domain can be covalently linked by a flexible peptide linker such that the light and heavy chains can associate in a "dimeric" structure analogous to that in a two-chain Fv species. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the VH-VL dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

[0081] The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab′ fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab′-SH is the designation herein for Fab′ in which the cysteine residue(s) of the constant domains bear a free thiol group. F(ab′)2 antibody fragments originally were produced as pairs of Fab′ fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

[0082] The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

[0083] Depending on the amino acid sequences of the constant domains of their heavy chains, antibodies (immunoglobulins) can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known and described generally in, for example, Abbas et al. Cellular and Mol. Immunology, 4th ed. (2000). An antibody may be part of a larger fusion molecule, formed by covalent or non-covalent association of the antibody with one or more other proteins or peptides.

[0084] The terms "full length antibody," "intact antibody" and "whole antibody" are used herein interchangeably, to refer to an antibody in its substantially intact form, not antibody fragments as defined below. The terms particularly refer to an antibody with heavy chains that contain the Fc region.

[0085] "Antibody fragments" comprise only a portion of an intact antibody, wherein the portion retains at least one, and as many as most or all, of the functions normally associated with that portion when present in an intact antibody. In one embodiment, an antibody fragment comprises an antigen binding site of the intact antibody and thus retains the ability to bind antigen. In another embodiment, an antibody fragment, for example one that comprises the Fc region, retains at least one of the biological functions normally associated with the Fc region when present in an intact antibody, such as FcRn binding, antibody half life modulation, ADCC function and complement binding. In one embodiment, an antibody fragment is a monovalent antibody that has an in vivo half life substantially similar to an intact antibody. For example, such an antibody fragment may comprise an antigen binding arm linked to an Fc sequence capable of conferring in vivo stability to the fragment.

[0086] The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies. Such monoclonal antibody typically includes an antibody comprising a polypeptide sequence that binds a target, wherein the target-binding polypeptide sequence was obtained by a process that includes the selection of a single target binding polypeptide sequence from a plurality of polypeptide sequences. For example, the

selection process can be the selection of a unique clone from a plurality of clones, such as a pool of hybridoma clones, phage clones or recombinant DNA clones. It should be understood that the selected target binding sequence can be further altered, for example, to improve affinity for the target, to humanize the target binding sequence, to improve its production in cell culture, to reduce its immunogenicity in vivo, to create a multispecific antibody, etc., and that an antibody comprising the altered target binding sequence is also a monoclonal antibody of this invention. In contrast to polyclonal antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen. In addition to their specificity, the monoclonal antibody preparations are advantageous in that they are typically uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by a variety of techniques, including, for example, the hybridoma method (e.g., Kohler et al., Nature, 256: 495 (1975); Harlow et al., Antibodies: A Laboratory Manual, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling et al., in: Monoclonal Antibodies and T-Cell hybridomas 563-681 (Elsevier, N.Y., 1981)), recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567), phage display technologies (See, e.g., Clackson et al., Nature, 352: 624-628 (1991); Marks et al., J. Mol. Biol. 222: 581-597 (1992); Sidhu et al., J. Mol. Biol. 338(2): 299-310 (2004); Lee et al., J. Mol. Biol. 340(5): 1073-1093 (2004); Fellouse, Proc. Natl. Acad. Sci. USA 101(34): 12467-12472 (2004); and Lee et al., J. Immunol. Methods 284(1-2): 119-132 (2004), and technologies for producing human or human-like antibodies in animals that have parts or all of the human immunoglobulin loci or genes encoding human immunoglobulin sequences (see, e.g., WO98/24893; WO96/34096; WO96/33735; WO91/10741; Jakobovits et al., Proc. Natl. Acad. Sci. USA 90: 2551 (1993); Jakobovits et al., Nature 362: 255-258 (1993); Bruggemann et al., Year in Immunol. 7:33 (1993); U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016; Marks et al., Bio. Technology 10: 779-783 (1992); Lonberg et al., Nature 368: 856-859 (1994); Morrison, Nature 368: 812-813 (1994); Fishwild et al., Nature Biotechnol. 14: 845-851 (1996); Neuberger, Nature Biotechnol. 14: 826 (1996) and Lonberg and Huszar, Intern. Rev. Immunol. 13: 65-93 (1995).

[0087] The monoclonal antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody

class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; and Morrison et al., Proc. Natl. Acad. Sci. USA 81:6851-6855 (1984)).

[0088] See also the following review articles and references cited therein: Vaswani and Hamilton, Ann. Allergy, Asthma & Immunol. 1:105-115 (1998); Harris, Biochem. Soc. Transactions 23:1035-1038 (1995); Hurle and Gross, Curr. Op. Biotech. 5:428-433 (1994).

[0089] The term "hypervariable region", "HVR", or "HV", when used herein refers to the regions of an antibody variable domain which are hypervariable in sequence and/or form structurally defined loops. Generally, antibodies comprise six hypervariable regions; three in the VH (H1, H2, H3), and three in the VL (L1, L2, L3). A number of hypervariable region delineations are in use and are encompassed herein. The Kabat Complementarity Determining Regions (CDRs) are based on sequence variability and are the most commonly used (Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)). Chothia refers instead to the location of the structural loops (Chothia and Lesk J. Mol. Biol. 196:901-917 (1987)). The AbM hypervariable regions represent a compromise between the Kabat CDRs and Chothia structural loops, and are used by Oxford Molecular's AbM antibody modeling software. The "contact" hypervariable regions are based on an analysis of the available complex crystal structures. The residues from each of these hypervariable regions are noted below.

Loop Kabat AbM Chothia Contact

L1 L24-L34 L24-L34 L26-L32 L30-L36

L2 L50-L56 L50-L56 L50-L52 L46-L55

L3 L89-L97 L89-L97 L91-L96 L89-L96

H1 H31-H35B H26-H35B H26-H32 H30-H35B

(Kabat Numbering)

H1 H31-H35 H26-H35 H26-H32 H30-H35

(Chothia Numbering)

H2 H50-H65 H50-H58 H53-H55 H47-H58

H3 H95-H102 H95-H102 H96-H101 H93-H101

[0090] Hypervariable regions may comprise "extended hypervariable regions" as follows: 24-36 or 24-34 (L1), 46-56 or 50-56 or 49-56 (L2) and 89-97 or 89-96 (L3) in the VL and 26-35 (H1), 50-65 or 49-65 (H2) and 93-102, 94-102, or 95-102 (H3) in the VH. The variable domain residues are numbered according to Kabat et al., supra, for each of these definitions.

[0091] "Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined.

[0092] The term "variable domain residue numbering as in Kabat" or "amino acid position numbering as in Kabat," and variations thereof, refers to the numbering system used for heavy chain variable domains or light chain variable domains of the compilation of antibodies in Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991). Using this numbering system, the actual linear amino acid sequence may contain fewer or additional amino acids corresponding to a shortening of, or insertion into, a FR or HVR of the variable domain. For example, a heavy chain variable domain may include a single amino acid insert (residue 52a according to Kabat) after residue 52 of H2 and inserted residues (e.g. residues 82a, 82b, and 82c, etc. according to Kabat) after heavy chain FR residue 82. The Kabat numbering of residues may be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a "standard" Kabat numbered sequence.

[0093] "Single-chain Fv" or "scFv" antibody fragments comprise the VH and VL domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the scFv polypeptide further comprises a polypeptide linker between the VH and VL domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv see Pluckthun, in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

[0094] The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (VH) connected to a light-chain variable domain (VL) in the same polypeptide chain (VH-VL). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO93/1161; and Hollinger et al., Proc. Natl. Acad. Sci. USA 90: 6444-6448 (1993).

[0095] A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human and/or has been made using any of the techniques for making human antibodies as disclosed herein. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues.

[0096] An "affinity matured" antibody is one with one or more alterations in one or more HVRs thereof which result in an improvement in the affinity of the antibody for antigen, compared to a parent antibody which does not possess those alteration(s). In one embodiment, an affinity matured antibody has nanomolar or even picomolar affinities for the target antigen. Affinity matured antibodies are produced by procedures known in the art. Marks et al. Bio/Technology 10:779-783 (1992) describes affinity maturation by VH and VL domain shuffling. Random mutagenesis of CDR and/or framework residues is described by: Barbas et al. Proc Nat. Acad. Sci. USA 91:3809-3813 (1994); Schier et al. Gene 169:147-155 (1995); Yelton et al. J. Immunol. 155:1994-2004 (1995); Jackson et al., J. Immunol. 154(7):3310-9 (1995); and Hawkins et al, J. Mol. Biol. 226:889-896 (1992).

[0097] A "blocking" antibody or an "antagonist" antibody is one which inhibits or reduces biological activity of the antigen it binds. Certain blocking antibodies or antagonist antibodies substantially or completely inhibit the biological activity of the antigen.

[0098] An "agonist antibody", as used herein, is an antibody which mimics at least one of the functional activities of a polypeptide of interest.

[0099] A "disorder" is any condition that would benefit from treatment with an antibody of the invention. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include cancer.

[00100] The terms "cell proliferative disorder" and "proliferative disorder" refer to disorders that are associated with some degree of abnormal cell proliferation. In one embodiment, the cell proliferative disorder is cancer.

[00101] "Tumor," as used herein, refers to all neoplastic cell growth and proliferation, whether malignant or benign, and all pre-cancerous and cancerous cells and tissues. The terms "cancer," "cancerous," "cell proliferative disorder," "proliferative disorder" and "tumor" are not mutually exclusive as referred to herein.

[00102] The terms “cancer” and “cancerous” generally refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth/proliferation. Examples of cancer include, but are not limited to, carcinoma, lymphoma (e.g., Hodgkin's and non-Hodgkin's lymphoma), blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, leukemia and other lymphoproliferative disorders, and various types of head and neck cancer.

[00103] As used herein, the term “antigen” is defined as any substance capable of eliciting an immune response.

[00104] As used herein, the term “antigen specific” refers to a property of a cell population such that supply of a particular antigen, or a fragment of the antigen, results in specific cell proliferation.

[00105] The term "CD20 expressing cancer" as used herein refers to all cancers in which the cancer cells show an expression of the CD20 antigen. Preferably CD20 expressing cancer as used herein refers to lymphomas (preferably B-Cell Non-Hodgkin's lymphomas (NHL)) and lymphocytic leukemias. Such lymphomas and lymphocytic leukemias include e.g. a) follicular lymphomas, b) Small Non-Cleaved Cell Lymphomas/ Burkitt's lymphoma (including endemic Burkitt's lymphoma, sporadic Burkitt's lymphoma and Non-Burkitt's lymphoma) c) marginal zone lymphomas (including extranodal marginal zone B cell lymphoma (Mucosa-associated lymphatic tissue lymphomas, MALT), nodal marginal zone B cell lymphoma and splenic marginal zone lymphoma), d) Mantle cell lymphoma (MCL), e) Large Cell Lymphoma (including B-cell diffuse large cell lymphoma (DLCL), Diffuse Mixed Cell Lymphoma, Immunoblastic Lymphoma, Primary Mediastinal B-Cell Lymphoma, Angiocentric Lymphoma-Pulmonary B-Cell Lymphoma) f) hairy cell leukemia, g) lymphocytic lymphoma, Waldenstrom's macroglobulinemia, h) acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL)/ small lymphocytic lymphoma (SLL), B-cell prolymphocytic leukemia, i) plasma cell neoplasms, plasma cell myeloma, multiple myeloma, plasmacytoma j) Hodgkin's disease. More preferably the CD20 expressing cancer is a B-Cell Non-Hodgkin's lymphomas (NHL). Especially the CD20 expressing cancer is a Mantle cell lymphoma (MCL), acute

lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), B-cell diffuse large cell lymphoma (DLCL), Burkitt's lymphoma, hairy cell leukemia, follicular lymphoma, multiple myeloma, marginal zone lymphoma, post transplant lymphoproliferative disorder (PTLD), HIV associated lymphoma, Waldenstrom's macro globulinemia, or primary CNS lymphoma.

[00106] As used herein, "treatment" refers to clinical intervention in an attempt to alter the natural course of the individual or cell being treated, and can be performed either for prophylaxis or during the course of clinical pathology. Desirable effects of treatment include preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, preventing or decreasing inflammation and/or tissue/organ damage, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis. In some embodiments, antibodies of the invention are used to delay development of a disease or disorder.

[00107] An "individual" or a "subject" is a vertebrate. In certain embodiments, the vertebrate is a mammal. Mammals include, but are not limited to, farm animals (such as cows), sport animals, pets (such as cats, dogs, and horses), primates, mice and rats. In certain embodiments, the vertebrate is a human.

[00108] "Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. In certain embodiments, the mammal is human.

[00109] An "effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic or prophylactic result.

[00110] A "therapeutically effective amount" of a substance/molecule of the invention may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the substance/molecule, to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the substance/molecule are outweighed by the therapeutically beneficial effects. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically but not necessarily, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount would be less than the therapeutically effective amount.

[00111] The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include

radioactive isotopes (e.g., At211, I131, I125, Y90, Re186, Re188, Sm153, Bi212, P32, Pb212 and radioactive isotopes of Lu), chemotherapeutic agents (e.g., methotrexate, adriamicin, vinca alkaloids (vincristine, vinblastine, etoposide), doxorubicin, melphalan, mitomycin C, chlorambucil, daunorubicin or other intercalating agents, enzymes and fragments thereof such as nucleolyticenzymes, antibiotics, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof, and the various antitumor or anticancer agents disclosed below. Other cytotoxic agents are described below. A tumoricidal agent causes destruction of tumor cells.

[00112] A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and CYTOXAN® cyclosphosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethiylenethiophosphoramide and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); delta-9-tetrahydrocannabinol (dronabinol, MARINOL®); beta-lapachone; lapachol; colchicines; betulinic acid; a camptothecin (including the synthetic analogue topotecan (HYCAMTIN®), CPT-11 (irinotecan, CAMPTOSAR®), acetylcamptothecin, scopolectin, and 9-aminocamptothecin); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); podophyllotoxin; podophyllinic acid; teniposide; cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlomaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimnustine; antibiotics such as the enediyne antibiotics (e.g., calicheamicin, especially calicheamicin gamma1I and calicheamicin omegaI1 (see, e.g., Agnew, Chem. Intl. Ed. Engl., 33: 183-186 (1994)); dynemicin, including dynemicin A; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, caminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, ADRIAMYCIN® doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin,

olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; 2-ethylhydrazide; procarbazine; PSK® polysaccharide complex (JHS Natural Products, Eugene, Oreg.); razoxane; rhizoxin; sizofuran; spirogermanium; tenuazonic acid; triaziquone; 2,2′,2″-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine (ELDISINE®, FILDESIN®); dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); thiotepa; taxoids, e.g., TAXOL® paclitaxel (Bristol-Myers Squibb Oncology, Princeton, N.J.), ABRAXANE™ Cremophor-free, albumin-engineered nanoparticle formulation of paclitaxel (American Pharmaceutical Partners, Schaumberg, Ill.), and TAXOTERE® doxetaxel (Rhône-Poulenc Rorer, Antony, France); chloranbucil; gemcitabine (GEMZAR®); 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine (VELBAN®); platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine (ONCOVIN®); oxaliplatin; leucovovin; vinorelbine (NAVELBINE®); novantrone; edatrexate; daunomycin; aminopterin; ibandronate; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids such as retinoic acid; capecitabine (XELODA®); pharmaceutically acceptable salts, acids or derivatives of any of the above; as well as combinations of two or more of the above such as CHOP, an abbreviation for a combined therapy of cyclophosphamide, doxorubicin, vincristine, and prednisolone, and FOLFOX, an abbreviation for a treatment regimen with oxaliplatin (ELOXATIN™) combined with 5-FU and leucovovin.

[00113] As used herein, "treatment" refers to clinical intervention in an attempt to alter the natural course of the individual or cell being treated, and can be performed either for

prophylaxis or during the course of clinical pathology. Desirable effects of treatment include preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, preventing or decreasing inflammation and/or tissue/organ damage, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis. In some embodiments, antibodies of the invention are used to delay development of a disease or disorder.

[00114] An "individual" or a "subject" is a vertebrate. In certain embodiments, the vertebrate is a mammal. Mammals include, but are not limited to, farm animals (such as cows), sport animals, pets (such as cats, dogs, and horses), primates, mice and rats. In certain embodiments, the vertebrate is a human.

[00115] "Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. In certain embodiments, the mammal is human.

[00116] An "effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic or prophylactic result.

[00117] A "therapeutically effective amount" of a substance/molecule of the invention may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the substance/molecule, to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the substance/molecule are outweighed by the therapeutically beneficial effects. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically but not necessarily, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount would be less than the therapeutically effective amount.

[00118] The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g., At211, I131, I125, Y90, Re186, Re188, Sm153, Bi212, P32, Pb212 and radioactive isotopes of Lu), chemotherapeutic agents (e.g., methotrexate, adriamicin, vinca alkaloids (vincristine, vinblastine, etoposide), doxorubicin, melphalan, mitomycin C, chlorambucil, daunorubicin or other intercalating agents, enzymes and fragments thereof such as nucleolyticenzymes, antibiotics, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants

thereof, and the various antitumor or anticancer agents disclosed below. Other cytotoxic agents are described below. A tumoricidal agent causes destruction of tumor cells.

[00119] "Treating" or "treatment" or "alleviation" refers to both therapeutic treatment and prophylactic or preventative measures; wherein the object is to prevent or slow down (lessen) the targeted pathologic condition or disorder. Those in need of treatment include those already with the disorder as well as those prone to have the disorder or those in whom the disorder is to be prevented. A subject or mammal is successfully "treated" for an infection if, after receiving a therapeutic amount of an antibody according to the methods of the present invention, the patient shows observable and/or measurable reduction in or absence of one or more of the following: reduction in the number of infected cells or absence of the infected cells; reduction in the percent of total cells that are infected; and/or relief to some extent, one or more of the symptoms associated with the specific infection; reduced morbidity and mortality, and improvement in quality of life issues. The above parameters for assessing successful treatment and improvement in the disease are readily measurable by routine procedures familiar to a physician.

[00120] The term "therapeutically effective amount" refers to an amount of an antibody or a drug effective to "treat" a disease or disorder in a subject or mammal See preceding definition of "treating."

[00121] Administration "in combination with" one or more further therapeutic agents includes simultaneous (concurrent) and consecutive administration in any order.

[00122] "Carriers" as used herein include pharmaceutically acceptable carriers, excipients, or stabilizers that are nontoxic to the cell or mammal being exposed thereto at the dosages and concentrations employed. Often the physiologically acceptable carrier is an aqueous pH buffered solution. Examples of physiologically acceptable carriers include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptide; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as TWEEN™ polyethylene glycol (PEG), and PLURONICS™.

Glycoantibodies

[00123] The glycosylation of recombinant proteins produced from mammalian cells in culture is an important process in ensuring the effective use of therapeutic antibodies (Goochee et al., 1991; Jenkins and Curling, 1994). Mammalian cell culture delivers a heterogeneous mixture of glycosylation patterns which do not all have the same properties. Properties like safety, efficacy and the serum half-life of therapeutic proteins can be affected by these glycosylation patterns. We have successfully addressed the glycoform heterogeneity problem by the development of a novel class of monoclonal antibodies, named "glycoantibodies".

[00124] Glycoantibodies may be generated from monoclonal antibodies (preferably, therapeutic monoclonal antibodies) commercially available or in the development. Monoclonal antibodies for therapeutic use can be humanized, human or chimeric. Examples of approved monoclonal antibodies for therapeutic use include, but not limited to, Muromomab, Abciximab, Rituximab, Daclizumab, Basiliximab, Palivizumab, Infliximab,Trastuzumab, Etanercept, Gemtuzumab, Alemtuzumab, Ibritomomab, Adalimumab, Alefacept, Omalizumab, Efalizumab, Cetuximab, Bevacizumab, Natalizumab, Ranibizumab, Panitumumab, Eculizumab and Certolizumab.

[00125] Described herein are the functionally active glycoantibodies derived from therapeutic monoclonal antibodies by Fc glycoengineering. The glycoantibodies with optimized glycoforms exhibit more potent biological activities compared to the therapeutic monoclonal antibodies. It is contemplated that the glycoantibodies with optimized glycoforms may provide an alternative for therapeutic use.

Anti-CD20 Glycoantibodies (Anti-CD20 GAb)

[00126] The "CD20" antigen is a non-glycosylated, transmembrane phosphoprotein with a molecular weight of approximately 35 kD that is found on the surface of greater than 90% of B cells from peripheral blood or lymphoid organs. CD20 is expressed during early pre-B cell development and remains until plasma cell differentiation; it is not found on human stem cells, lymphoid progenitor cells or normal plasma cells. CD20 is present on both normal B cells as well as malignant B cells. Other names for CD20 in the literature include "B-lymphocyte-restricted differentiation antigen" and "Bp35". The CD20 antigen is described in, for example, Clark and Ledbetter, *Adv. Can Res.* 52:81-149 (1989) and Valentine et al. *J. Biol. Chem.* 264(19):11282-11287 (1989).

