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(71) Applicant *(for all designated States except US)*: **THE ROCKEFELLER UNIVERSITY** [US/US]; 1230 York Avenue, New York, NY 10021-6399 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **RAVETCH, Jeffrey** [US/US]; 500 Park Avenue, New York, NY 10022 (US). **NIMMERJAHN, Falk** [DE/DE]; Bismarckstr. 17, 91054 Erlangen (DE). **KANEKO, Yoshikatsu** [JP/JP]; 1-48-9 Horiwari-cho, Niigata City, Niigata, 9518154 (JP).

(74) Agents: **NORTON, Gerard, P.** et al.; Fox Rothschild LLP, 997 Lenox Drive, Bldg. #3, Lawrenceville, NJ 08648 (US).

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(54) Title: POLYPEPTIDES WITH ENHANCED ANTI-INFLAMMATORY AND DECREASED CYTOTOXIC PROPERTIES AND RELATING METHODS

(57) Abstract: The invention provides a polypeptide containing at least one IgG Fc region, wherein said at least one IgG Fc region is glycosylated with at least one galactose moiety connected to a respective terminal sialic acid moiety by a α 2,6 linkage, and wherein said polypeptide having a higher anti-inflammatory activity as compared to an unpurified antibody.

POLYPEPTIDES WITH ENHANCED ANTI-INFLAMMATORY AND DECREASED CYTOTOXIC PROPERTIES AND RELATING METHODS

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Application Number 11/957,015 filed December 14, 2007, which is a continuation-in-part patent application of PCT Patent Application Number PCT/US 07/08396, filed on April 3, 2007, which claims the benefit of United States Provisional Patent Application Number 60/789,384, filed on April 5, 2006, all of which are incorporated herein by reference. This application is also a continuation-in-part patent application of PCT Patent Application Number PCT/US07/72771 filed on July 3, 2007, which claims the benefit of PCT/US06/41791, filed on October 27, 2006 which claims the benefit of United States Provisional Patent Application Number 60/734,196, filed on November 7, 2005, all of which are also incorporated herein by reference.

STATEMENT REGARDING FEDERALLY FUNDED RESEARCH

The Research leading to the present invention was supported in part, by National Institutes of Health Grant No. AI 034662. Accordingly, the U.S. Government may have certain rights in this invention.

FIELD OF THE INVENTION

The present invention relates to a novel method for designing therapeutic polypeptides for treatment of inflammatory diseases.

BACKGROUND OF INVENTION

Although cellular receptors for immunoglobulins were first identified nearly 40 years ago, their central role in the immune response was only discovered in the last decade. They are key players in both the afferent and efferent phase

of an immune response, setting thresholds for B cell activation and antibody production, regulating the maturation of dendritic cells and coupling the exquisite specificity of the antibody response to effector pathways, such as phagocytosis, antibody dependent cellular cytotoxicity and the recruitment and activation of inflammatory cells. Their central role in linking the humoral immune system to innate effector cells has made them attractive immunotherapeutic targets for either enhancing or restricting the activity of antibodies *in vivo*.

The interaction of antibodies and antibody-antigen complexes with cells of the immune system effects a variety of responses, including antibody dependent cell-mediated cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC), phagocytosis, inflammatory mediator release, clearance of antigen, and antibody half-life (reviewed in Daron, *Annu Rev Immunol*, 15, 203-234 (1997); Ward and Ghetie, *Therapeutic Immunol*, 2, 77-94 (1995); Ravetch and Kinet, *Annu Rev Immunol*, 9, 457-492 (1991)), each of which is incorporated herein by reference).

Antibody constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions. Depending on the amino acid sequence of the constant region of their heavy chains, antibodies or 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, and IgG4; IgA1 and IgA2. The heavy chain constant regions that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ, respectively. Of the various human

immunoglobulin classes, human IgG1 and IgG3 mediate ADCC more effectively than IgG2 and IgG4.

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. The Fc region is central to the effector functions of antibodies. The crystal structure of the human IgG Fc region has been determined (Deisenhofer, *Biochemistry*, 20, 2361-2370 (1981), which is incorporated herein by reference). In human IgG molecules, the Fc region is generated by papain cleavage N-terminal to Cys, 226.

IgG has long been appreciated to mediate both pro- and anti-inflammatory activities through interactions mediated by its Fc fragment. Thus, while Fc-FcyR interactions are responsible for the pro-inflammatory properties of immune complexes and cytotoxic antibodies, intravenous gamma globulin (IVIG) and its Fc fragments are anti-inflammatory and are widely used to suppress inflammatory diseases. The precise mechanism of such paradoxical properties is unclear but it has been proposed that glycosylation of IgG is crucial for regulation of cytotoxicity and inflammatory potential of IgG.