[00127] The present disclosure features a novel class of anti-CD20 antibodies, termed "anti-CD20 glycoantibodies" ("anti- CD20 GAb") . The anti-CD20 glycoantibodies can be generated from anti-CD20 monoclonal antibodies by Fc glycoengineering. The individual anti-CD20

glycoantibodies comprising the homogeneous population are identical and contain the same Fc glycan with a well-defined glycan structure and sequence. The anti-CD20 GAb according to the present invention specifically binds to the same epitope of a human CD20 antigen on a cell membrane as its patent antibody.

[00128] The term "parental antibody" as used herein refers to the anti-CD20 monoclonal antibody used to produce an anti-CD20 glycoantibody.

[00129] The parental antibodies can be obtained by cell culturing such as mammalian cell culture, Pichia pastoris or insect cell lines. Preferrably, the parental antibodies are produced in mammalian cell culture. The parental antibodies may be FDA approved or in development. Exemplary parental antibodies include, but not limited to, Rituximab, Ofatumumab, Tositumomab, Ocrelizumab, 11B8 or 7D8 (disclosed in W02004/035607), an anti-CD20 antibody disclosed in WO 2005/103081 such as C6, an anti-CD antibody disclosed in W02003/68821 such as IMMU-106 (from Immunomedics), an anti-CD20 antibody disclosed in W02004/103404 such as AME-133 (from Applied Molecular Evolution/Lilly), and anti-CD20 antibody disclosed in US 2003/0118592 such as TRU-015 (from Trubion Pharmaceuticals Inc), 90Y-labeled 2B8 murine antibody designated "Y2B8" (ZEVALIN®) (Biogen-Idec, Inc.) (e.g., U.S. Pat. No. 5,736,137, Anderson et al.; ATCC deposit HB11388); murine and chimeric 2H7 antibody (e.g., U.S. Pat. No. 5,677,180, Robinson et al.); humanized 2H7 antibodies such as rhuMAb2H7 and other versions (Genentech, Inc.) (e.g., WO 2004/056312, Adams et al., and other references noted below); human monoclonal antibodies against CD20 (GenMab A/S/Medarex, Inc.) (e.g., WO 2004/035607 and WO 2005/103081, Teeling et al.); a chimerized or humanized monoclonal antibody binding to an extracellular epitope of CD20 (Biomedics Inc.) (e.g., WO 2006/106959, Numazaki et al.); humanized LL2 and similar antibodies (Immunomedics, Inc.) (e.g., U.S. Pat. No. 7,151,164 and US 2005/0106108, Hansen); A20 antibodies (Immunomedics, Inc.) such as chimeric A20 (cA20) or humanized A20 antibody (hA20, IMMUN-106T, veltuzumab) (e.g., US 2003/0219433, Hansen et al.); fully human antibodies against CD20 (Amgen/AstraZeneca) (e.g., WO 2006/130458, Gazit et al.); antibodies against CD20 (Avestha Gengraine Technologies Pvt Ltd.) (e.g., WO 2006/126069, Morawala); and chimeric or humanized B-Ly1 antibodies to CD20 (Roche/GlycArt Biotechnology AG) such as GA101 (e.g., WO 2005/044859; US 2005/0123546; US 2004/0072290; and US 2003/0175884, Umana et al.).

[00130] In some embodiments, the exemplary anti-CD20 GAb described herein comprise a heavy chain having the amino acid sequence set forth in SEQ ID NO: 1, and a light chain having

the amino acid sequence set forth in SEQ ID NO: 2. In a preferred embodiment, the anti-CD20 GAb comprises a light chain sequence and a heavy chain sequence of Rituximab.

[00131] Table 1 below shows the heavy chain and the light chain sequences of Rituximab.

TABLE 1

Rituximab

Accession Number: DB00073

Source: http://www.drugbank.ca/drugs/DB00073

>Rituximab heavy chain
QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLEWIGAIYPGNGDTSY
NQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYCARSTYYGGDWYFNVWGAGTTVTVS
AASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQS
SGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLG
GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY
NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRD
ELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSR
WQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
(SEQ ID:2)

>Rituximab light chain
QIVLSQSPAILSASPGEKVTMTCRASSSVSYIHWFQQKPGSSPKPWIYATSNLASGVPVR
FSGSGSGTSYSLTISRVEAEDAATYYCQQWTSNPPTFGGGTKLEIKRTVAAPSVFIFPPS
DEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTL
SKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
(SEQ ID:1)

[00132] In some embodiments, the N-glycan is attached to the Asn-297 of the Fc region.

[00133] The N-glycans according to the invention have a common pentasaccharide core of $Man_3GlcNAc_2$ which is also referred to as "trimannose core" or "pentasaccharide core", wherein "Man" refers to mannose, "Glc" refers to glucose, "NAc" refers to N-acetyl, and GlcNAc refers to N-acetylglucosamine.

[00134] In some embodiments, the N-glycan has a biantennary structure.

[00135] The N-glycan described herein may have intrachain substitutions comprising "bisecting" GlcNAc. When a glycan comprises a bisecting GlcNAc on the trimannose core, the structure is represented as $Man_3GlcNAc_3$. When a glycan comprises a core fucose attached to the trimannose core, the structure is represented as $Man_3GlcNAc_2(F)$. The N-glycan may comprise one or more termial sialic acids (e.g. N-acetylneuraminic acid). The structure

represented as "Sia" refers to a termial sialic acid. Sialylation may occur on either the α1–3 or α1–6 arm of the biantennary structures.

[00136] In some embodiments, the N-glycan described herein comprises at least one α2–6 terminal sialic acid. In certain embodiments, the N-glycan comprises one α2–6 terminal sialic acid. In a preferred embodiment, the N-glycan comprises two α2–6 terminal sialic acids.

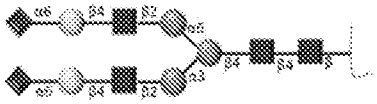
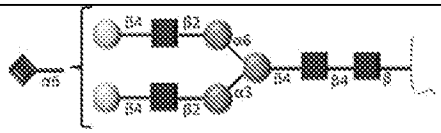
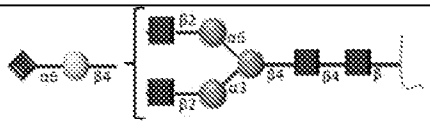
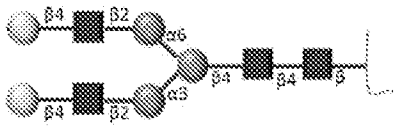
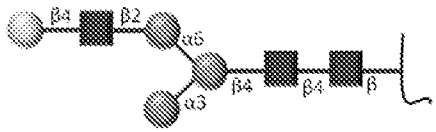
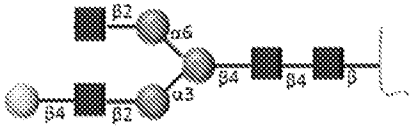
[00137] In some embodiments, the N-glycan described herein comprises at least one α2–3 terminal sialic acid. In certain embodiments, the N-glycan comprises one α2–3 terminal sialic acid. In a preferred embodiment, the N-glycan comprises two α2–3 terminal sialic acids.

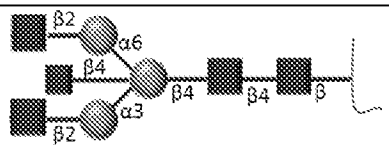
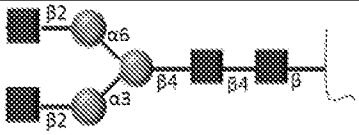
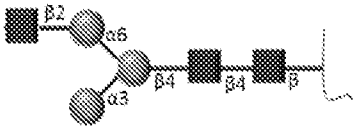
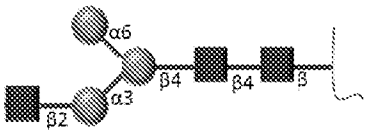
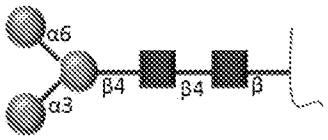
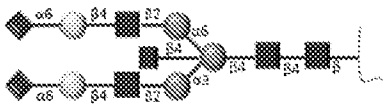
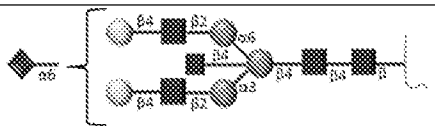
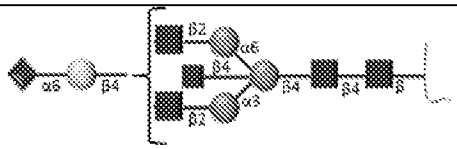
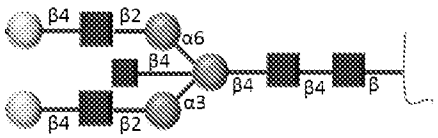
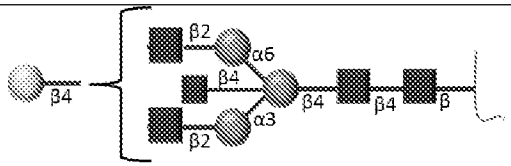
[00138] In some embodiments, the N-glycan described herein comprises at least one galactose. In certain embodiments, the N-glycan comprises one galactose. In a preferred embodiment, the N-glycan comprises two galactoses.

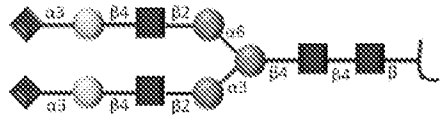
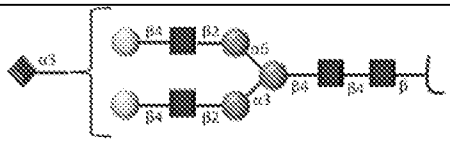
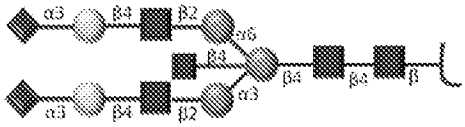
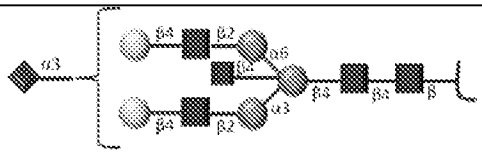
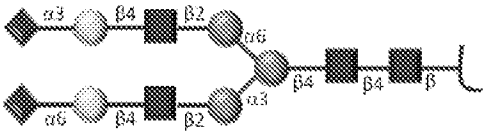
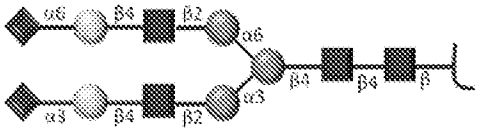
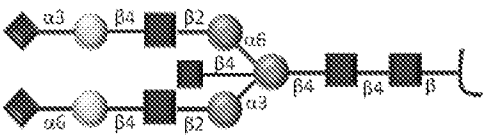
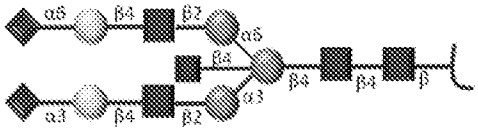
[00139] Preferrably, the N-glycan according to the disclosure is free of core fucose.

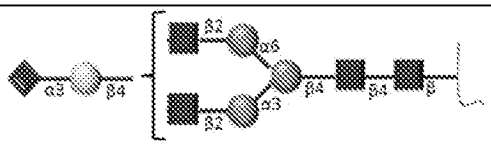
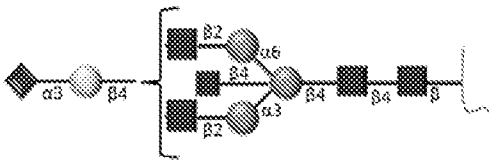
[00140] Table 2 lists exemplary N-glycans in anti-CD20 glycoantibodies. Embodiments of the present disclosure may include or exclude any of the N-glycans listed herein.

Table 2.

GAb	Glycan structure	Glycan sequence
101		$Sia_2(\alpha2\text{-}6)Gal_2GlcNAc_2Man_3GlcNAc_2$
102		$Sia(\alpha2\text{-}6)Gal_2GlcNAc_2Man_3GlcNAc_2$
103		$Sia(\alpha2\text{-}6)GalGlcNAc_2Man_3GlcNAc_2$
104		$Gal_2GlcNAc_2Man_3GlcNAc_2$
105		$GalGlcNAcMan_3GlcNAc_2$
106		$GalGlcNAc_2Man_3GlcNAc_2$

107		$GlcNAc_3Man_3GlcNAc_2$
108		$GlcNAc_2Man_3GlcNAc_2$
109		$GlcNAcMan_3GlcNAc_2$
110		$GlcNAcMan_3GlcNAc_2$
111		$Man_3GlcNAc_2$
112		$Sia_2(\alpha 2\text{-}6)Gal_2GlcNAc_3Man_3GlcNAc_2$
113		$Sia(\alpha 2\text{-}6)Gal_2GlcNAc_3Man_3GlcNAc_2$
114		$Sia(\alpha 2\text{-}6)GalGlcNAc_3Man_3GlcNAc_2$
115		$Gal_2GlcNAc_3Man_3GlcNAc_2$
116		$GalGlcNAc_3Man_3GlcNAc_2$

117		$Sia_2(\alpha 2\text{-}3)Gal_2GlcNAc_2Man_3GlcNAc_2$
118		$Sia(\alpha 2\text{-}3)Gal_2GlcNAc_2Man_3GlcNAc_2$
119		$Sia_2(\alpha 2\text{-}3)Gal_2GlcNAc_3Man_3GlcNAc_2$
120		$Sia(\alpha 2\text{-}3)Gal_2GlcNAc_3Man_3GlcNAc_2$
121		$Sia_2(\alpha 2\text{-}3/\alpha 2\text{-}6)Gal_2GlcNAc_2Man_3GlcNAc_2$
122		$Sia_2(\alpha 2\text{-}6/\alpha 2\text{-}3)Gal_2GlcNAc_2Man_3GlcNAc_2$
123		$Sia_2(\alpha 2\text{-}3/\alpha 2\text{-}6)Gal_2GlcNAc_3Man_3GlcNAc_2$
124		$Sia_2(\alpha 2\text{-}6/\alpha 2\text{-}3)Gal_2GlcNAc_3Man_3GlcNAc_2$

125		Sia(α2-3)GalGlcNAc$_2$Man$_3$GlcNAc$_2$
126		Sia(α2-3)GalGlcNAc$_3$Man$_3$GlcNAc$_2$

Biological Characteristic of Anti-CD20 Glycoantibodies

[00141] Glycosylation on Fc can affect a variety of immunoglobulin effector-mediated functions, including ADCC, CDC and circulating half-life. ADCC enhancement is a key strategy for improving therapeutic antibody drug efficacy. It has the potential of lowering effective drug dosage for benefits of lower drug cost. The anti-CD20 glycoantibodies described herein can be characterized by functional properties. The anti-CD20 GAb has cell growth inhibitory activities including apoptosis against human CD20 expressing cells. In some embodiments, the anti-CD20 GAb exhibits more potent cell growth inhibitory activities as compared to its patent antibody.

ADCC Activities of anti-CD20 glycoantibodies

[00142] The increased ADCC activity of the glycoantibody according to the invention is at least about 5 fold, including but not limited to, at least about 6 fold, about 7 fold, about 8 fold, about 9 fold about 10 fold, about 15 fold, about 20 fold, about 25 fold, about 30 fold, about 35 fold, about 40 fold, about 50 fold, about 60 fold, and about 80 fold or at least about a value in the range between any of the two numbers listed herein compared to the ADCC activity of the parental antibody.

[00143] Table 3 lists exemplary enhanced ADCC activities of anti-CD20 GAbs as compared to Rituximab. Exemplary assays are described in the examples.

Table 3.

Anti-CD20	Rituximab	GAb101	GAb104	GAb105	GAb107	GAb108	GAb111
ADCC (fold)	1	>50	>50	30~50	>50	10~30	5~10

[00144] A number of anti-CD20 GAbs described herein, in particular GAb101, and GAb104, exhibit enhanced ADCC activity compared to it parental antibody, Rituximab. It is contemplated that the glycoantibodies of the invention may exhibit superior effect as therapeutic agents for B

cell-mediated malignant tumors and immunological diseases in which B cells or antibodies produced by B cells are involved, and an object of the present invention is to use the anti-CD20 GAb in development of therapeutic agents.

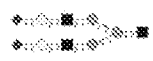
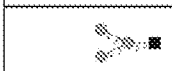
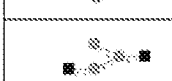
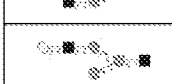
CDC Activities of anti-CD20 glycoantibodies

[00145] The glycoantibody described herein is surprisingly able to provide improved ADCC without affecting CDC. Exemplary CDC assays are described in the examples. In exemplary embodiments, ADCC of the glycoantibody is increased but other immunoglobulin-type effector functions such as complement-dependent cytoxicity (CDC) remain similar or are not significantly affected.

Binding between FcγRIII and anti-CD20 glycoantibodies

[00146] Table 4 lists exemplary FcγRIIIA binding of anti-CD20 GAbs and Rituximab. FcγRIIIA binding may be measured using assays known in the art. Exemplary assays are described in the examples. The Fc receptor binding may be determined as the relative ratio of anti-CD20 GAb vs Rituximab. Fc receptor binding in exemplary embodiments is increased by at least 1.2-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 15-fold or 20-fold, 30-fold, 40-fold, 50-fold, 100-fold or higher.

Table 4.

Binding Constant of FcRIIIA to variable glycoantibodies by SPR					
	Curve	KD (nM)	Rmax (RU)	Fold	Note
	Rituxan	100~300	49.29		
	GAb101	1~25	90.48	A	Category A: increase >30X Category B: increase 15~30X Category C: increase 5~10X
	GAb104	1~25	93.4	A	
	GAb111	40~130	56.28	C	
	GAb108	40~130	67.01	C	
	GAb107	7~30	76.02	B	
	GAb109	40~130	51.03	C	
	GAb110	40~130	38.43	C	
	GAb105	1~25	72.12	A	
	GAb106	7~30	70.8	B	
	GAb102	1~25	67.52	A	

[00147] As compared to Rituximab, the binding data showed that the anti-CD20 GAbs, in particular GAb101 and GAb104, exhibit stronger binding affinity for the target molecule CD20.

[00148] Taken together, anti-CD20 Gabs, exhibit enhanced ADCC activity and stronger FcγRIIIA binding affinity as compared to Rituximab. It is contemplated that the glycoantibodies of the invention may provide a superior clinical response either alone or, in a composition comprising two or more such antibodies, and optionally in combination with other treatments such as chemotherapy. It is contemplated that the ADCC-enhanced anti-CD20 glycoantibody may provide an alternative therapeutic for B-cell lymphoma and other diseases. The glycoantibodies of the present invention advantageously can be used to alter current routes of administration and current therapeutic regimens, as their increased effector function means they can be dosed at lower concentrations and with less frequency, thereby reducing the potential for antibody toxicity and/or development of antibody tolerance. Furthermore, the improved effector function yields new approaches to treating clinical indications that have previously been resistant or refractory to treatment with the corresponding anti-CD20 monoclonal antibody produced in recombinant host systems.

Preparation of anti-CD20 GAb

[00149] The anti-CD20 glycoantibodies of the invention can be produced by Fc glycoengineering from anti-CD20 monoclonal antibodies ("parental antibodies") commercially available or in the preclinical or clinical development. Preferrably, the monoclonal antibodies are therapeutic monoclonal antibodies. Fc glycoengineering may be performed enzymatically or chemoenzymatically. In a preferred embodiment, the parental antibody is Rituximab.

[00150] The N-glycans in the glycoantibodies of the invention are preferrably defucosylated.

[00151] Defucosylation of N-glycans is a process to remove core fucoses in N-glycans of the Fc domains. Defucosylation can be employed enzymatically. Since N-glycans are embedded between two Fc domains, the enzymatic defucosylation efficiency is much lower due to steric hindrance, i.e., access of fucosidase to fucose residues is blocked by potions of the Fc domains.

[00152] Many α-fucosidases are known in the art. Examples include α-fucosidases from Turbo cornutus, Charonia lampas, Bacillus fulminans, Aspergillus niger, Clostridium perfringens, Bovine kidney (Glyko), chicken liver (Tyagarajan et al., 1996, Glycobiology 6:83-93) and α-fucosidase II from Xanthomonas manihotis (Glyko, PROzyme). Many varieties of fucosidase are also commercially available (Glyko, Novato, Calif.; PROzyme, San Leandro, Calif.; Calbiochem-Novabiochem Corp., San Diego, Calif.; among others). However, none of α-fucosidases are known to efficiently remove the core fucose from N-linked glycans.

[00153] WO 2013/12066 disclosed the defucosylation of (Fucαl ,6)GlcNAc-Rituximab by an α-fucosidase from bovine kidney. As described in WO 2013/12066, a reaction mixture of (Fuc αl, 6)GlcNAc-Rituximab was incubated with α-fucosidase from bovine kidney (commercially available from Prozyme) at 37°C for 20 days to completely remove the fucose in (Fucαl ,6)GlcNAc-Rituximab.

[00154] Thermal instability of immunoglobulin has been reported (Vermeer et al., Biophys J. Jan 78: 394–404 (2000)). The Fab fragment is most sensitive to heat treatment, whereas the Fc fragment is most sensitive to decreasing pH. To examine the thermal stability and functional activity of the antibody, we performed the same experiment as described in WO 2013/12066, and found the antibody lost about 10% binding affinity to CD20 after thermal treatment at 37°C for 3 days. Furthermore, we found the antibody lost about 20% binding affinity to CD20 after thermal treatment at 37°C for 7 days. It is contemplated that the antibody will significantly lose the binding affinity to CD20 after prolonged thermal treatment, such as at 37°C for 20 days, as described in WO 2013/12066.

[00155] In our attempts to synthesize the glycoantibodies with improved therapeutic values, we unexpectedly discovered a *Bacteroides fragilis* α-fucosidase (GenBank accession no. YP_212855.1) that is capable of efficiently removing fucose residues from N-linked glycans. Efficient defucosylation has been successfully achieved using the specific enzyme. Importantly, the efficiency of making the glycoantibodies of the invention has been valuably improved by the use of the specific α-fucosidase that yields a facile defucosylation of N-glycans, as illustrated in Figure 1.

[00156] Accordingly, the present invention provides a compostion of the α-fucosidase, and an improved method for removing core fucoses of N-glycans using the α-fucosidase. The α-fucosidase comprises a polypeptide having an amino acid sequence having at least 80%, 85% 90%, 95%, 98% or 99% identity to the sequences of SEQ ID NO: 5 or variants thereof. The improved method of defucosylation comprises contacting an antibody with an α-fucosidase, and in which the α-fucosidase comprises a polypeptide having an amino acid sequence having at least 80%, 85%, 90%, 95%, 98% or 99% identity to the sequences of SEQ ID NO: 5, a variant or a fragment thereof.

[00157] Described herein includes an improved method for making an anti-CD20 glycoantibody, the method comprising the steps of (a) contacting an anti-CD20 monoclonal antibody with an α-fucosidase and at least one endoglycosidase, thereby yielding a

defucosylated antibody having a single N-acetylglucosamine (GlcNAc), and (b) adding a carbohydrate moiety to GlcNAc under suitable conditions.

[00158] In some embodiments, the anti-CD20 monoclonal antibody according to the method of the invention is Rituximab.

[00159] Endoglycosidase is used to trim off the variable portions of an oligosaccharide in N-glycan. Examples of endoglycosidases used herein include, but not limited to, EndoA, EndoF, EndoF1, EndoF2, EndoF3, EndoH, EndoM, EndoS, EndoS2 and variants thereof.

[00160] The α-fucosidase according to the method of the invention comprises a polypeptide having an amino acid sequence having at least 85% identity to the sequences of SEQ ID NO: 5, a functional variant thereof.

[00161] In some embodiments, the α-fucosidase comprises a polypeptide having an amino acid sequence having at least 90% or 95% identity to the sequences of SEQ ID NO: 5, a variant or a fragment thereof.

[00162] In certain embodiments, the α-fucosidase is a recombinant *Bacteroides* α-fucosidase.

TABLE 5

QQKYQPTEANLKARSEFQDNKFGIFLHWGLYAMLATGEWT
MTNNNLNYKEYAKLAGGFYPSKFDADKWVAAIKASGAKYICFTTRHHEGFSMFDTKYSDY
NIVKATPFKRDVVKELADACAKHGIKLHFYYSHIDWYREDAPQGRTGRRTGRPNPKGDWK
SYYQFMNNQLTELLTNYGPIGAIWFDGWWDQDINPDFDWELPEQYALIHRLQPACLVGNN
HHQTPFAGEDIQIFERDLPGENTAGLSGQSVSHLPLETCETMNGMWGYKITDQNYKSTKT
LIHYLVKAAGKDANLLMNIGPQPDGELPEVAVQRLKEVGEWMSKYGETIYGTRGGLVAPH
DWGVTTQKGNKLYVHILNLQDKALFLPIVDKKVKKAVVFADKTPVRFTKNKEGIVLELAK
VPTDVDYVVELTID
(SEQ ID: 5)

[00163] Step (a) in the method of the invention leads to a defucosylated antibody having a single N-acetylglucosamine (GlcNAc). Subsequent enzyme-mediated glycosylation using a transglycosylase is performed to add a designated carbohydrate moiety to GlcNAc and extend the sugar chain. A homogenous population of glycoantibodies can therefore be produced. Examples of transglycosylases as described herein include, but not limited to, EndoA, EndoF, EndoF1, EndoF2, Endo F3, EndoH, EndoM, EndoS, Endo S2 and variants thereof.

[00164] In some embodiments, the carbohydrate moiety according to the method the invention is slected from the group consisting of $Sia_2(\alpha 2\text{-}6)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia_2(\alpha 2\text{-}6)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia_2(\alpha 2\text{-}3)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia_2(\alpha 2\text{-}$

3)$Gal_2GlcNAc_3Man_3GlcNAc_2$, Sia_2(α2-3/α2-6)$Gal_2GlcNAc_2Man_3GlcNAc_2$, Sia_2(α2-6/α2-3)$Gal_2GlcNAc_2Man_3GlcNAc_2$, Sia_2(α2-3/α2-6)$Gal_2GlcNAc_3Man_3GlcNAc_2$, Sia_2(α2-6/α2-3)$Gal_2GlcNAc_3Man_3GlcNAc_2$, Sia(α2-6)$Gal_2GlcNAc_2Man_3GlcNAc_2$, Sia(α2-3)$Gal_2GlcNAc_2Man_3GlcNAc_2$, Sia(α2-6)$Gal_2GlcNAc_3Man_3GlcNAc_2$, Sia(α2-3)$Gal_2GlcNAc_3Man_3GlcNAc_2$, Sia(α2-6)$GalGlcNAc_2Man_3GlcNAc_2$, Sia(α2-3)$GalGlcNAc_2Man_3GlcNAc_2$, Sia(α2-6)$GalGlcNAc_3Man_3GlcNAc_2$, Sia(α2-3)$GalGlcNAc_3Man_3GlcNAc_2$, $Gal_2GlcNAc_2Man_3GlcNAc_2$, $GalGlcNAcMan_3GlcNAc_2$, $Gal_2GlcNAc_3Man_3GlcNAc_2$, $GalGlcNAc_2Man_3GlcNAc_2$, $GalGlcNAc_3Man_3GlcNAc_2$, $GlcNAc_3Man_3GlcNAc_2$, $GlcNAc_2Man_3GlcNAc_2$, $GlcNAcMan_3GlcNAc_2$ and $Man_3GlcNAc_2$.

[00165] In preferred embodiments, the carbohydrate moiety is selected from the group consisting of Sia_2(α2-6)$Gal_2GlcNAc_2Man_3GlcNAc_2$, Sia_2(α2-6)$Gal_2GlcNAc_3Man_3GlcNAc_2$, Sia_2(α2-3)$Gal_2GlcNAc_2Man_3GlcNAc_2$, Sia_2(α2-3)$Gal_2GlcNAc_3Man_3GlcNAc_2$, Sia_2(α2-3/α2-6)$Gal_2GlcNAc_2Man_3GlcNAc_2$, Sia_2(α2-6/α2-3)$Gal_2GlcNAc_2Man_3GlcNAc_2$, Sia_2(α2-3/α2-6)$Gal_2GlcNAc_3Man_3GlcNAc_2$, Sia_2(α2-6/α2-3)$Gal_2GlcNAc_3Man_3GlcNAc_2$, Sia(α2-6)$Gal_2GlcNAc_2Man_3GlcNAc_2$, Sia(α2-3)$Gal_2GlcNAc_2Man_3GlcNAc_2$, Sia(α2-6)$Gal_2GlcNAc_3Man_3GlcNAc_2$, Sia(α2-3)$Gal_2GlcNAc_3Man_3GlcNAc_2$, Sia(α2-6)$GalGlcNAc_2Man_3GlcNAc_2$, Sia(α2-3)$GalGlcNAc_2Man_3GlcNAc_2$, Sia(α2-6)$GalGlcNAc_3Man_3GlcNAc_2$, Sia(α2-3)$GalGlcNAc_3Man_3GlcNAc_2$, $Gal_2GlcNAc_2Man_3GlcNAc_2$, $GalGlcNAcMan_3GlcNAc_2$ and $Gal_2GlcNAc_3Man_3GlcNAc_2$.

[00166] Step (b) in the method of the invention leads to sugar chain extension. One method for sugar chain extension is through an enzyme-catalyzed glycosylation reaction. It is well known in the art that glycosylation using a sugar oxazoline as the sugar donor among the enzyme-catalyzed glycosylation reactions is useful for synthesizing oligosaccharides because the glycosylation reaction is an addition reaction and advances without any accompanying elimination of acid, water, or the like. (Fujita, et al., *Biochim. Biophys. Acta* 2001, 1528, 9–14)

[00167] In some embodiments, the carbohydrate moiety is a sugar oxazoline.

[00168] Suitable conditions also include incubation of the reaction mixture for at least 20 minutes, 30 minutes, 40 minutes, 50 minutes, 60 minutes, 70 minutes, 80 minutes, 90 minutes or 100 minutes, preferably less than 60 minutes. Incubation preferably takes place at room temperature, more preferably at approximately 20°C, 25 °C, 30°C, 35 °C, 40°C or 45 °C, and most preferably at approximately 37°C.

[00169] It will be understood that the polypeptide of the α-fucosidase of the invention may be derivatized or modified to assist with their isolation or purification. Thus, in one embodiment of

the invention, the polypeptide for use in the invention is derivatized or modified by addition of a ligand which is capable of binding directly and specifically to a separation means. Alternatively, the polypeptide is derivatized or modified by addition of one member of a binding pair and the separation means comprises a reagent that is derivatized or modified by addition of the other member of a binding pair. Any suitable binding pair can be used. In a preferred embodiment where the polypeptide for use in the invention is derivatized or modified by addition of one member of a binding pair, the polypeptide is preferably histidine-tagged or biotin-tagged. Typically the amino acid coding sequence of the histidine or biotin tag is included at the gene level and the proteins are expressed recombinantly in *E. coli*. The histidine or biotin tag is typically present at one end of the polypeptide, either at the N-terminus or at the C-terminus. The histidine tag typically consists of six histidine residues, although it can be longer than this, typically up to 7, 8, 9, 10 or 20 amino acids or shorter, for example 5, 4, 3, 2 or 1 amino acids. Furthermore, the histidine tag may contain one or more amino acid substitutions, preferably conservative substitutions as defined above.

[00170] Variant polypeptide as described herein are those for which the amino acid sequence varies from that in SEQ ID NO: 5, but exhibit the same or similar function of the enzyme comprising the polypeptide having an amino acid sequence of SEQ ID NO: 5.

[00171] As used herein percent (%) sequence identity with respect to a sequence is defined as the percentage of amino acid residues in a candidate polypeptide sequence that are identical with the amino acid residues in the reference polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining percent sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

[00172] Some preferred embodiments of the invention are demonstrated in the examples.

[00173] Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an “import” variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-327 (1988); Verhoeyen et al., Science, 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding

sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567), 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 FR residues are substituted by residues from analogous sites in rodent antibodies.

[00174] The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody (Sims et al., J. Immunol., 151:2296 (1993); Chothia et al., J. Mol. Biol., 196:901 (1987)). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., Proc. Natl. Acad Sci. USA, 89:4285 (1992); Prestaetal., J. Immnol., 151:2623 (1993)).

[00175] It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i. e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

[00176] Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (JH) gene in chimeric and

germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., Proc. Natl. Acad. Sci. USA, 90:2551 (1993); Jakobovits et al., Nature, 362:255-258 (1993); Bruggermann et al., Year in Immuno., 7:33 (1993). Human antibodies can also be derived from phage-display libraries (Hoogenboom et al., J. Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol., 222:581-597 (1991)).

Pharmaceutical Formulations

[00177] Therapeutic formulations comprising an antibody of the invention are prepared for storage by mixing the antibody having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980)), in the form of aqueous solutions, lyophilized or other dried formulations. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, histidine and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g., Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

[00178] The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, including, but not limited to those with complementary activities that do not adversely affect each other. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

[00179] The active ingredients may also be entrapped in microcapsule prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsule and poly-(methylmethacylate) microcapsule, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres,

microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980).

[00180] The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

[00181] Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the immunoglobulin of the invention, which matrices are in the form of shaped articles, e.g., films, or microcapsule. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(−)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated immunoglobulins remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37° C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S—S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

[00182] The amount of antibody in the pre-lyophilized formulation is determined taking into account the desired dose volumes, mode(s) of administration etc. Where the protein of choice is an intact antibody (a full-length antibody), from about 2 mg/mL to about 50 mg/mL, preferably from about 5 mg/mL to about 40 mg/mL and most preferably from about 20-30 mg/mL is an exemplary starting protein concentration. The protein is generally present in solution. For example, the protein may be present in a pH-buffered solution at a pH from about 4-8, and preferably from about 5-7. Exemplary buffers include histidine, phosphate, Tris, citrate, succinate and other organic acids. The buffer concentration can be from about 1 mM to about 20 mM, or from about 3 mM to about 15 mM, depending, for example, on the buffer and the desired isotonicity of the formulation (e.g. of the reconstituted formulation). The preferred

buffer is histidine in that, as demonstrated below, this can have lyoprotective properties. Succinate was shown to be another useful buffer.

[00183] The lyoprotectant is added to the pre-lyophilized formulation. In preferred embodiments, the lyoprotectant is a non-reducing sugar such as sucrose or trehalose. The amount of lyoprotectant in the pre-lyophilized formulation is generally such that, upon reconstitution, the resulting formulation will be isotonic. However, hypertonic reconstituted formulations may also be suitable. In addition, the amount of lyoprotectant must not be too low such that an unacceptable amount of degradation/aggregation of the protein occurs upon lyophilization. Where the lyoprotectant is a sugar (such as sucrose or trehalose) and the protein is an antibody, exemplary lyoprotectant concentrations in the pre-lyophilized formulation are from about 10 mM to about 400 mM, and preferably from about 30 mM to about 300 mM, and most preferably from about 50 mM to about 100 mM.

[00184] The ratio of protein to lyoprotectant is selected for each protein and lyoprotectant combination. In the case of an antibody as the protein of choice and a sugar (e.g., sucrose or trehalose) as the lyoprotectant for generating an isotonic reconstituted formulation with a high protein concentration, the molar ratio of lyoprotectant to antibody may be from about 100 to about 1500 moles lyoprotectant to 1 mole antibody, and preferably from about 200 to about 1000 moles of lyoprotectant to 1 mole antibody, for example from about 200 to about 600 moles of lyoprotectant to 1 mole antibody.

[00185] In preferred embodiments of the invention, it has been found to be desirable to add a surfactant to the pre-lyophilized formulation. Alternatively, or in addition, the surfactant may be added to the lyophilized formulation and/or the reconstituted formulation. Exemplary surfactants include nonionic surfactants such as polysorbates (e.g. polysorbates 20 or 80); poloxamers (e.g. poloxamer 188); Triton; sodium dodecyl sulfate (SDS); sodium laurel sulfate; sodium octyl glycoside; lauryl-, myristyl-, linoleyl-, or stearyl-sulfobetaine; lauryl-, myristyl-, linoleyl- or stearyl-sarcosine; linoleyl-, myristyl-, or cetyl-betaine; lauroamidopropyl-, cocamidopropyl-, linoleamidopropyl-, myristamidopropyl-, palnidopropyl-, or isostearamidopropyl-betaine (e.g lauroamidopropyl); myristamidopropyl-, palmidopropyl-, or isostearamidopropyl-dimethylamine; sodium methyl cocoyl-, or disodium methyl oleyl-taurate; and the MONAQUATTM series (Mona Industries, Inc., Paterson, N.J.), polyethyl glycol, polypropyl glycol, and copolymers of ethylene and propylene glycol (e.g. Pluronics, PF68 etc). The amount of surfactant added is such that it reduces aggregation of the reconstituted protein and minimizes the formation of particulates after reconstitution. For example, the surfactant may be present in

the pre-lyophilized formulation in an amount from about 0.001-0.5%, and preferably from about 0.005-0.05%.

[00186] In certain embodiments of the invention, a mixture of the lyoprotectant (such as sucrose or trehalose) and a bulking agent (e.g. mannitol or glycine) is used in the preparation of the pre-lyophilization formulation. The bulking agent may allow for the production of a uniform lyophilized cake without excessive pockets therein etc.

[00187] Other pharmaceutically acceptable carriers, excipients or stabilizers such as those described in Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980) may be included in the pre-lyophilized formulation (and/or the lyophilized formulation and/or the reconstituted formulation) provided that they do not adversely affect the desired characteristics of the formulation. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed and include; additional buffering agents; preservatives; co-solvents; antioxidants including ascorbic acid and methionine; chelating agents such as EDTA; metal complexes (e.g. Zn-protein complexes); biodegradable polymers such as polyesters; and/or salt-forming counterions such as sodium.

[00188] The pharmaceutical compositions and formulations described herein are preferably stable. A "stable" formulation/composition is one in which the antibody therein essentially retains its physical and chemical stability and integrity upon storage. Various analytical techniques for measuring protein stability are available in the art and are reviewed in Peptide and Protein Drug Delivery, 247-301, Vincent Lee Ed., Marcel Dekker, Inc., New York, N.Y., Pubs. (1991) and Jones, A. Adv. Drug Delivery Rev. 10: 29-90 (1993). Stability can be measured at a selected temperature for a selected time period.

[00189] The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to, or following, lyophilization and reconstitution. Alternatively, sterility of the entire mixture may be accomplished by autoclaving the ingredients, except for protein, at about 120° C. for about 30 minutes, for example.

[00190] After the protein, lyoprotectant and other optional components are mixed together, the formulation is lyophilized. Many different freeze-dryers are available for this purpose such as Hull50® (Hull, USA) or GT20® (Leybold-Heraeus, Germany) freeze-dryers. Freeze-drying is accomplished by freezing the formulation and subsequently subliming ice from the frozen content at a temperature suitable for primary drying. Under this condition, the product temperature is below the eutectic point or the collapse temperature of the formulation. Typically, the shelf temperature for the primary drying will range from about -30 to 25° C. (provided the

product remains frozen during primary drying) at a suitable pressure, ranging typically from about 50 to 250 mTorr. The formulation, size and type of the container holding the sample (e.g., glass vial) and the volume of liquid will mainly dictate the time required for drying, which can range from a few hours to several days (e.g. 40-60hrs). A secondary drying stage may be carried out at about 0-40° C., depending primarily on the type and size of container and the type of protein employed. However, it was found herein that a secondary drying step may not be necessary. For example, the shelf temperature throughout the entire water removal phase of lyophilization may be from about 15-30° C. (e.g., about 20° C.). The time and pressure required for secondary drying will be that which produces a suitable lyophilized cake, dependent, e.g., on the temperature and other parameters. The secondary drying time is dictated by the desired residual moisture level in the product and typically takes at least about 5 hours (e.g. 10-15 hours). The pressure may be the same as that employed during the primary drying step. Freeze-drying conditions can be varied depending on the formulation and vial size.

[00191] In some instances, it may be desirable to lyophilize the protein formulation in the container in which reconstitution of the protein is to be carried out in order to avoid a transfer step. The container in this instance may, for example, be a 3, 5, 10, 20, 50 or 100 cc vial. As a general proposition, lyophilization will result in a lyophilized formulation in which the moisture content thereof is less than about 5%, and preferably less than about 3%.

[00192] At the desired stage, typically when it is time to administer the protein to the patient, the lyophilized formulation may be reconstituted with a diluent such that the protein concentration in the reconstituted formulation is at least 50 mg/mL, for example from about 50 mg/mL to about 400 mg/mL, more preferably from about 80 mg/mL to about 300 mg/mL, and most preferably from about 90 mg/mL to about 150 mg/mL. Such high protein concentrations in the reconstituted formulation are considered to be particularly useful where subcutaneous delivery of the reconstituted formulation is intended. However, for other routes of administration, such as intravenous administration, lower concentrations of the protein in the reconstituted formulation may be desired (for example from about 5-50 mg/mL, or from about 10-40 mg/mL protein in the reconstituted formulation). In certain embodiments, the protein concentration in the reconstituted formulation is significantly higher than that in the pre-lyophilized formulation. For example, the protein concentration in the reconstituted formulation may be about 2-40 times, preferably 3-10 times and most preferably 3-6 times (e.g. at least three fold or at least four fold) that of the pre-lyophilized formulation.