IgG contains a single, N-linked glycan at Asn^{297} in the CH2 domain on each of its two heavy chains. The covalently-linked, complex carbohydrate is composed of a core, biantennary penta-polysaccharide containing N-acetylglucosamine (GIcNAc) and mannose (man). Further modification of the core carbohydrate structure is observed in serum antibodies with the presence of fucose, branching GIcNAc, galactose (gal) and terminal sialic acid (sa) moieties variably found. Over 40 different glycoforms have

thus been detected to be covalently attached to this single glycosylation site. Fujii et al., *J. Biol. Chem* 265, 6009 (1990). Glycosylation of IgG has been shown to be essential for binding to all FcγRs by maintaining an open conformation of the two heavy chains. Jefferis and Lund, *Immune.l Lett.* 82, 57 (2002), Sondermann et al., *J. Mol. Biol.* 309, 737 (2001). This absolute requirement of IgG glycosylation for FcγR binding accounts for the inability of deglycosylated IgG antibodies to mediate in vivo triggered inflammatory responses, such as ADCC, phagocytosis and the release of inflammatory mediators. Nimmerjahn and Ravetch, *Immunity* 24, 19 (2006). Further observations that individual glycoforms of IgG may contribute to modulating inflammatory responses has been suggested by the altered affinities for individual FcγRs reported for IgG antibodies containing or lacking fucose and their consequential affects on cytotoxicity. Shields et al., *J. Biol. Chem.* 277, 26733 (2002), Nimmerjahn and Ravetch, *Science* 310, 1510 (2005). A link between autoimmune states and specific glycosylation patterns of IgG antibodies has been observed in patients with rheumatoid arthritis and several autoimmune vasculities in which decreased galactosylation and sialylation of IgG antibodies have been reported. Parekh et al., *Nature* 316, 452 (1985), Rademacher et al., *Proc. Natl. Acad. Sci. USA* 91, 6123 (1994), Matsumoto et al., 128, 621 (2000), Holland et al., Biochim. Biophys. Acta Dec 27; [Epub ahead of print] 2005. Variations in IgG glycoforms have also been reported to be associated with aging and upon immunization, although the in vivo significance of these alterations have not been determined. Shikata et al., *Glycoconj. J.* 15, 683 (1998), Lastra, et al., *Autoimmunity* 28, 25 (1998).

Accordingly, there is a need for the development of methods for the generation of polypeptides that would account for the disparate observations of IVIG properties *in vivo*.

SUMMARY OF INVENTION

The present invention fills the foregoing need by providing such methods and molecules. In one aspect, the invention provides an isolated polypeptide containing at least one IgG Fc region, having altered properties compared to an unpurified antibody preparation, wherein sialylation of the isolated polypeptide is higher than the sialylation of the unpurified antibody preparation. In one embodiment, the isolated polypeptide containing at least one IgG Fc region is glycosylated with at least one galactose moiety connected to a respective terminal sialic acid moiety by a α 2,6 linkage, and wherein said polypeptide having a higher anti-inflammatory activity as compared to an unpurified antibody. In one embodiment the isolated polypeptide containing at least one IgG Fc region is glycosylated with at least one galactose moiety connected to a respective terminal sialic acid moiety by a α 2,6 linkage, and wherein said polypeptide having a reduced binding to an Fc activating receptor as compared to an unpurified antibody preparation. In a further embodiment the Fc activating receptor is selected from the group consisting of FcγRIIA, FcγRIIC and FcγRIIIA.

In one aspect, the isolated polypeptide is derived from a recombinant source.

In another aspect, the instant invention provides a pharmaceutical formulation comprising a polypeptide containing at least one Fc region having a higher anti-

inflammatory activity, in combination with a suitable carrier or diluent.

In another aspect, the invention provides a method of modulating properties of a polypeptide comprising an Fc region comprising altering the sialylation of the polysaccharide chain of the Fc region.

In one embodiment the method comprises: providing an unpurified source of the polypeptide containing at least one Fc region, said unpurified source of the polypeptide containing at least one Fc region comprising a plurality of the polypeptides containing at least one Fc region having a polysaccharide chain comprising a terminal sialic acid connected to a galactose moiety through a α 2,6 linkage, and a plurality of the polypeptides containing at least one Fc region lacking a polysaccharide chain comprising a terminal sialic acid connected to a galactose moiety through the α 2,6 linkage; and increasing the ratio of the plurality of the polypeptides containing at least one Fc region having the polysaccharide chain comprising the terminal sialic acid connected to the galactose moiety through the α 2,6 linkage to the plurality of the polypeptide containing at least one Fc region lacking the polysaccharide chain comprising the terminal sialic acid connected to the galactose moiety through the α 2,6 linkage.