[00193] Reconstitution generally takes place at a temperature of about 25° C. to ensure complete hydration, although other temperatures may be employed as desired. The time required

for reconstitution will depend, e.g., on the type of diluent, amount of excipient(s) and protein. Exemplary diluents include sterile water, bacteriostatic water for injection (BWFI), a pH buffered solution (e.g. phosphate-buffered saline), sterile saline solution, Ringer's solution or dextrose solution. The diluent optionally contains a preservative. Exemplary preservatives have been described above, with aromatic alcohols such as benzyl or phenol alcohol being the preferred preservatives. The amount of preservative employed is determined by assessing different preservative concentrations for compatibility with the protein and preservative efficacy testing. For example, if the preservative is an aromatic alcohol (such as benzyl alcohol), it can be present in an amount from about 0.1-2.0% and preferably from about 0.5-1.5%, but most preferably about 1.0-1.2%. Preferably, the reconstituted formulation has less than 6000 particles per vial which are >10 μm m size.

Therapeutic Applications

[00194] The glycoantibody described herein may be used for treating a patient having a cancer. The method of the treatment comprises administering to the patient an effective amount of a glycoantibody or a pharmaceutical composition described herein. Examples of the cancers include, but are not limited to, B cell lymphomas, NHL, precursor B cell lymphoblastic leukemia/lymphoma and mature B cell neoplasms, B cell chronic lymphocytic leukemia (CLL)/small lymphocytic lymphoma (SLL), B cell prolymphocytic leukemia, lymphoplasmacytic lymphoma, mantle cell lymphoma (MCL), follicular lymphoma (FL), low-grade, intermediate-grade and high-grade (FL), cutaneous follicle center lymphoma, marginal zone B cell lymphoma, MALT type marginal zone B cell lymphoma, nodal marginal zone B cell lymphoma, splenic type 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 (ALCL).

[00195] In certain embodiments, the cancer is B-cell lymphoma such as non-Hodgkin's lymphoma.

[00196] Further, the glycoantibody described herein may be used for treating a patient having an autoimmune or inflammatory disease. The method of the treatment comprises administering to the patient an effective amount of a glycoantibody or a pharmaceutical composition described herein. Examples of the autoimmune or inflammatory disease include, but are not limited to, rheumatoid arthritis, juvenile rheumatoid arthritis, systemic lupus erythematosus (SLE), Wegener's disease, inflammatory bowel disease, idiopathic thrombocytopenic purpura (ITP), thrombotic thrombocytopenic purpura (TTP), autoimmune thrombocytopenia, multiple sclerosis,

psoriasis, IgA nephropathy, IgM polyneuropathies, myasthenia gravis, vasculitis, diabetes mellitus, Reynaud's syndrome, Crohn's diasease, ulcerative colitis, gastritis, Hashimoto's thyroiditis, ankylosing spondylitis, hepatitis C-associated cryoglobulinemic vasculitis, chronic focal encephalitis, bullous pemphigoid, hemophilia A, membranoproliferative glomerulnephritis, adult and juvenile dermatomyositis, adult polymyositis, chronic urticaria, primary biliary cirrhosis, neuromyelitis optica, Graves' dysthyroid disease, bullous pemphigoid, membranoproliferative glonerulonephritis, Churg-Strauss syndrome, asthma, psoriatic arthritis, dermatitis, respiratory distress syndrome, meningitis, encephalitits, uveitis, eczema, atherosclerosis, leukocyte adhesion deficiency, juvenile onset diabetes, Reiter's disease, Behcet's disease, hemolytic anemia, atopic dermatitis, Wegener's granulomatosis, Omenn's syndrome, chronic renal failure, acute infectious mononucleosis, HIV and herpes-associated disease, systemic sclerosis, Sjorgen's syndrome and glomerulonephritis, dermatomyositis, ANCA, aplastic anemia, autoimmune hemolytic anemia (AIHA), factor VIII deficiency, hemophilia A, autoimmune neutropenia, Castleman's syndrome, Goodpasture's syndrome, solid organ transplant rejection, graft versus host disease (GVHD), autoimmune hepatitis, lymphoid interstitial pneumonitis (HIV), bronchiolitis obliterans (non-transplant), Guillain-Barre Syndrome, large vessel vasculitis, giant cell (Takayasu's) arteritis, medium vessel vasculitis, Kawasaki's Disease, and polyarteritis nodosa.

[00197] In certain embodiments, the autoimmune or inflammatory disease is rheumatoid arthritis.

[00198] In these treatment methods, the anti-CD20 glycoantibody can be administered alone or in conjunction with a second therapeutic agent such as a second antibody, or a chemotherapeutic agent or an immunosuppressive agent. The second antibody can be one that binds CD20 or a different B cell antigen, or a NK or T cell antigen.

[00199] An antibody of the invention (and adjunct therapeutic agent) can be administered by any suitable means, including parenteral, subcutaneous, intraperitoneal, intrapulmonary, and intranasal, and, if desired for local treatment, intralesional administration. Parenteral infusions include intramuscular, intravenous, intraarterial, intraperitoneal, or subcutaneous administration. In addition, the antibody is suitably administered by pulse infusion, particularly with declining doses of the antibody. Dosing can be by any suitable route, e.g. by injections, such as intravenous or subcutaneous injections, depending in part on whether the administration is brief or chronic.

[00200] The location of the binding target of an antibody of the invention may be taken into consideration in preparation and administration of the antibody. When the binding target is an

intracellular molecule, certain embodiments of the invention provide for the antibody or antigen-binding fragment thereof to be introduced into the cell where the binding target is located.

[00201] In another embodiment, internalizing antibodies are provided. Antibodies can possess certain characteristics that enhance delivery of antibodies into cells, or can be modified to possess such characteristics. Techniques for achieving this are known in the art. For example, cationization of an antibody is known to facilitate its uptake into cells (see, e.g., U.S. Pat. No. 6,703,019). Lipofections or liposomes can also be used to deliver the antibody into cells. Where antibody fragments are used, the smallest inhibitory fragment that specifically binds to the binding domain of the target protein is generally advantageous. For example, based upon the variable-region sequences of an antibody, peptide molecules can be designed that retain the ability to bind the target protein sequence. Such peptides can be synthesized chemically and/or produced by recombinant DNA technology. See, e.g., Marasco et al., Proc. Natl. Acad. Sci. USA, 90: 7889-7893 (1993).

[00202] Entry of modulator polypeptides into target cells can be enhanced by methods known in the art. For example, certain sequences, such as those derived from HIV Tat or the Antennapedia homeodomain protein are able to direct efficient uptake of heterologous proteins across cell membranes. See, e.g., Chen et al., Proc. Natl. Acad. Sci. USA (1999), 96:4325-4329.

[00203] When the binding target is located in the brain, certain embodiments of the invention provide for the antibody or antigen-binding fragment thereof to traverse the blood-brain barrier. Certain neurodegenerative diseases are associated with an increase in permeability of the blood-brain barrier, such that the antibody or antigen-binding fragment can be readily introduced to the brain. When the blood-brain barrier remains intact, several art-known approaches exist for transporting molecules across it, including, but not limited to, physical methods, lipid-based methods, and receptor and channel-based methods.

[00204] Physical methods of transporting the antibody or antigen-binding fragment across the blood-brain barrier include, but are not limited to, circumventing the blood-brain barrier entirely, or by creating openings in the blood-brain barrier. Circumvention methods include, but are not limited to, direct injection into the brain (see, e.g., Papanastassiou et al., Gene Therapy 9: 398-406 (2002)), interstitial infusion/convection-enhanced delivery (see, e.g., Bobo et al., Proc. Natl. Acad. Sci. USA 91: 2076-2080 (1994)), and implanting a delivery device in the brain (see, e.g., Gill et al., Nature Med. 9: 589-595 (2003); and Gliadel Wafers™, Guildford Pharmaceutical). Methods of creating openings in the barrier include, but are not limited to, ultrasound (see, e.g., U.S. Patent Publication No. 2002/0038086), osmotic pressure (e.g., by administration of

hypertonic mannitol (Neuwelt, E. A., Implication of the Blood-Brain Barrier and its Manipulation, Vols 1 & 2, Plenum Press, N.Y. (1989))), permeabilization by, e.g., bradykinin or permeabilizer A-7 (see, e.g., U.S. Pat. Nos. 5,112,596, 5,268,164, 5,506,206, and 5,686,416), and transfection of neurons that straddle the blood-brain barrier with vectors containing genes encoding the antibody or antigen-binding fragment (see, e.g., U.S. Patent Publication No. 2003/0083299).

[00205] Lipid-based methods of transporting the antibody or antigen-binding fragment across the blood-brain barrier include, but are not limited to, encapsulating the antibody or antigen-binding fragment in liposomes that are coupled to antibody binding fragments that bind to receptors on the vascular endothelium of the blood-brain barrier (see, e.g., U.S. Patent Application Publication No. 20020025313), and coating the antibody or antigen-binding fragment in low-density lipoprotein particles (see, e.g., U.S. Patent Application Publication No. 20040204354) or apolipoprotein E (see, e.g., U.S. Patent Application Publication No. 20040131692).

[00206] Receptor and channel-based methods of transporting the antibody or antigen-binding fragment across the blood-brain barrier include, but are not limited to, using glucocorticoid blockers to increase permeability of the blood-brain barrier (see, e.g., U.S. Patent Application Publication Nos. 2002/0065259, 2003/0162695, and 2005/0124533); activating potassium channels (see, e.g., U.S. Patent Application Publication No. 2005/0089473), inhibiting ABC drug transporters (see, e.g., U.S. Patent Application Publication No. 2003/0073713); coating antibodies with a transferrin and modulating activity of the one or more transferrin receptors (see, e.g., U.S. Patent Application Publication No. 2003/0129186), and cationizing the antibodies (see, e.g., U.S. Pat. No. 5,004,697).

[00207] The antibody composition of the invention would be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners. The antibody need not be, but is optionally formulated with one or more agents currently used to prevent or treat the disorder in question. The effective amount of such other agents depends on the amount of antibodies of the invention present in the formulation, the type of disorder or treatment, and other factors discussed above. These are generally used in the same dosages and with administration routes as described herein, or about

from 1 to 99% of the dosages described herein, or in any dosage and by any route that is empirically/clinically determined to be appropriate.

[00208] For the prevention or treatment of disease, the appropriate dosage of an antibody of the invention (when used alone or in combination with other agents such as chemotherapeutic agents) will depend on the type of disease to be treated, the type of antibody, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments. Depending on the type and severity of the disease, about 1 μg/kg to 15 mg/kg (e.g. 0.1 mg/kg-10 mg/kg) of antibody can be an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. One typical daily dosage might range from about 1 μg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment would generally be sustained until a desired suppression of disease symptoms occurs. One exemplary dosage of the antibody would be in the range from about 0.05 mg/kg to about 10 mg/kg. Thus, one or more doses of about 0.5 mg/kg, 2.0 mg/kg, 4.0 mg/kg or 10 mg/kg (or any combination thereof) may be administered to the patient. Such doses may be administered intermittently, e.g. every week or every three weeks (e.g. such that the patient receives from about two to about twenty, or e.g. about six doses of the antibody). An initial higher loading dose, followed by one or more lower doses may be administered. An exemplary dosing regimen comprises administering an initial loading dose of about 4 mg/kg, followed by a weekly maintenance dose of about 2 mg/kg of the antibody. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

Articles of Manufacture

[00209] In another aspect of the invention, an article of manufacture containing materials useful for the treatment, prevention and/or diagnosis of the disorders described above is provided. The article of manufacture comprises a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is by itself or when combined with another composition effective for treating, preventing and/or diagnosing the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper

pierceable by a hypodermic injection needle). At least one active agent in the composition is an antibody of the invention. The label or package insert indicates that the composition is used for treating the condition of choice. Moreover, the article of manufacture may comprise (a) a first container with a composition contained therein, wherein the composition comprises an antibody of the invention; and (b) a second container with a composition contained therein, wherein the composition comprises a further cytotoxic or otherwise therapeutic agent. The article of manufacture in this embodiment of the invention may further comprise a package insert indicating that the compositions can be used to treat a particular condition. Alternatively, or additionally, the article of manufacture may further comprise a second (or third) container comprising a pharmaceutically-acceptable buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

Anti-CD20 GAb Formulations and Stability

[00210] There is a need for formulating a shear and temperature stable pharmaceutical formulation comprising an antibody which is suitable for therapeutic use.

[00211] In one embodiment, the invention relates to an anti-CD20 antibody formulation comprising a therapeutically effective amount of an anti-CD20 antibody, wherein the formulation further comprises 10 to 100 mM sodium acetate, 25 to 100 mM sodium chloride, 0.5 to 5% arginine free base, 0.02 to 0.2 mM EDTA, 0.01 to 0.2% polysorbate 80 and adjusted to pH 5.0 to 7.0.

[00212] In another embodiment, the invention relates to an anti-CD20 antibody formulation comprising an anti-CD20 antibody in the concentration range of 20-300 mg/mL, wherein the formulation further comprises 50 mM sodium acetate, 51 mM sodium chloride, 1% arginine free base, 0.05 mM EDTA, 0.02% polysorbate 80, and adjusted to pH 5.5.

[00213] In one embodiment, the invention relates to an anti-CD20 glycoantibody formulation comprising a therapeutically effective amount of glycoantibody, wherein the formulation further comprises 10 to 100 mM sodium acetate, 25 to 100 mM sodium chloride, 0.5 to 5% arginine free base, 0.02 to 0.2 mM EDTA, 0.01 to 0.2% polysorbate 80 and adjusted to pH 5.0 to 7.0.

[00214] In one embodiment, the invention relates to a glycoantibody formulation comprising a glycoantibody in the concentration range of 20-300 mg/mL, wherein the formulation further comprises 50 mM sodium acetate, 51 mM sodium chloride, 1% arginine free base, 0.05 mM EDTA, 0.02% polysorbate 80, and adjusted to pH 5.5.

[00215] In yet another embodiment, the invention relates to an anti-CD20 antibody formulation wherein the formulation is stable for at least 2 years. In another embodiment, the invention relates to an anti-CD20 antibody formulation wherein the formulation is stable at temperatures up to at least 55° C. In another embodiment, the invention relates to an anti-CD20 antibody formulation wherein the formulation is stable at a temperature of about 5° C. for at least 2 years. In another embodiment, the invention relates to an anti-CD20 antibody formulation wherein the formulation is stable at a temperature of about 25° C for at least 3 months. In another embodiment, the invention relates to an anti-CD20 antibody formulation wherein the formulation is stable at a temperature of about 40°C . for at least 1 month. In another embodiment, the invention relates to an anti-CD20 antibody formulation wherein the formulation is stable at a temperature of about 55° C for at least 1 day.

[00216] In another embodiment, the invention relates to an anti-CD20 antibody formulation wherein the antibody is present in an amount of about 20-300 mg/mL, 50-300 mg/mL, 100-300 mg/mL, 150-300 mg/mL, 200-300 mg/mL, or 250-300 mg/mL.

[00217] In another embodiment, the invention relates to an anti-CD20 antibody formulation wherein polysorbate 80 is present in an amount of about 0.02%, 0.015%, or 0.025%. In other embodiments, the polysorbate 80 may be present in an amount of 0.01-0.2%, 0.01-0.15%, 0.02-0.2%, 0.02-0.15%, 0.01-0.25%, or 0.01-0.05%.

[00218] The details of one or more embodiments of the invention are set forth in the description below. Other features or advantages of the present invention will be apparent from the following drawings and detailed description of several embodiments, and also from the appending claims.

[00219] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLES

EXAMPLE 1: General procedure for analysis of N-glycosylation of anti-CD20 antibodies

[00220] We developed a mass spectrometric method to monitor the yield of oligosaccharide–derived fragment ions (oxonium ions) over a collision induced dissociation (CID) energy applied to a glycopeptides precursor. Multiple Reaction Monitoring (MRM) of oxonium ions

method could fulfill the regulatory requirement on the routine quality control analysis of forthcoming biosimilar therapeutics.

[00221] 5 ug of Rituximab (purchased from Genentech) was dissolved in 25 ul of 2M Guanidine-HCl, and dithiothreitol (DTT) were added to a final concentration of 5 mM. After 10 minutes incubation in 110 °C, reduced cysteine residues were alkylated in 10 mM Iodoacetamide (IAA) at 37 °C for 1 hour. Add 5 mM DTT to quench excess IAA at RT for 10 minutes. The product was diluted 15 times in 50 mM ammonium bicarbonate before microcentrifugation with spin column (10kDa protein MW cut-off). The trypsin digestion was performed for 4 hours at 37 °C using an enzyme: protein ratio of 1: 25 (w/w). Sample was frozen at -20 °C for LC-MS/MS analysis.

Instrumentation

[00222] The glycopeptide quantification by m/z 204 oxonium ion (HexNAc) monitoring was performed using a 4000 QTrap triple quadrupole mass spectrometer (AB Sciex) with Aglient 1200 HPLC system. For relative quantification of glycopeptide microheterogeneity, precursor ion m/z was derived in-silico, covering all possible glycan compositions, and a single quantitative transition was monitored for each precursor ion (Q3 m/z= 204).

MS data analysis

[00223] The acquired raw data was processed with Analyst 1.5 (AB Sciex). The mass chromatogram of each transition was integrated and quantified by peak area. The percentage composition of each component was calculated with respect to the sum of all components combined.

[00224] Analysis of N-glycan compositions revealed there are more than 50 glycoforms present in Rituximab. Results showed the biantennary complex type glycans are the major N-glcans. Greater than 90% of N-glycans are fucosylated.

EXAMPLE 2: Generation of anti-CD20 GAbs

Anti-CD20 GAb301

[00225] The complete removal of N-linked glycan at Asn^{297} from Fc region of Rituximab (Rituxan) is achieved by means of PNGase F, and evaluated with 4-12% Bis-Tris NeuPAGE and LC-MS/MS analysis of tryptic glycopeptides from modified and unmodified IgG. The molecular weights of tryptic glycopeptides helps to determine the potential site of N-linked glycosylation at each asparagine and to elucidate the species of predominant glycans.

Anti-CD20 GAb200

[00226] Commercial or home-made heterogeneous mAb was used as the starting material and modified with selected glycosidases. Application of Endoglycosidase (Endo F2, Endo F3, or

Endo H) can yield a homogenous di-sugar mAb of GlcNAc-Fuc at its Fc N-linked glycosylation site (GAb200). Subsequently a homogeneous mono-sugar mAb can be obtained with application of fucosidase; or the mono-sugar species can also be obtained with combination of Endo F3 and fucosidase in one step as shown with Rituximab. The product was trypsinized, and the glycopeptides, TKPREEQYNSTYR (SEQ ID No) and EEQYNSTYR (SEQ ID No), were analyzed using nanospray LC/MS to confirm the glycosylation pattern of GAb200.

Anti-CD20 GAb201

[00227] Rituximab (2.5 mg) in a sodium phosphate buffer (50 mM, pH 7.0, 1.25 mL) was incubated with Endo S (125 μg) and BfFucH (2.5 mg) at 37 °C for 22 h. LC-MS and SDS-PAGE analyses indicated the complete cleavage of the N-glycans on the heavy chain. The reaction mixture was subject to affinity chromatography on a column of protein A-agarose resin (1 mL) that was pre-equilibrated with a sodium phosphate buffer (20 mM, pH 7.0). The column was washed with a sodium phosphate buffer (20 mM, pH 7.0, 10 mL). The bound IgG was released with glycine-HCl (50 mM, pH 3.0, 10 mL), and the elution fractions were immediately neutralized with Tris-Cl buffer (1.0 M, pH 8.3). The fractions containing the Fc fragments were combined and concentrated by centrifugal filtration (Amicon Ultra centrifugal filter, Millipore, Billerica, MA) to give anti-CD20 GAb201 (1.93 mg). The product was trypsinized, and the glycopeptides, TKPREEQYNSTYR and EEQYNSTYR, were analyzed using nanospray LC/MS to confirm the glycosylation pattern of GAb201.

Anti-CD20 GAb101

[00228] Isolation of the sialylglycopeptide (SGP) from hen's egg yolk was according to the published method. Briefly, the phenol extraction of hen's egg yolk was centrifuged, filtrated, and purified by the chromatographic columns, including Sephadex G-50, Sephadex G-25, DEAE-Toyoperarl 650M, CM-Sephadex C-25 and Sephadex G-25. A solution of sialylglycopeptide (SGP) (52 mg) in a sodium phosphate buffer (50 mM, pH 6.0, 5mM) was incubated with the Endo M (53 μg) at 37 °C. After 7 hour, the reaction mixture was subjected to gel filtration chromatography on a Sephadex G-25 column eluted by water. The fractions containing the product were combined and lyophilized to give the product (glycan-101) as a white powder (30 mg, yield 82%).

[00229] A solution of glycan-101 ($Sia_2Gal_2GlcNAc_2Man_3GlcNAc$) (30 mg), 2-chloro-1,3-dimethylimidazolinium chloride (DMC) (62.7 mg) and Et_3N (89 μL) in water (593 μL) was stirred at 4°C for 1 h. The reaction mixture was subjected to gel filtration chromatography on a Sephadex G-25 column eluted by 0.05% aqueous Et_3N. The fractions containing the product

(glycan oxazoline-101) were combined and lyophilized to give a white powder (26 mg, yield 87.4%).

[00230] Glycan oxazoline-101 was added to a mixture of endoglycosidase and GAb201 in 50 mM Tris buffer (pH 7.8) and incubated for an hour at room temperature. The reaction mixture was purified with protein A affinity column, followed by amanion exchange column capto Q to collect the desired product, anti-CD20 GAb101. The product was trypsinized, and the glycopeptides, TKPREEQYNSTYR and EEQYNSTYR, were analyzed using nanospray LC/MS to confirm the glycosylation pattern of GAb101.

Anti-CD20 GAb102

[00231] Glycan-102 ($SiaGal_2GlcNAc_2Man_3GlcNAc$) was prepared in a similar manner as the process for making glycan-101 described above. A solution of glycan-102 ($SiaGal_2GlcNAc_2Man_3GlcNAc$) (30 mg), 2-chloro-1,3-dimethylimidazolinium chloride (DMC) (62.7 mg) and Et_3N (89 μL) in water was stirred at 4°C for 1 h. The reaction mixture was subjected to gel filtration chromatography on a Sephadex G-25 column and eluted by 0.05% aqueous Et_3N. The fractions containing the product (glycan oxazoline-102) were combined and lyophilized to give a white powder.

[00232] Glycan oxazoline-102 was added to a mixture of endoglycosidase and GAb201 in 50 mM Tris buffer (pH 7.8) and incubated for an hour at room temperature. The reaction mixture was purified with protein A affinity column, followed by amanion exchange column capto Q to collect the desired product, anti-CD20 GAb102. The product was trypsinized, and the glycopeptides, TKPREEQYNSTYR and EEQYNSTYR, were analyzed using nanospray LC/MS to confirm the glycosylation pattern of GAb102.

Anti-CD20 GAb103

[00233] A solution of glycan-103 ($SiaGalGlcNAc_2Man_3GlcNAc$) (30 mg), 2-chloro-1,3-dimethylimidazolinium chloride (DMC) (62.7 mg) and Et_3N (89 μL) in water (593 μL) was stirred at 4°C for 1 h. The reaction mixture was subjected to gel filtration chromatography on a Sephadex G-25 column and eluted by 0.05% aqueous Et_3N. The fractions containing the product (glycan oxazoline-103) were combined and lyophilized to give a white powder.

[00234] Glycan oxazoline-103 was added to a mixture of endoglycosidase and GAb 201 in 50 mM Tris buffer (pH 7.8) and incubated for an hour at room temperature. The reaction mixture was purified with protein A affinity column, followed by amanion exchange column capto Q to collect the desired product, anti-CD20 GAb103. The product was trypsinized, and the glycopeptides, TKPREEQYNSTYR and EEQYNSTYR, were analyzed using nanospray LC/MS to confirm the glycosylation pattern of GAb103.

Anti-CD20 GAb104

[00235] A solution of glycan-104 ($Gal_2GlcNAc_2Man_3GlcNAc$) (30 mg), 2-chloro-1,3-dimethylimidazolinium chloride (DMC) (62.7 mg) and Et_3N (89 μL) in water was stirred at 4°C for 1 h. The reaction mixture was subjected to gel filtration chromatography on a Sephadex G-25 column and eluted by 0.05% aqueous Et_3N. The fractions containing the product (glycan oxazoline-104) were combined and lyophilized to give a white powder.

[00236] Glycan oxazoline-104 was added to a mixture of endoglycosidase and GAb 201 in 50 mM Tris buffer (pH 7.8) and incubated for an hour at room temperature. The reaction mixture was purified with protein A affinity column, followed by amanion exchange column capto Q to collect the desired product, anti-CD20 GAb104. The product was trypsinized, and the glycopeptides, TKPREEQYNSTYR and EEQYNSTYR, were analyzed using nanospray LC/MS to confirm the glycosylation pattern of GAb104.

Anti-CD20 GAb 105

[00237] A solution of coupling glycan-105 ($GalGlcNAc_2Man_3GlcNAc$) (30 mg), 2-chloro-1,3-dimethylimidazolinium chloride (DMC) (62.7 mg) and Et_3N (89 μL) in water was stirred at 4°C for 1 h. The reaction mixture was subjected to gel filtration chromatography on a Sephadex G-25 column and eluted by 0.05% aqueous Et_3N. The fractions containing the product (glycan oxazoline-105) were combined and lyophilized to give a white powder.

[00238] Glycan oxazoline-105 was added to a mixture of endoglycosidase and GAb 201 in 50 mM Tris buffer (pH 7.8) and incubated for an hour at room temperature. The reaction mixture was purified with protein A affinity column, followed by amanion exchange column capto Q to collect the desired product, GAb105. The product was trypsinized, and the glycopeptides, TKPREEQYNSTYR and EEQYNSTYR, were analyzed using nanospray LC/MS to confirm the glycosylation pattern of GAb105.

Anti-CD20 GAb 106

[00239] A solution of coupling glycan-106 ($GalGlcNAcMan_3GlcNAc$) (30 mg), 2-chloro-1,3-dimethylimidazolinium chloride (DMC) (62.7 mg) and Et_3N (89 μL) in water (593 μL) was stirred at 4°C for 1 h. The reaction mixture was subjected to gel filtration chromatography on a Sephadex G-25 column and eluted by 0.05% aqueous Et_3N. The fractions containing the product (glycan oxazoline-106) were combined and lyophilized to give a white powder.

[00240] Glycan oxazoline-106 was added to a mixture of endoglycosidase and GAb 201 in 50 mM Tris buffer (pH 7.8) and incubated for an hour at room temperature. The reaction mixture was purified with protein A affinity column, followed by amanion exchange column capto Q to collect the desired product, anti-CD20 GAb106. The product was trypsinized, and the

glycopeptides, TKPREEQYNSTYR and EEQYNSTYR, were analyzed using nanospray LC/MS to confirm the glycosylation pattern of GAb106.

Anti-CD20 GAb107

[00241] A solution of coupling glycan-107 ($GlcNAc_3Man_3GlcNAc$) (30 mg), 2-chloro-1,3-dimethylimidazolinium chloride (DMC) (62.7 mg) and Et_3N (89 μL) in water was stirred at 4°C for 1 h. The reaction mixture was subjected to gel filtration chromatography on a Sephadex G-25 column and eluted by 0.05% aqueous Et_3N. The fractions containing the product (glycan oxazoline-107) were combined and lyophilized to give a white powder.

[00242] Glycan oxazoline-107 was added to a mixture of endoglycosidase and GAb 201 in 50 mM Tris buffer (pH 7.8) and incubated for an hour at room temperature. The reaction mixture was purified with protein A affinity column, followed by amanion exchange column capto Q to collect the desired product, anti-CD20 GAb107. The product was trypsinized, and the glycopeptides, TKPREEQYNSTYR and EEQYNSTYR, were analyzed using nanospray LC/MS to confirm the glycosylation pattern of GAb107.

Anti-CD20 GAb 108

[00243] A solution of coupling glycan-108 ($GlcNAc_2Man_3GlcNAc$) (30 mg), 2-chloro-1,3-dimethylimidazolinium chloride (DMC) (62.7 mg) and Et_3N (89 μL) in water was stirred at 4°C for 1 h. The reaction mixture was subjected to gel filtration chromatography on a Sephadex G-25 column and eluted by 0.05% aqueous Et_3N. The fractions containing the product (glycan oxazoline-108) were combined and lyophilized to give a white powder.

[00244] Glycan oxazoline-108 was added to a mixture of endoglycosidase and GAb 201 in 50 mM Tris buffer (pH 7.8) and incubated for an hour at room temperature. The reaction mixture was purified with protein A affinity column, followed by amanion exchange column capto Q to collect the desired product, anti-CD20 GAb108. The product was trypsinized, and the glycopeptides, TKPREEQYNSTYR and EEQYNSTYR, were analyzed using nanospray LC/MS to confirm the glycosylation pattern of GAb108.

Anti-CD20 GAb109

[00245] A solution of coupling glycan-109 ($GlcNAcMan_3GlcNAc$) (30 mg), 2-chloro-1,3-dimethylimidazolinium chloride (DMC) (62.7 mg) and Et_3N (89 μL) in water was stirred at 4°C for 1 h. The reaction mixture was subjected to gel filtration chromatography on a Sephadex G-25 column and eluted by 0.05% aqueous Et_3N. The fractions containing the product (glycan oxazoline-109) were combined and lyophilized to give a white powder.

[00246] Glycan oxazoline-109 was added to a mixture of endoglycosidase and GAb 201 in 50 mM Tris buffer (pH 7.8) and incubated for an hour at room temperature. The reaction mixture

was purified with protein A affinity column, followed by amanion exchange column capto Q to collect the desired product, anti-CD20 GAb109. The product was trypsinized, and the glycopeptides, TKPREEQYNSTYR and EEQYNSTYR, were analyzed using nanospray LC/MS to confirm the glycosylation pattern of GAb109.

Anti-CD20 GAb110

[00247] A solution of coupling glycan-110 ($GlcNAcMan_3GlcNAc$) (30 mg), 2-chloro-1,3-dimethylimidazolinium chloride (DMC) (62.7 mg) and Et_3N (89 μL) in water was stirred at 4°C for 1 h. The reaction mixture was subjected to gel filtration chromatography on a Sephadex G-25 column and eluted by 0.05% aqueous Et_3N. The fractions containing the product (glycan oxazoline-110) were combined and lyophilized to give a white powder.

[00248] Glycan oxazoline-110 was added to a mixture of endoglycosidase and GAb 201 in 50 mM Tris buffer (pH 7.8) and incubated for an hour at room temperature. The reaction mixture was purified with protein A affinity column, followed by amanion exchange column capto Q to collect the desired product, anti-CD20 GAb110. The product was trypsinized, and the glycopeptides, TKPREEQYNSTYR and EEQYNSTYR, were analyzed using nanospray LC/MS to confirm the glycosylation pattern of GAb110.

Anti-CD20 GAb111

[00249] A solution of coupling glycan-110 ($Man_3GlcNAc$) (30 mg), 2-chloro-1,3-dimethylimidazolinium chloride (DMC) (62.7 mg) and Et_3N (89 μL) in water was stirred at 4°C for 1 h. The reaction mixture was subjected to gel filtration chromatography on a Sephadex G-25 column and eluted by 0.05% aqueous Et_3N. The fractions containing the product (glycan oxazoline-111) were combined and lyophilized to give a white powder.

[00250] Glycan oxazoline-111 was added to a mixture of endoglycosidase and GAb 201 in 50 mM Tris buffer (pH 7.8) and incubated for an hour at room temperature. The reaction mixture was purified with protein A affinity column, followed by amanion exchange column capto Q to collect the desired product, anti-CD20 GAb111. The product was trypsinized, and the glycopeptides, TKPREEQYNSTYR and EEQYNSTYR, were analyzed using nanospray LC/MS to confirm the glycosylation pattern of GAb111.

EXAMPLE 3: Characterization of GAb301

[00251] Anti-CD20 GAb301 was tested for its antigenic binding and induced functions using B-lymphoma Ramos cells. The sugar-free Rituximab variant retained a full strength in both CD20 binding activity and induction of apoptosis, and reserved a 35% of CDC effect as compared to the Rituximab's maximum values; however, GAb301 lost almost completely the

ADCC effect. These results indicate the presence of carbohydrates is essential to the induction of ADCC; the CDC activity is much impaired.

EXAMPLE 4: Characterization of GAb200 and GAb201

[00252] The antigenic binding and the induced apoptosis, CDC and ADCC effects were evaluated using Ramos cells. Neither antigenic binding nor apoptosis effect were affected by enzyme digestion with endoglycosidase and/or fucosidase, as GAb200 and GAb201 exhibited both biological activities approximately the same as Rituximab. However, the induced CDC effect was much impaired with mono-sugar variant, GAb201, while the di-sugar variant, GAb200, was able to retain about 50% of the Rituximab's maximum activity. As to the ADCC using PBMC as effector cells, the mono- and di-sugar variants retained about 60% and 80% of the Rituximab's maximum effect, respectively. Compared to mono-sugar variant (GAb201), the di-sugar variant (GAb200) performs better in both CDC and ADCC.

EXAMPLE 5: Characterization of anti-CD20 GAbs

Purified anti-CD20 GAbs 101, 102, 104, 105, 106, 107, 108, 109, 110, 111 and 201 were confirmed by SDS-PAGE (Figure 2), and the molecular weight of the IgG molecule of anti-CD20 GAb101 was confirmed by MS. The N-glycan profiling was performed by trypsin digestion, and analyzed by nanospray LC/MS based on the cleavaged glycopeptide TKPREEQYNSTYR. Results of N-glycan profiling for anti-CD20 GAbs 101,102, 104, 105, 106, 107, 108, 109, 110 and 111 are shown in Figure 3.

EXAMPLE 6: Binding to B-lymphoma cells

[00253] The binding activities of exemplary of GAbs, GAb101, GAb117 and GAb104 to Ramos cells, SKW6.4, Raji and SU-DHL4 cells were examined, and the results showed that they have similar binding activities as Rituximab (Fig. 4).

EXAMPLE 7: Apoptosis to B-lymphoma cells

[00254] The apoptotic effects induced by GAb101 and GAb104 to Ramos cells (Fig. 5, left panel) & SKW6.4 cells (Fig.5, right panel) were also examined and the results showed that they have similar apoptotic effects as Rituximab.

EXAMPLE 8: CDC to B-lymphoma cells

[00255] The CDC effects of GAb101, GAb104, GAb108 and GAb117 were tested using Ramos cells. Results in Fig. 6A showed that GAb101 induced similar CDC effect as Rituximab, while GAb 117 displayed a slightly lower activity. This indicates that 2,6 sialic acid linkage glycoantibody is more potent than 2,3 sialic acid linkage glycoantibody in the induction of CDC.

[00256] The role of galactose residue on CDC induction was also examined with our GAbs. Consistent with previous report that terminal galactose can positively influence CDC activity,

GAb101 and GAb104 showed better EC_{50} than GAb108 which is a G0 variant. Of interest to note, the comparable CDC induced by GAb101 and GAb104 suggests that the presence of terminal symmetric sialic acids did not affect the "galactose"-dependent complement-dependent cytotoxicity.

EXAMPLE 9: Ex vivo ADCC activity

The most dramatic effect upon de-fucosylation and homogenous reglycosylation occurred in the induction of ADCC. The ADCC activities of GAbs to Ramos and SKW6.4 cells were evaluated using effector PBMC cells from three different donors (FcRIII alloptype not determined). As shown in Fig. 7 (A~C), the induced ADCC effects by GAb101, GAb117 and GAb104 were more potent than Rituximab estimated by both EC_{50} and % Maximal killing. In general, GAb101, GAb117 and GAb104 required 10~100 fold lower concentrations to achieve the maximum killing effect of Rituximab. It is noteworthy that, in all cases GAb101, GAb117 and GAb104 can induce significant ADCC effect (10-20% cell lysis above background) at ultra-low concentrations (below 1.0 ng/mL), while little effect was observed for Rituximab.

[00257] Similar results were observed in SKW6.4 cells. Despite that the maximum ADCC was not as high as in Ramos after anti-CD20 treatment (Fig.7B and 7C), the enhancement by GAbs relative to Rituximab was more obvious and estimated to be 100~1000 folds. Taken together, the ADCC effects in Ramos and SKW6.4 cells induced by GAb101, GAb117 and GAb104 are approximately 10~1000 folds higher compared to Rituximab.

EXAMPLE 10: Depletion of human B cells

[00258] The depletion of human B cells was conducted using human PBMC cells freshly prepared from human blood. The cells at $2x10^6$ in RPMI 1640-5%FBS cultured on microplates were incubated, in the absence or presence of 15% autologous plasma, at 37 °C for 4 hr with the anti-CD20 GAbs 101, 102, 105, 106, 107, 108, 109, 110, 111, 201 and Rituximab at different concentrations. The cells after wash were stained with anti-CD2-PE and anti-CD19-FITC on ice for 5 min. B cells depletion was analyzed on FACS, based on the $CD19^+$ $CD2^-$ B cells. (Figure 8)

EXAMPLE 11: Binding affinity of anti-CD20 GAbs to FcγRIIIA

[00259] FcγRIIIA was transfected into HEK-293 cell line to express recombinant protein. The secreted FcγRIIIA recombinant protein was purified and then diluted to serial concentration in HBS-EP buffer (200 nM, 100 nM, 50 nM, 25 nM, and 12.5 nM). Each of anti-CD20 GAbs101, 102, 104, 105, 106, 107, 108, 109, 110 and 111was diluted in HBS-EP buffer to the concentration of 10 ug/ml, and then captured to the CM5 chip in which anti-human Fab domain antibodies were pre-immobilized. A serial titration of FcγRIIIA was injected and bound at the

flow rate of 30 ml/min. Single cycle kinetics data was fitted into 1:1 binding model using Biacore T200 evaluation software to measure the equilibrium constant (Ka/Kd). The results were shown in Table 4. Anti-CD20 GAbs exhibit stronger FcγRIIIA binding affinity as compared to Rituximab.

EXAMPLE 12: Tumor suppression by GAb101

[00260] Anti-tumor activity of anti-CD20 Glycoantibodies was evalauted in the Ramos lymphoma-bearing SCID mice. GAb101 was used as an exemplary Ab in this study. In contrast to the aggressive growth of tumor in the PBS group, GAb101 administered twice weekly for total 7 injections at 1 mg/kg and 10 mg/kg effectively suppressed tumor growth ($p<0.01$) (Fig. 9).

Materials and Methods

Exemplary General Procedures

Method A: Glycosylation by Thio-glycan Donor

[00261] To activate molecular sieves MS-4Å for glycosylation, it was connected to vacuum system and heated for 1 hour. After the activated molecular sieves was cooled to room temperature, it was added to a flask containing *Donor* (*1.5~2.0 eq.* for one position glycosylation) and *Acceptor* (*1.0 eq.*). Dichloromethane was added to the mixture, and then the solution was stirred at room temperature for 3 h. *N*-iodosuccinimide (NIS, 1.7~2.2 eq.) and trimethylsilyl trifluoromethanesulfonate (TMSOTf, 0.1 eq.) were added to the solution on -78°C, and then the solution was stirred at -20 °C. Reaction was monitored by thin-layer chromatography (TLC) analysis, which was carried out on glass-backed silica gel plates (Merck DC Kieselgel $60F_{254}$) and visualized by UV light (254 nm) and acidic ceric ammonium molybdate. After the acceptor was consumed completely, the reaction was quenched with sat. $NaHCO_{3(aq)}$, and 20% $Na_2S_2O_3$, and then the mixture was filtered through a pad of celite. After the aqueous layer was extracted with two portions of dichloromethane, the combined organic layers were washed with brine, dried over $MgSO_4$, and concentrated. The crude was purified by silica gel column chromatography (toluene/ethyl acetate as elution system) to give product (the yield was shown on the scheme).

Method B: Glycosylation by Fluoride-glycan Donor

[00262] A mixture of silver triflate (5 eq.), bis (cyclopentadienyl) hafnium dichloride (3.5 eq.) and 4Å activated molecular sieves in dry toluene was stirred at room temperature for 1 h. The reaction mixture was then cooled to -50°C, a solution of *acceptor* (*1.0 eq.*) and donor (*1.2~1.5 eq.*) in toluene was added. The mixture was stirred at -10° C for 2-8 h. After TLC

indicated complete consumption of acceptor, the reaction was quenched with Et_3N, diluted with EtOAc and filtered through Celite. The filtrate was washed with aqueous $NaHCO_3$, and a brine solution. The organic layers was dried over Na_2SO_4 and concentrated in *vacuo*. The crude was purified by silica gel column chromatography (toluene/ethyl acetate as elution system) to give product (the yield was shown on the scheme).