In yet another embodiment the invention provides a method of treating an inflammatory disease comprising administering to a subject in need thereof a therapeutic composition comprising a plurality of isolated polypeptides, each containing at least one IgG Fc region, wherein a first portion of the respective Fc regions comprises respective carbohydrate chains having galactose moieties connected to respective terminal sialic acid moieties by 2,6 linkage; a

dose of the therapeutic composition is smaller than a dose of a second composition which comprises a plurality of isolated polypeptides, each containing at least one IgG Fc region, having a second portion of the respective Fc regions comprising respective carbohydrate chains having galactose moieties connected to respective terminal sialic acid moieties by 2,6 linkage; and either the first portion is greater than the second portion, whereby the dose of the therapeutic composition and the dose of the second composition suppress inflammation to substantially the same extent, or the first portion is greater than the second portion, whereby the therapeutic composition suppresses inflammation to substantially a greater extent than an equal dose of the second composition.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is an illustration of MALDI-Tof analysis of SNA^+ FC linkages.

Figure 2 summarizes experiments demonstrating that enrichment of α 2,6 linkages between sialic acid and galactose improves anti-inflammatory properties of IVIG Fc fragments.

Figure 3 summarizes experiments demonstrating that removal of α 2,6 linkages between sialic acid and galactose attenuates anti-inflammatory properties of IVIG Fc fragments.

Fig. 4 demonstrates that reduced cytotoxicity does not depend on the linkage between galactose and sialic acid.

Fig. 5 demonstrates that the in vivo anti-inflammatory activity of the 2,6 sialylated IgG Fc is solely a property of the IgG Fc glycan.

DETAILED DESCRIPTION OF THE INVENTION

The inventors have surprisingly found that the cytotoxic and anti-inflammatory response of the IgG Fc domain results from the differential sialylation of the Fc-linked core polysaccharide. The cytotoxicity of IgG antibodies is reduced upon sialylation; conversely, the anti-inflammatory activity of IVIG is enhanced. IgG sialylation is shown to be regulated upon the induction of an antigen-specific immune response, thus providing a novel means of switching IgG from an innate, anti-inflammatory molecule in the steady-state, to a adaptive, pro-inflammatory species upon antigenic challenge. The Fc-sialylated IgGs bind to a unique receptor on macrophages that in turn upregulates an inhibitory Fcγ receptor (FcγR) thereby protecting against autoantibody-mediated pathology. See, generally, Ravetch and Nimmerjahn, J. Experim. Medicine 24(1): 11-15 (2007). The inventors have further surprisingly discovered that the anti-inflammatory response depends on the nature of the linkage between galactose and sialic acid moieties. The observation that the anti-inflammatory activity of IVIG is dependent on a precise glycan structure on the Fc further supports the model that the inventors have previously advanced (Y. Kaneko, F. Nimmerjahn, J. V. Ravetch, *Science* 313, 670 (2006); F. Nimmerjahn, J. V. Ravetch, *J Exp Med* 204, 11 (2007)) that a specific lectin receptor, and not a canonical Fc receptor, is involved in this pathway. The data underlying this invention support a model in which binding of the 2,6 sialylated Fc to its cognate lectin receptor expressed on a population of regulatory myeloid cells results in the trans upregulation of the inhibitory IgG Fc on effector macrophages, located at sites of inflammation, such as the inflamed joint, thus raising the threshold required for

cytotoxic IgGs to engage activation FcRs and trigger inflammatory responses(F. Nimmerjahn, J. V. Ravetch, *Science* 310, 1510 (2005)).

Accordingly, the instant disclosure provides an advantageous strategy of creating and selecting IgGs with desired cytotoxic and anti-inflammatory potential.

DEFINITIONS

Throughout the present specification and claims, the numbering of the residues in an immunoglobulin heavy chain is that of the EU index as in Kabat et al., *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991), which is expressly incorporated herein by reference. The "EU index as in Kabat" refers to the residue numbering of the human IgG1 EU antibody.

The term "native" or "parent" refers to an unmodified polypeptide comprising an Fc amino acid sequence. The parent polypeptide may comprise a native sequence Fc region or an Fc region with pre-existing amino acid sequence modifications (such as additions, deletions and/or substitutions).