Method C: Deprotection of *O*-Acetyl

[00263] *NaOMe* (*0.25 eq.*) was added to solution of starting material (*1.0 eq.*) in THF/Methanol (2/3). Reaction was stirred at room temperature and monitored by TLC analysis. After the acetyl group was de-protected completely, the solution was neutralized by IR-120, filtered, and concentrated. The crude was purified by silica gel column chromatography (hexanes/ethyl acetate as elution system) to give product (the yield was shown on the scheme).

Method D: Deprotection of *O*-Troc

[00264] *Zn powder* (*20 eq.*) and *acetic acid* (*0.2 eq.*) were added to solution of starting material (*1.0 eq.*) in THF. Reaction was stirred at room temperature and monitored by thin-layer chromatography (TLC) analysis. After the Troc group was de-protected completely, the solution was filtered, and concentrated. The crude was purified by silica gel column chromatography (hexanes/ethyl acetate as elution system) to give product (the yield was shown on the scheme).

Method E: Deprotection of Benzylidene

[00265] *p-Toluenesulfonic acid* (*pTSA*, *1.5 eq.*) was added to solution of starting material (*1.0 eq.*) in ACN/MeOH (2/1). Reaction was stirred at room temperature and monitored by thin-layer chromatography (TLC) analysis. After the benzylidene group was removed completely, the reaction was quenched by trimethylamine and then concentrated. The crude was purified by silica gel column chromatography (hexanes/ethyl acetate as elution system) to give product (the yield was shown on the scheme).

Method F: Global Deprotection

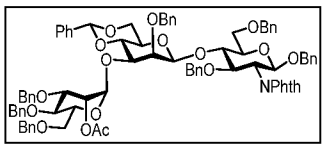
[00266] A mixture of protected oligosaccharides (50 mmol) and 10 mL of ethylene diamine: *n*BuOH (1/4) were stirred at 90 °C overnight. Volatiles were evaporated, and crude was reacted with 10 mL Ac_2O/pyridine (1/2) overnight. The solvents were removed using high vacuum, and the product was purified by flash column chromatography (acetone/toluene as elute system). The products were de-acetylated using sodium methoxide in MeOH (10 mL) overnight. Reactions were neutralized by using IR-120, then, filtered and concentrated in vacuum. The

residues were purified by flash column chromatography (acetone/toluene as elute system). The products were dissolved in 10 mL MeOH : H_2O : HCOOH (6/3/1), $Pd(OH)_2$ (50% by weight) was added, and the reactions were hydrogenated overnight. The reaction mixtures were filtered through celite and concentrated in *vacuo*. The residues were purified by G-15 gel column chromatography using water as eluent. The products were lypholysed to get white color powders (the yield was shown on the scheme).

Method G: Enzymatic (2,6)-Sialylation

[00267] Starting materials (5 μmol), CTP (1 μmol), Neu5Ac (9.5 μmol), PEP (10 μmol), α-2,6 sialyltransferase (200 μL, estimated concentration of 2 mg/L), CMK (80 units), PK (40 units), and PPA (40 units) were dissolved in 50 μmol sodium cacodylate (pH 7.4) containing 1% BSA (130 μL). The reactions were incubated at 37 °C with gentle agitation for 2d. The products were purified by using G-15 gel chromatography (eluent H_2O) to afford the desired products as white solid after lyophilization.

Building Blocks:



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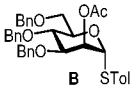

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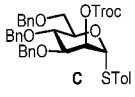

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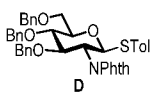

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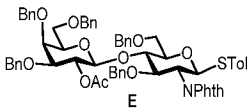

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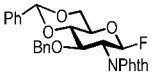

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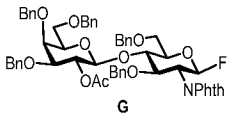
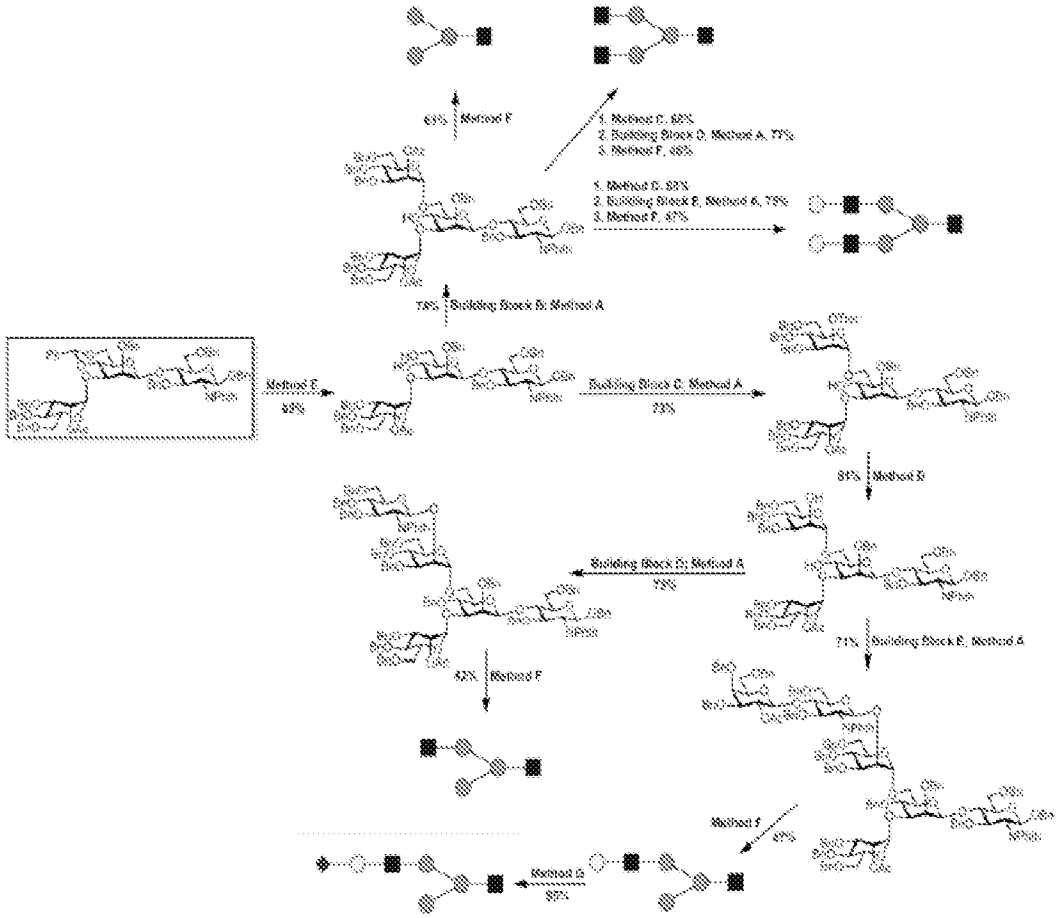


G

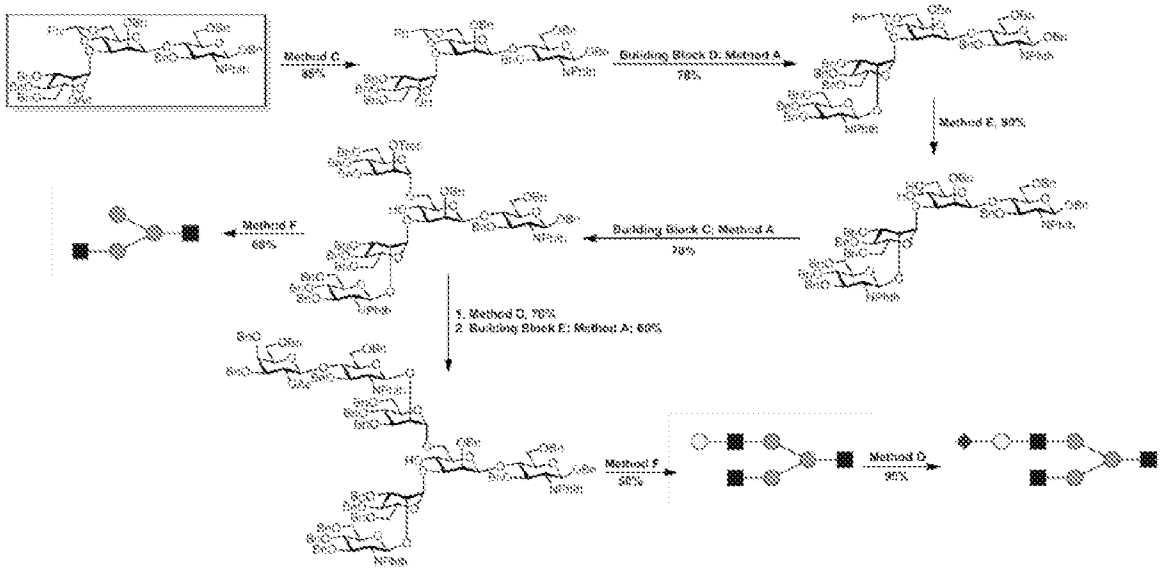
Experimental Procedures for Synthesizing Asymmetric N-Glycans

Scheme 1



Experimental Procedures for Synthesizing Asymmetric N-Glycans

Scheme 2



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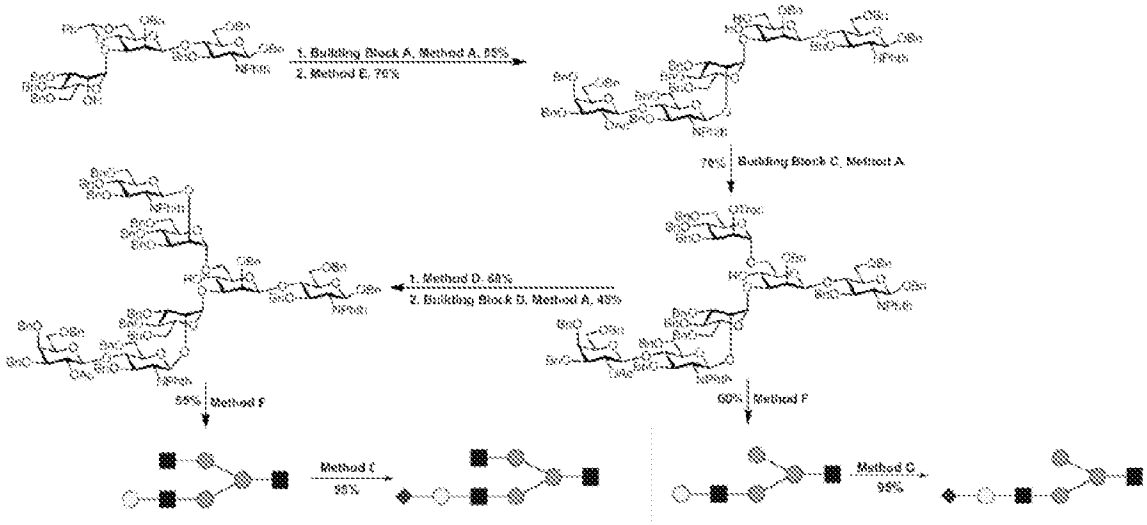
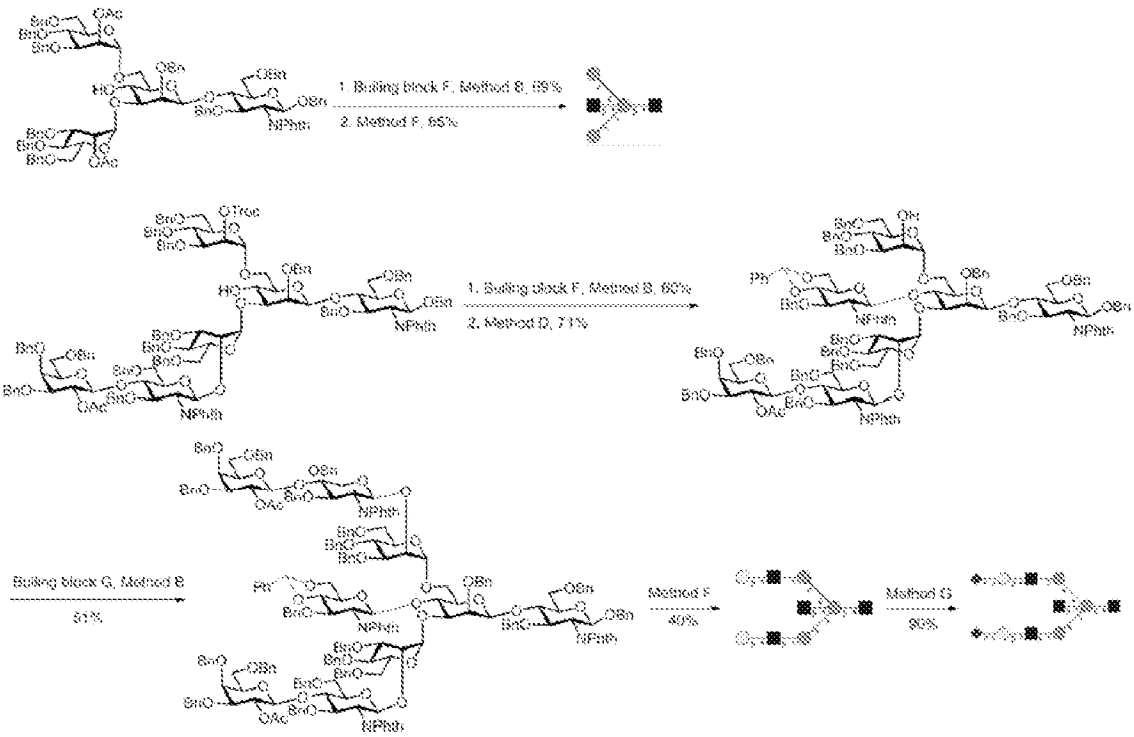


Experimental Procedures for Synthesizing Bisecting-GlcNAc N-Glycans

Scheme 4



[00268] ***Binding to B-lymphoma cells.*** The binding of the antibodies was investigated in $CD20^+$ B lymphoma cell lines (Ramos and SKW6.4) and analyzed on flow cytometry. The cells in PBS containing 1% fetal bovine serum at $2x10^5$/well on microplate were incubated on ice for 1 hr with antibodies of interest at different concentrations. The cells are washed, re-suspended in the PBS buffer, and incubated with the detecting goat anti-hIgG-Fcγ-PE on ice for 30 min. The cells are washed and subjected to analysis on FACS.

[00269] ***Binding to FcRIIIa-expressing CHO cells.*** The binding of the antibodies to the FcRIIIa receptors (CD16a), which is a precursor event known to be correlative with the induction of antibody-dependent cellular cytotoxicity (ADCC), was investigated in CHO cells transfected with the high-affinity CD16a (158Val) and analyzed on flow cytometry. The cells in PBS containing 1% fetal bovine serum at $1x10^5$/well on microplate were incubated on ice for 1 hr with antibodies of interest at different concentrations. The cells are washed, re-suspended in the PBS buffer, and incubated with the detecting goat anti-hIgG-Fcγ-PE on ice for 30 min. The cells are washed and subjected to analysis on FACS.

[00270] ***Apoptosis to B-lymphoma cells.*** The apoptotic activity induced by the antibodies were investigated in $CD20^+$ B lymphoma cell lines (Ramos and SKW6.4) and analyzed on flow

cytometry. The cells in RPMI 1640-10%FBS culture medium at $5x10^4$/well on microplates were incubated at 37 °C for 24 hr with antibodies of interest at different concentrations. The apoptosis was induced in the presence of the cross linker Goat $F(ab)'_2$ anti-hIgG-Fcγ, in concentrations at 1:1 ratio with the antibodies. The cells were washed and incubated in the dark for 5 min with Annexin V-FITC/PI reagents. The apoptotic deaths detected were analyzed on FACS.

[00271] ***Complement-dependent cytotoxicity (CDC) to B-lymphoma cells.*** The CDC effect induced by the antibodies were investigated in $CD20^+$ B lymphoma cell lines (Ramos and SKW6.4) and analyzed on flow cytometry. The cells in RPMI 1640 culture medium at $2.0x10^5$/well on microplates were incubated on ice for 30 min with antibodies of interest at different concentrations. The cells were washed and incubated at 37 °C for 30 min with 10% human serum in RPMI 1640. The cells were washed and incubated in the dark for 5 min with the PI reagent. The cell deaths by CDC were analyzed on FACS.

[00272] ***Antibody-dependent cellular cytotoxicity (ADCC) to B-lymphoma cells.*** The ADCC effect induced by the glyco-antibodies were investigated in CD20-containing B lymphoma cell lines (Ramos and SKW6.4), using freshly prepared human PBMC as effector cells, and the results analyzed on flow cytometry. The target B cells in PBS-0.1%BSA were first labeled with CFSE at 37 °C for 5 min. After wash the CFSE-labeled cells in RPMI 1640 medium were incubated at 37 °C for 4 hr on microplates with the glyco-antibodies of interest at different concentrations and PBMC effector cells. The ratio of target cells to effector cells was set at 25:1. The resultant mixtures were stained in the dark for 5 min with the PI reagent. The cell deaths by ADDC were analyzed on FACS.

[00273] ***Depletion of human B cells.*** The depletion of human B cells was conducted using human PBMC cells freshly prepared from human blood. The cells at $2x10^6$ in RPMI 1640-5%FBS cultured on microplates were incubated, in the absence or presence of 15% autologous plasma, at 37 °C for 4 hr with the antibodies of interest at different concentrations. The cells after wash were stained with anti-CD2-PE and anti-CD19-FITC on ice for 5 min. B cells depletion was analyzed on FACS, based on the $CD19^+$ $CD2^-$ B cells.

[00274] ***Lymphoma xenograft models.*** B lymphoma mouse xenograft studies were performed at, and according to procedures approved by, the Southern Research Institute's Institutional Animal Care and Use Committee (IACUC). In brief, 6~8-week-old female C.B-17 severe combined immunodeficient (SCID) mice (BioLASCO Taiwan, Co.) were implanted s.c. with 5 × 10^6 Ramos lymphoma cells. Mice were randomly placed into three groups (n = 6/group) when tmor grew to ~60 mm^3 in average and started antibody treatment. On day 9 post-tumor cell

implant, mice were injected i.v. with 1, or 10 mg/kg of GAb101 or PBS twice weekly for 7 injections. Palpable tumors were measured twice per week with calipers; tumor volumes were calculated as (length × width^2) / 2.

CLAIMS

We claim:

1. A composition of anti-CD20 glycoantibodies or anti-CD20 binding fragments comprising an isolated homogeneous population of anti-CD20 IgG molecules having the same N-glycan on each of the Fc region.

2. The composition of claim 1, wherein the anti-CD20 IgG molecule comprises a heavy chain having the amino acid sequence set forth in SEQ ID NO: 1, and a light chain having the amino acid sequence set forth in SEQ ID NO: 2.

3. The composition of claim 1, wherein the anti-CD20 IgG molecule comprises a light chain sequence and a heavy chain sequence of Rituximab.

4. The composition of claim 1, wherein the anti-CD20 glycoantibodies have improved ADCC activity than Rituximab.

5. The composition of claim 4, wherein the ADCC is improved by at least about 10 -fold.

6. The composition of claim 1, wherein the anti-CD20 glycoantibodies exhibit improved binding to FcγRIIIA as compared to Rituximab.

7. The composition of claim 1, wherein the N-glycan is attached to the Asn-297 of the Fc region.

8. The composition of claim 1, wherein the N-glycan is free of fucose.

9. The composition of claim 1, wherein the N-glycan comprises at least one α2–6 sialic acid.

10. The composition of claim 9, wherein the N-glycan comprises two α2–6 sialic acids.

11. The composition of claim 1, wherein the N-glycan is selected from the group consisting of $Sia_2(\alpha 2\text{-}6)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia_2(\alpha 2\text{-}6)Gal_2GlcNAc_3Man_3GlcNAc_2$,

$Sia_2(\alpha2\text{-}3)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}3)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}3/\alpha2\text{-}6)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}6/\alpha2\text{-}3)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}3/\alpha2\text{-}6)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}6/\alpha2\text{-}3)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia(\alpha2\text{-}6)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia(\alpha2\text{-}3)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia(\alpha2\text{-}6)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia(\alpha2\text{-}3)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia(\alpha2\text{-}6)GalGlcNAc_2Man_3GlcNAc_2$, $Sia(\alpha2\text{-}3)GalGlcNAc_2Man_3GlcNAc_2$, $Sia(\alpha2\text{-}6)GalGlcNAc_3Man_3GlcNAc_2$, $Sia(\alpha2\text{-}3)GalGlcNAc_3Man_3GlcNAc_2$, $Gal_2GlcNAc_2Man_3GlcNAc_2$, $GalGlcNAcMan_3GlcNAc_2$, $Gal_2GlcNAc_3Man_3GlcNAc_2$, $GalGlcNAc_2Man_3GlcNAc_2$, $GalGlcNAc_3Man_3GlcNAc_2$, $GlcNAc_3Man_3GlcNAc_2$, $GlcNAc_2Man_3GlcNAc_2$, $GlcNAcMan_3GlcNAc_2$ and $Man_3GlcNAc_2$.

12. The composition of claim 11, wherein the N-glycan is selected from the group consisting of $Sia_2(\alpha2\text{-}6)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}6)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}3)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}3)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}3/\alpha2\text{-}6)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}6/\alpha2\text{-}3)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}3/\alpha2\text{-}6)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}6/\alpha2\text{-}3)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia(\alpha2\text{-}6)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia(\alpha2\text{-}3)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia(\alpha2\text{-}6)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia(\alpha2\text{-}3)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia(\alpha2\text{-}6)GalGlcNAc_2Man_3GlcNAc_2$, $Sia(\alpha2\text{-}3)GalGlcNAc_2Man_3GlcNAc_2$, $Sia(\alpha2\text{-}6)GalGlcNAc_3Man_3GlcNAc_2$, $Sia(\alpha2\text{-}3)GalGlcNAc_3Man_3GlcNAc_2$, $Gal_2GlcNAc_2Man_3GlcNAc_2$, $GalGlcNAcMan_3GlcNAc_2$ and $Gal_2GlcNAc_3Man_3GlcNAc_2$.

13. A pharmaceutical formulation comprising substantially pure population of anti-CD20 glycoantibodies or antigen binding fragments thereof according to claims 1 to 12 and a pharmaceutically acceptable carrier.

14. A method for the treatment of cancer in a patient wherein the method comprising administering to the patient in need thereof an effective amount of said pharmaceutical formulation according to claim 13.

15. The method of claim 14, wherein the cancer is selected from the group consisting of B cell lymphomas, NHL, precursor B cell lymphoblastic leukemia/lymphoma and mature B cell neoplasms, B cell chronic lymphocytic leukemia (CLL)/small lymphocytic lymphoma (SLL), B cell prolymphocytic leukemia, lymphoplasmacytic lymphoma, mantle cell lymphoma

(MCL), follicular lymphoma (FL), low-grade, intermediate-grade and high-grade (FL), cutaneous follicle center lymphoma, marginal zone B cell lymphoma, MALT type marginal zone B cell lymphoma, nodal marginal zone B cell lymphoma, splenic type 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 (ALCL).

16. The method of claim 14, further comprising administering to the patient a therapeutic agent.

17. The method of claim 14, wherein the therapeutic agent comprises Rituximab.

18. A method for improving disease treatment based on B-cell depletion, wherein the method comprising administering a therapeutically effective amount of said pharmaceutical composition according to claim 13.

19. The method of claim 18, wherein the method comprising administering a therapeutically effective amount of said pharmaceutical composition according to claim 13 as coadministration or coformulation.

20. A method of making an anti-CD20 glycoantibody or antigen binding fragment of claim 1, the method comprising:

(a) contacting an anti-CD20monoclonal antibody with an α-fucosidase and at least one endoglycosidase, thereby yielding a defucosylated antibody having a single N-acetylglucosamine (GlcNAc), and

(b) adding a carbohydrate moiety to GlcNAc under suitable conditions.

21. The method of claim 20, wherein the anti-CD20 monoclonal antibody is Rituximab.

22. The method of claim 20, the anti-CD20 glycoantibodies are produced *in vitro*.

23. The method of claim 20, the anti-CD20 glycoantibodies are enzymatically engineered from an antibody obtained by cell culturing.

24. The method of claim 20, wherein the carbohydrate moiety is slected from the group consisting of $Sia_2(\alpha2\text{-}6)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}6)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}3)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}3)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}3/\alpha2\text{-}6)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}6/\alpha2\text{-}3)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}3/\alpha2\text{-}6)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}6/\alpha2\text{-}3)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia(\alpha2\text{-}6)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia(\alpha2\text{-}3)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia(\alpha2\text{-}6)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia(\alpha2\text{-}3)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia(\alpha2\text{-}6)GalGlcNAc_2Man_3GlcNAc_2$, $Sia(\alpha2\text{-}3)GalGlcNAc_2Man_3GlcNAc_2$, $Sia(\alpha2\text{-}6)GalGlcNAc_3Man_3GlcNAc_2$, $Sia(\alpha2\text{-}3)GalGlcNAc_3Man_3GlcNAc_2$, $Gal_2GlcNAc_2Man_3GlcNAc_2$, $GalGlcNAcMan_3GlcNAc_2$, $Gal_2GlcNAc_3Man_3GlcNAc_2$, $GalGlcNAc_2Man_3GlcNAc_2$, $GalGlcNAc_3Man_3GlcNAc_2$, $GlcNAc_3Man_3GlcNAc_2$, $GlcNAc_2Man_3GlcNAc_2$, $GlcNAcMan_3GlcNAc_2$ and $Man_3GlcNAc_2$.

25. The method of claim 24, wherein the carbohydrate moiety is slected from the group consisting of $Sia_2(\alpha2\text{-}6)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}6)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}3)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}3)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}3/\alpha2\text{-}6)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}6/\alpha2\text{-}3)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}3/\alpha2\text{-}6)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia_2(\alpha2\text{-}6/\alpha2\text{-}3)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia(\alpha2\text{-}6)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia(\alpha2\text{-}3)Gal_2GlcNAc_2Man_3GlcNAc_2$, $Sia(\alpha2\text{-}6)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia(\alpha2\text{-}3)Gal_2GlcNAc_3Man_3GlcNAc_2$, $Sia(\alpha2\text{-}6)GalGlcNAc_2Man_3GlcNAc_2$, $Sia(\alpha2\text{-}3)GalGlcNAc_2Man_3GlcNAc_2$, $Sia(\alpha2\text{-}6)GalGlcNAc_3Man_3GlcNAc_2$, $Sia(\alpha2\text{-}3)GalGlcNAc_3Man_3GlcNAc_2$, $Gal_2GlcNAc_2Man_3GlcNAc_2$, $GalGlcNAcMan_3GlcNAc_2$ and $Gal_2GlcNAc_3Man_3GlcNAc_2$.

26. The method of claim 20, wherein the adding in step (b) is performed by a transglycosylase.

27. The method of claim 20, wherein the endoglycosidase is EndoS, Endo S2, EndoH, EndoA, EndoM, EndoF, EndoF1, EndoF2 or EndoF3.

28. The method of claim 20, wherein the α-fucosidase comprises a polypeptide having an amino acid sequence at least 90% identical to SEQ ID NO: 5.

29. The method of claim 20, wherein the α-fucosidase is a recombinant *Bacteroides* alpha-L-fucosidase.

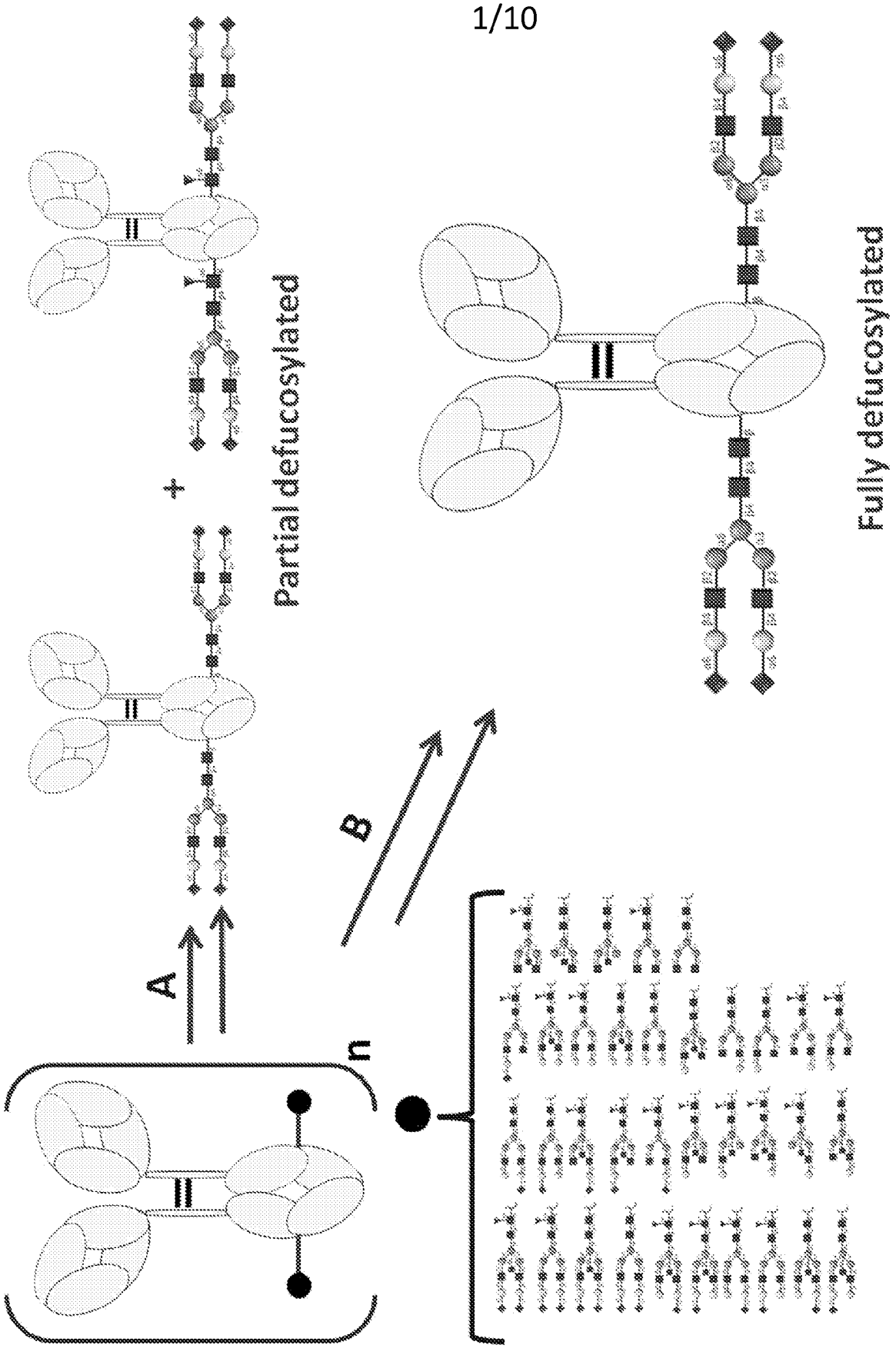

A
B
n
+
Partial defucosylated
Fully defucosylated
Highly heterogeneous

Figure 1

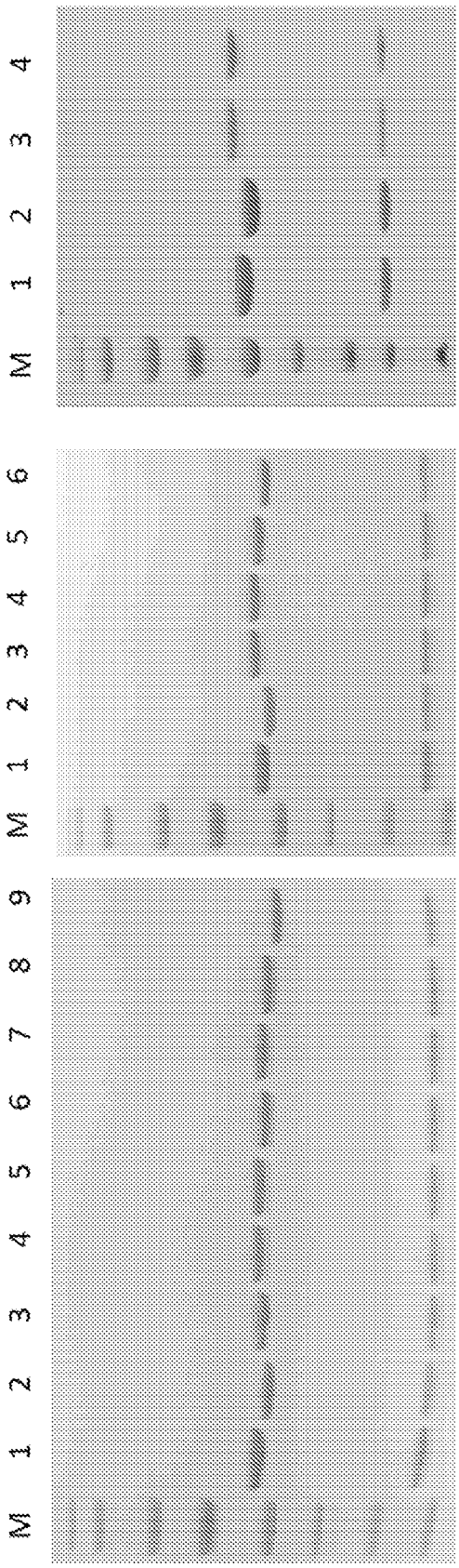

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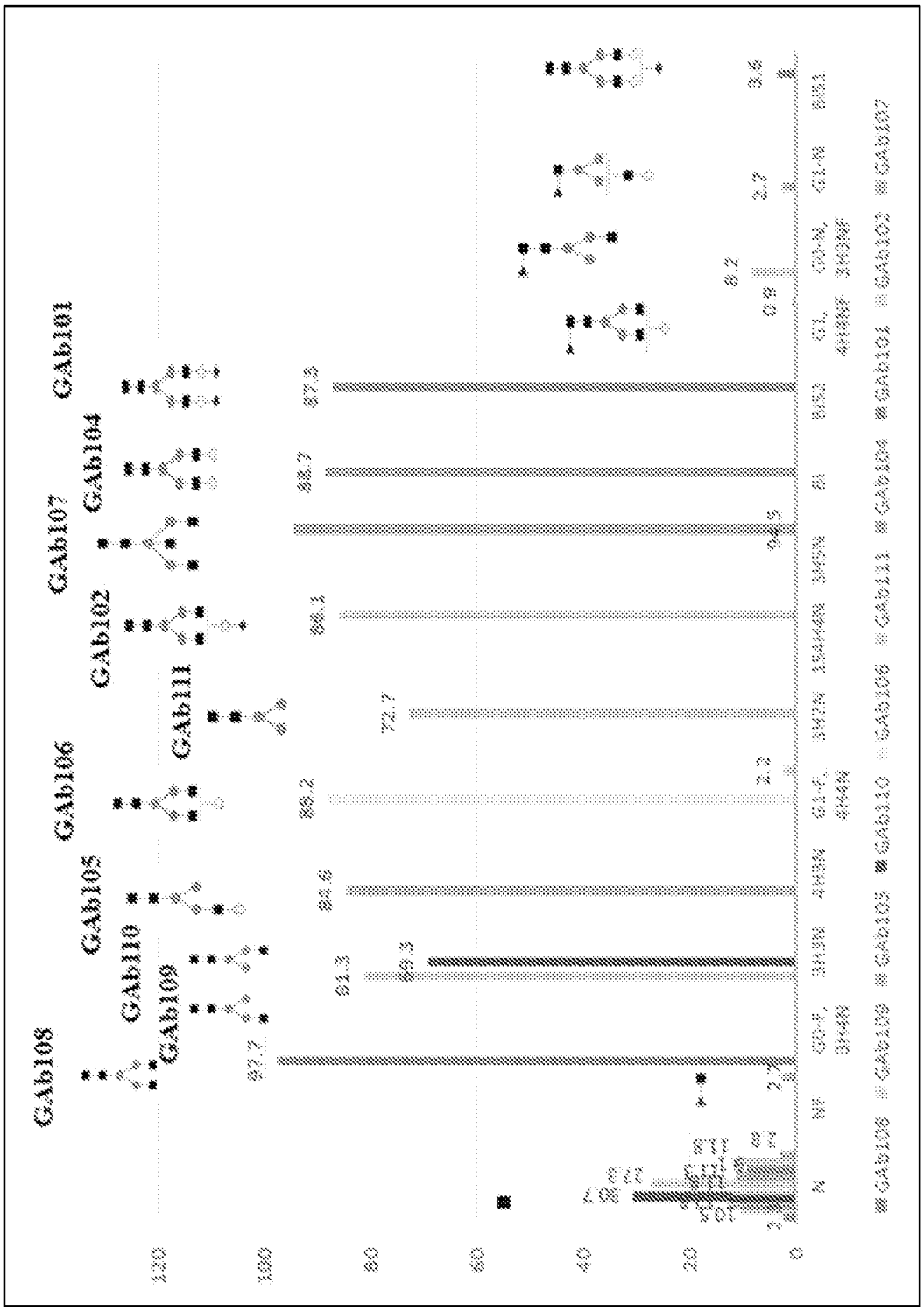
Figure 2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3H2N
1S4H4N
3H5N
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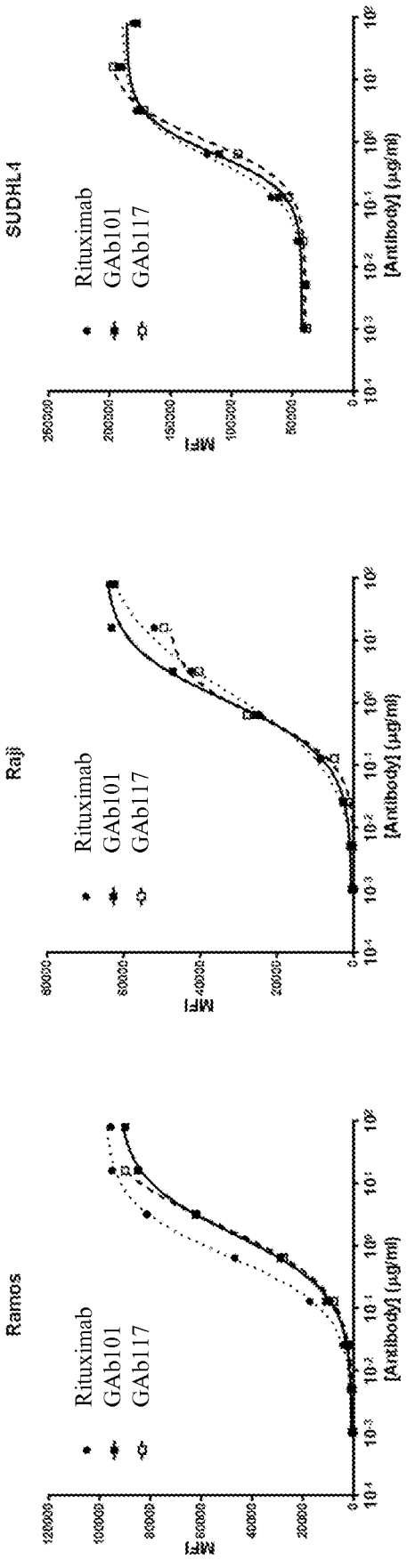
Figure 3


SUDHL4
Rituximab
GAb101
GAb117
MFI
[Antibody] (µg/ml)
Raji
Rituximab
GAb101
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Ramos
Rituximab
GAb101
GAb117
MFI
[Antibody] (µg/ml)