The term "polypeptide" refers to any fragment of a protein containing at least one IgG Fc region and fragments thereof, including, without limitation, fully functional proteins, such as, for example, antibodies, e.g., IgG antibodies. When a polypeptide of the invention is compared to an unpurified antibody preparation, such a preparation is typically a blood sample, serum sample, and/or IVIG sample, derived from a mammal, e.g., a human donor. The preparation may be unfractionated or partially fractionated but typically comprises only about 2-4% sialylated Fc containing proteins. Compositions of the invention enriched or

formulated to have immunosuppressive activity typically comprise at least about 5% sialylated Fc containing proteins or more (e.g., 5-10%, 10-30%, 30-50%, 50-100% or ranges or intervals thereof).

The term "Fc region" is used to define a C-terminal region of an immunoglobulin heavy chain. The "Fc region" may be a native sequence Fc region or a variant Fc region. Although the boundaries of the Fc region of an immunoglobulin heavy chain might vary, the human IgG heavy chain Fc region is usually defined to stretch from an amino acid residue at position Cys226, or from Pro230, to the carboxyl-terminus thereof.

The "CH2 domain" of a human IgG Fc region (also referred to as "Cγ2" domain) usually extends from about amino acid 231 to about amino acid 340. The CH2 domain is unique in that it is not closely paired with another domain. Rather, two N-linked branched carbohydrate chains are interposed between the two CH2 domains of an intact native IgG molecule. It has been speculated that the carbohydrate may provide a substitute for the domain-domain pairing and help stabilize the CH2 domain (Burton, *Mol Immunol*, 22, 161-206 (1985), which is incorporated herein by reference).

The "CH3 domain" comprises the stretch of residues C-terminal to a CH2 domain in an Fc region (i.e., from about amino acid residue 341 to about amino acid residue 447 of an IgG).

The term "hinge region" is generally defined as stretching from Glu216 to Pro230 of human IgG1 (Burton (1985). Hinge regions of other IgG isotypes may be aligned with the IgG1 sequence by placing the first and last cysteine residues forming inter-heavy chain S--S bonds in the same positions.

The term "binding domain" refers to the region of a polypeptide that binds to another molecule. In the case of an FcR, the binding domain can comprise a portion of a polypeptide chain thereof (e.g., the α chain thereof) which is responsible for binding an Fc region. One exemplary binding domain is the extracellular domain of an FcR chain.

A "functional Fc region" possesses at least a partial "effector function" of a native sequence Fc region. 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 generally require the Fc region to be combined with a binding domain (e.g., an antibody variable domain) and can be assessed using various assays as herein disclosed, for example. The term also includes Fc fragments provided the fragment contains at least one amino acid residue that is glycosylated or suitable for glycosylation as described herein.

A "native sequence Fc region" comprises an amino acid sequence identical to the amino acid sequence of an Fc region found in nature. A "variant Fc region" as appreciated by one of ordinary skill in the art comprises an amino acid sequence which differs from that of a native sequence Fc region by virtue of at least one "amino acid modification." Preferably, the variant Fc region has at least one amino acid substitution compared to a native sequence Fc region or to the Fc region of a parent polypeptide, e.g., from about one to about ten amino acid substitutions, and preferably from about one to about five amino acid substitutions in a native sequence Fc region or in the Fc region of the parent

polypeptide. The variant Fc region herein will preferably possess at least about 80% homology with a native sequence Fc region and/or with an Fc region of a parent polypeptide, and more preferably at least about 90% homology therewith, more preferably at least about 95% homology therewith, even more preferably, at least about 99% homology therewith.

The term "altered glycosylation" refers to a polypeptide, as defined above, be it native or modified, in which the carbohydrate addition to the heavy chain constant region is manipulated to either increase or decrease specific sugar components. For example, polypeptides, such as, for example, antibodies, prepared in specific cell lines, such as, for example, Lec2 or Lec3, may be deficient in the attachment of sugar moieties such as fucose and sialic acid.

The terms "Fc receptor" or "FcR" are used to describe a receptor that binds to the Fc region of an antibody. In one embodiment of the invention, FcR is a native sequence human FcR. In another embodiment, FcR, including human FcR, binds an IgG antibody (a gamma receptor) and includes receptors of the FcγRI, FcγRII, and FcγRIII 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 in Daron, *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), Nimmerjahn and Ravetch 2006, Ravetch Fc Receptors in Fundemental Immunology, ed William Paul 5th Ed. each of which is incorporated herein by reference).