Figure 4

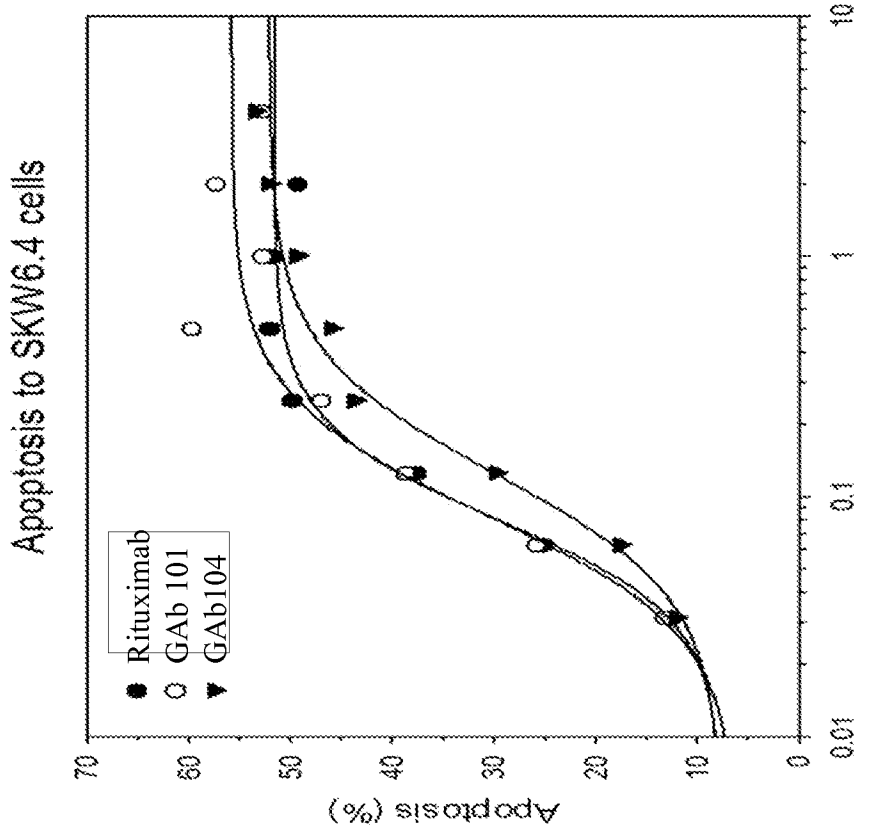

Figure 5B

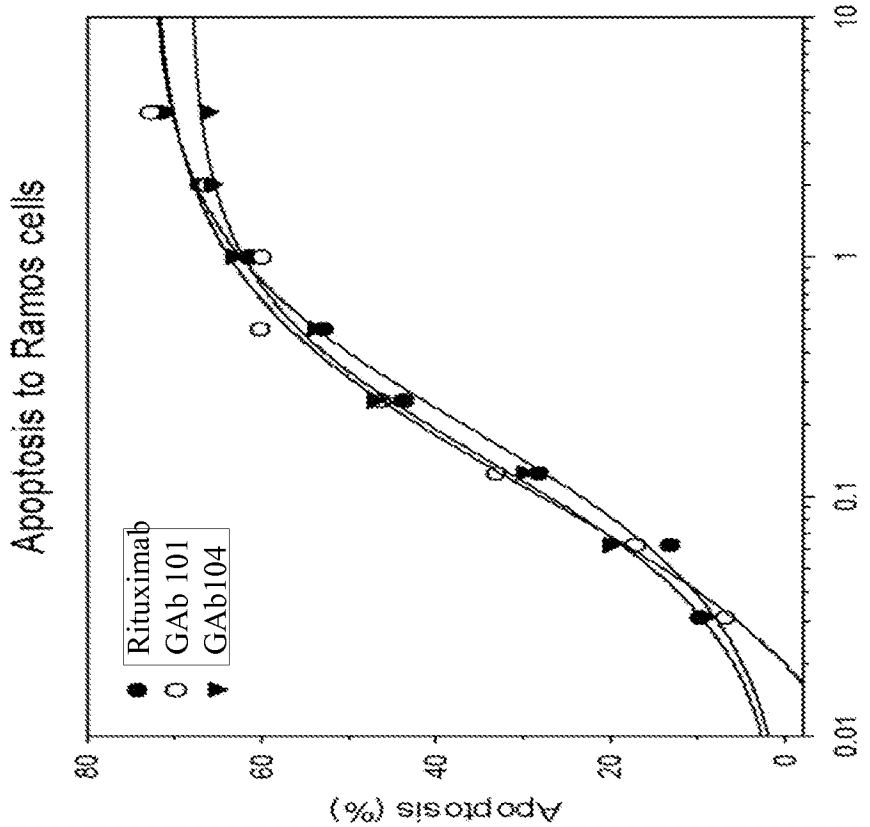

Figure 5A

CDC to Ramos cells

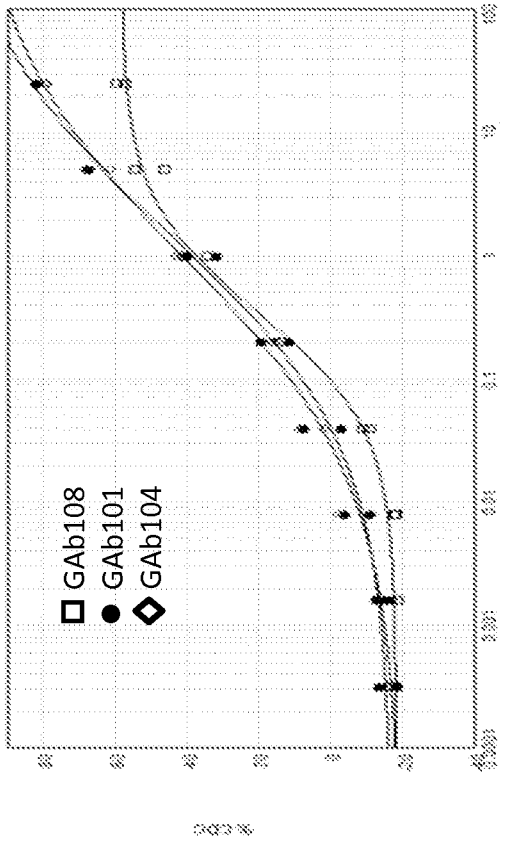

Figure 6B

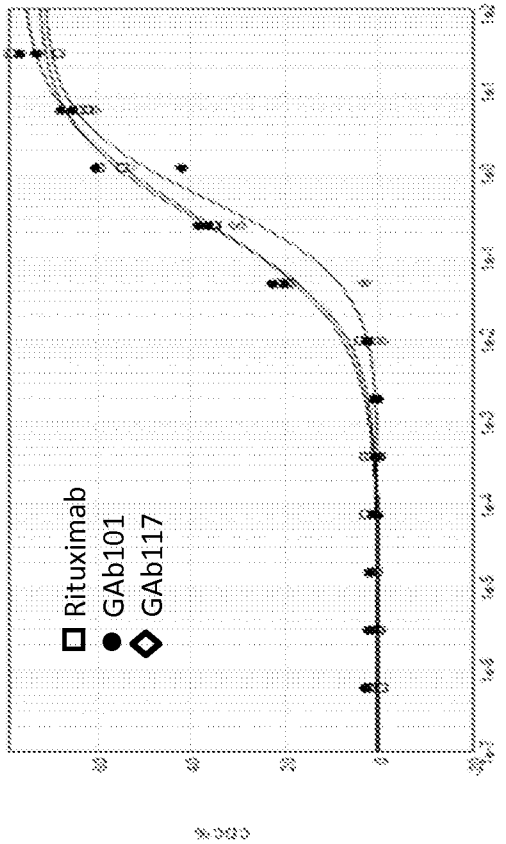

Figure 6A

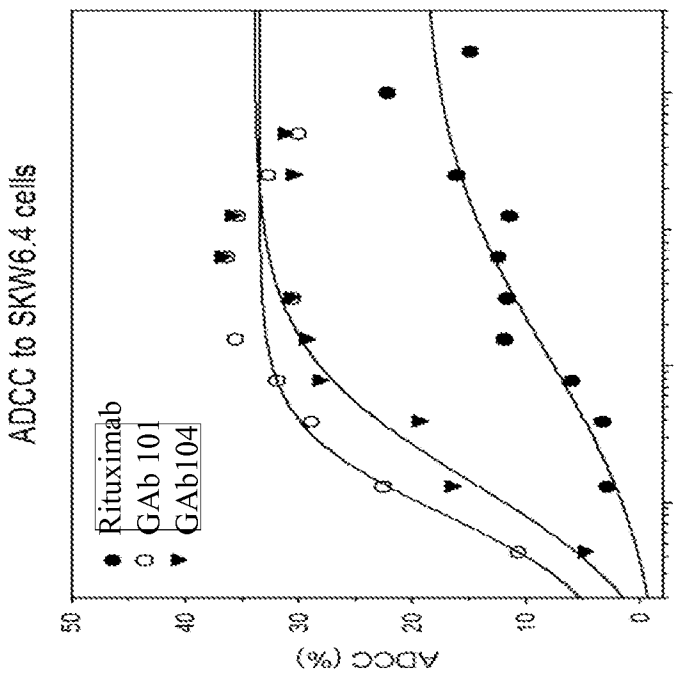

Figure 7B

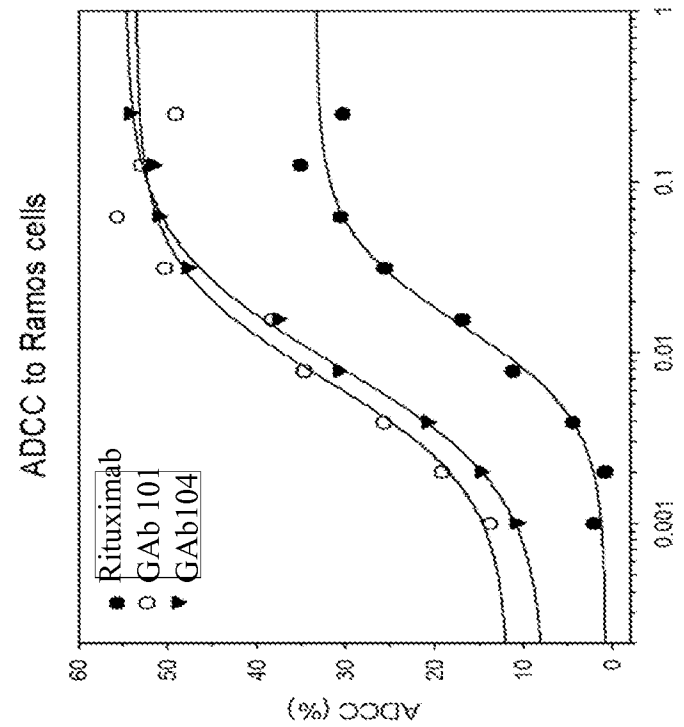

Figure 7A

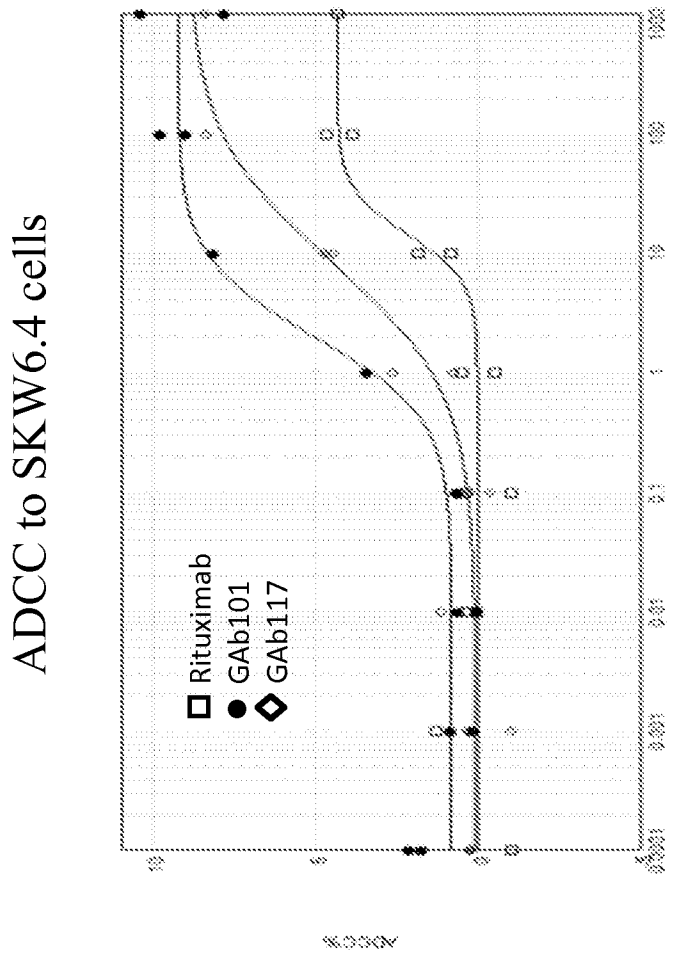
ADCC to SKW6.4 cells
Rituximab
GAb101
GAb117
%ADCC
concentration (ng/ml)

Figure 7C

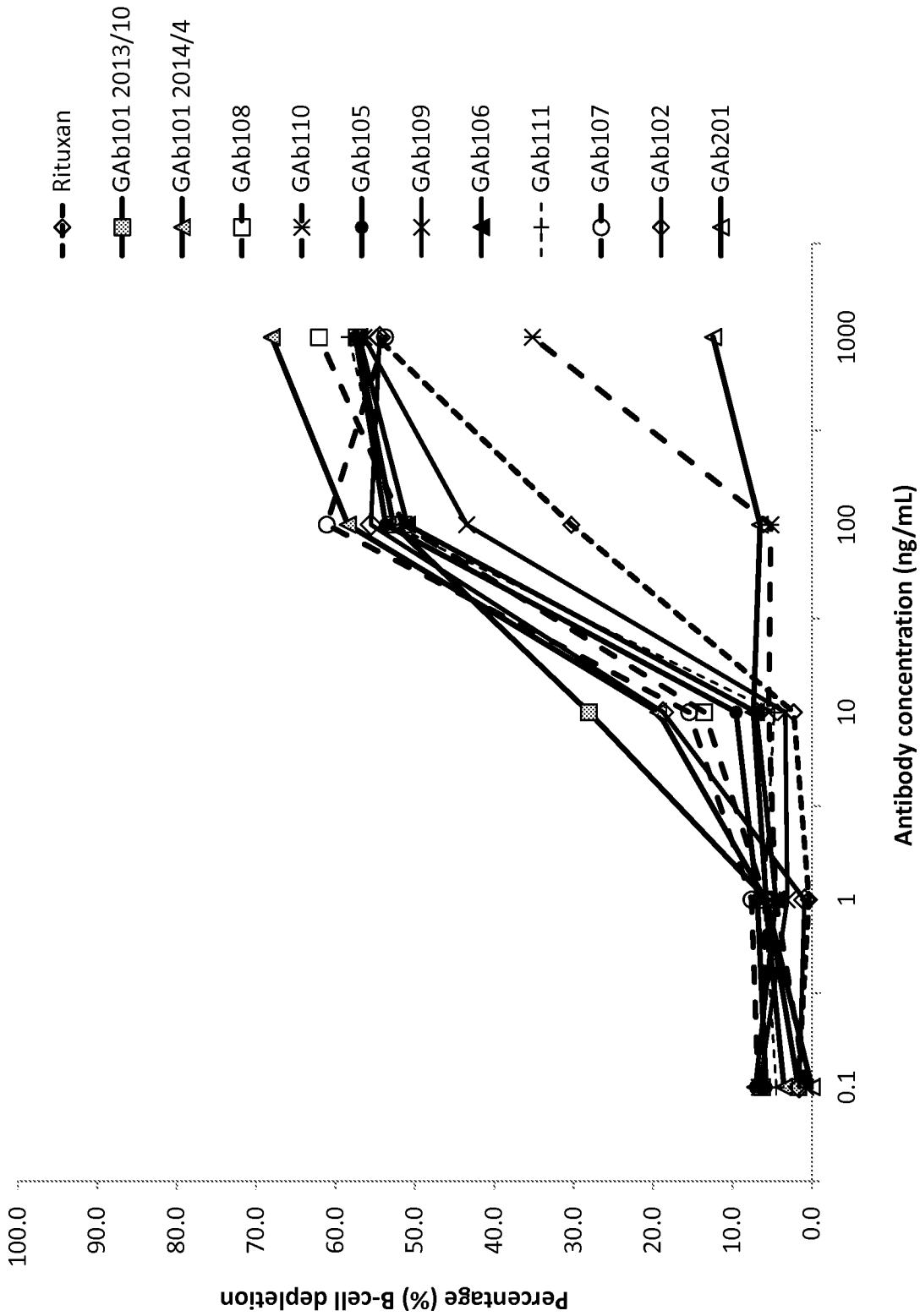
Rituxan
GAb101 2013/10
GAb101 2014/4
GAb108
GAb110
GAb105
GAb109
GAb106
GAb111
GAb107
GAb102
GAb201
Percentage (%) B-cell depletion
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0.1
1
10
100
1000
Antibody concentration (ng/mL)

Figure 8

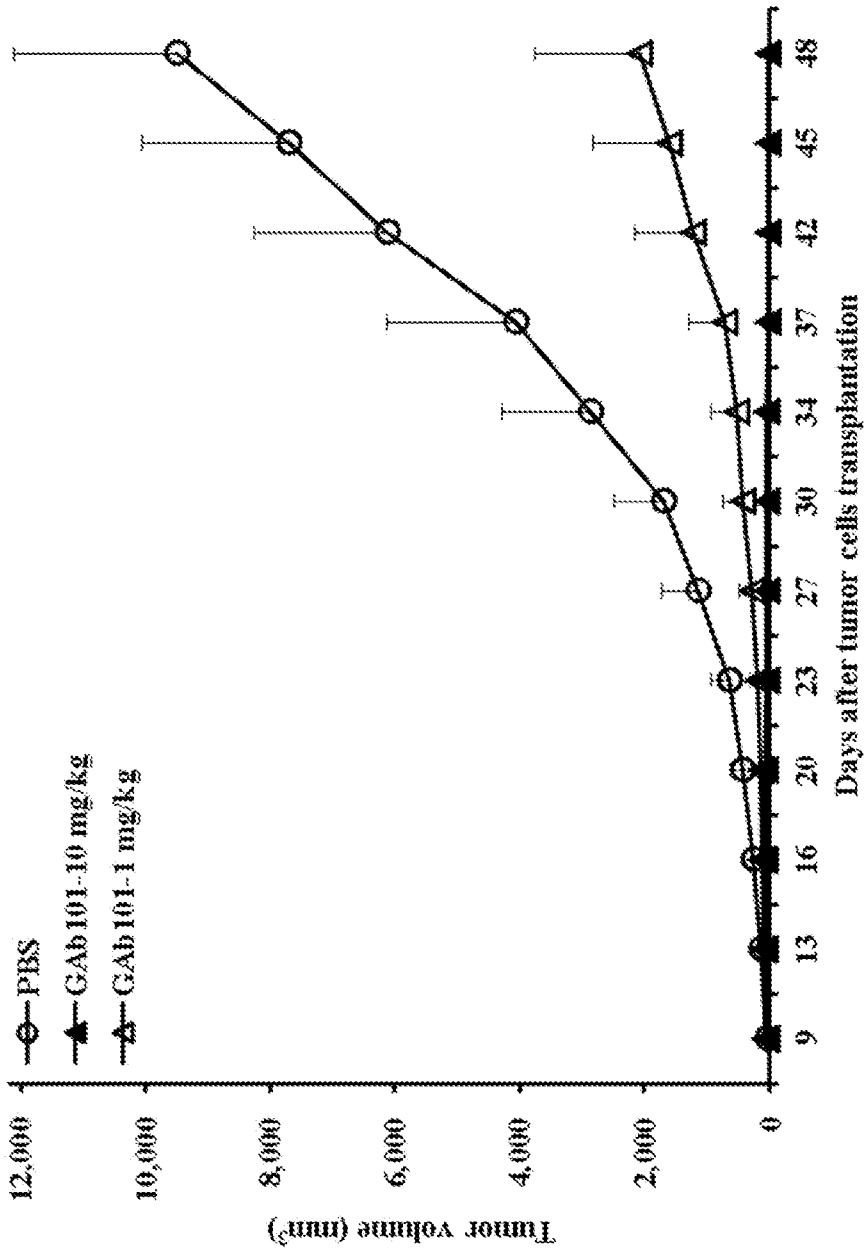
PBS
GAb101-10 mg/kg
GAb101-1 mg/kg
Tumor volume (mm³)
12,000
10,000
8,000
6,000
4,000
2,000
0
9
13
16
20
23
27
30
34
37
42
45
48
Days after tumor cells transplantation

Figure 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/032740

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C07K16/00 (2015.01)
CPC - C07K2317/41 (2015.09)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - A61P 35/00; C07K 16/00; C12P 21/00 (2015.01)
CPC - A61K 2039/505; C07K 16/00, 16/18, 2317/40, 2317/72, 2317/41, 2317/732; C12P 21/00, 21/005; C12Y 204/99, 302/01051, 302/01096 (2015.09)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC - A61K 2039/505; C07K 16/00, 16/18, 2317/40, 2317/72, 2317/41, 2317/732; C12P 21/00, 21/005; C12Y 204/99, 302/01051, 302/01096 (2015.09) (keyword delimited)
USPC - 435/68.1; 530/387.1

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Patents, PubMed

Search terms used: CD20 antibody homogeneous uniform glycosylation ASN-297 defucosylated fucosylated

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2011/0263828 A1 (WONG et al) 27 October 2011 (27.10.2011) entire document	1, 4-12, 20-27
--		----------
Y		2, 3, 28, 29
Y	US 2007/0224189 A1 (LAZAR et al) 27 September 2007 (27.09.2007) entire document	2, 3
Y	US 2014/0127241 A1 (ESPERANCE PHARMACEUTICALS, INC) 08 May 2014 (08.05.2014) entire document	2
Y	US 2006/0073161 A1 (BRETON) 06 April 2006 (06.04.2006) entire document	28, 29
A	US 2010/0173323 A1 (STROME et al) 08 July 2010 (08.07.2010) entire document	1-12, 20-29

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 September 2015	26 OCT 2015
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/032740

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☒ furnished subsequent to the international filing date for the purposes of international search only:

      ☒ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☒ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NOs: 1-5 were searched.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/032740

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 13-19
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.