"Antibody-dependent cell-mediated cytotoxicity" and "ADCC" refer to an in vitro or in vivo cell-mediated reaction in which cytotoxic cells that express FcRs (e.g., monocytic cells such as natural killer (NK) cells and macrophages) recognize bound antibody on a target cell and subsequently cause lysis of the target cell. In principle, any effector cell with an activating FcγR can be triggered to mediate ADCC. One such cell, the NK cell, expresses FcγRIII only, whereas monocytes, depending on their state of activation, localization, or differentiation, can express FcγRI, FcγRII, and FcγRIII. FcR expression on hematopoietic cells is summarized in Ravetch and Bolland, *Annu Rev Immunol*, (2001), which is incorporated herein by reference.

"Human effector cells" are leukocytes which express one or more FcRs and perform effector functions. Preferably, the cells express at least one type of an activating Fc receptor, such as, for example, FcγRIII and perform ADCC effector function. Examples of human leukocytes which mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, and neutrophils, with PBMCs and NK cells being preferred. The effector cells may be isolated from a native source thereof, e.g., from blood or PBMCs as described herein.

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and

antibody fragments so long as they exhibit the desired biological activity.

The phrase “sialic acid content” of an antibody refers both to the total number of sialic acid residues on an Fc region of a heavy chain of an antibody and to the ratio of sialylated antibodies to asialylated antibodies in an unpurified antibody preparation, unless the phrase is in a context clearly suggesting that another meaning is intended.

"Antibody fragments", as defined for the purpose of the present invention, comprise a portion of an intact antibody, generally including the antigen binding or variable region of the intact antibody or the Fc region of an antibody which retains FcR binding capability. Examples of antibody fragments include linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments. The antibody fragments preferably retain at least part of the hinge and optionally the CH1 region of an IgG heavy chain. More preferably, the antibody fragments retain the entire constant region of an IgG heavy chain, and include an IgG light chain.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. 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 the hybridoma method first described by Kohler and Milstein, Nature, 256, 495-497 (1975), which is incorporated herein by reference, or may be made by recombinant DNA methods (see, e.g., U.S. Patent No. 4,816,567, which is incorporated herein by reference). The monoclonal antibodies may also be isolated from phage antibody libraries using the techniques described in Clackson et al., *Nature*, 352, 624-628 (1991) and Marks et al., *J Mol Biol*, 222, 581-597 (1991), for example, each of which is incorporated herein by reference.

In other embodiments of the invention, the polypeptide containing at least one IgG Fc region may be fused with other protein fragments, including, without limitation, whole proteins. A person of ordinary skill in the art will undoubtedly appreciate that many proteins may be fused with the polypeptide of the present invention, including, without limitation, other immunoglobulins, especially, immunoglobulins lacking their respective Fc regions. Alternatively, other biologically active proteins or fragments thereof may be fused with the polypeptide of the present invention, as described, for example, in the U.S. Patent No. 6,660,843, which is incorporated herein by reference. This embodiment is especially advantageous for delivery of such biologically active proteins or fragments thereof to cells expressing Fc receptors. Further, different markers, such as, for example, GST tag or green fluorescent protein, or GFP, may be used.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) 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 (see U.S. Patent No. 4,816,567; Morrison et al., *Proc Natl Acad Sci USA*, 81, 6851-6855 (1984); Neuberger et al., *Nature*, 312, 604-608 (1984); Takeda et al., *Nature*, 314, 452-454 (1985); International Patent Application No. PCT/GB85/00392, each of which is incorporated herein by reference).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of 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 that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one,

and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FR residues are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature*, 321, 522-525 (1986); Riechmann et al., *Nature*, 332, 323-329 (1988); Presta, *Curr Op Struct Biol*, 2, 593-596 (1992); U.S. Patent No. 5,225,539, each of which is incorporated herein by reference.

The polypeptides of the instant invention may be recombinantly produced, for example, from a cDNA, such as, for example SEQ ID NO: 1. The polypeptides of different embodiments include Fc regions or functional fragments thereof.

The polypeptides containing at least one IgG Fc region include those in which specific amino acid substitutions, additions or deletions are introduced into a parental sequence through the use of recombinant DNA techniques to modify the genes encoding the heavy chain constant region. The introduction of these modifications follows well-established techniques of molecular biology, as described in manuals such as Molecular Cloning (Sambrook and Russel, (2001)). In addition, the polypeptides with at least one Fc region will include those polypeptides which have been selected to contain specific carbohydrate modifications, obtained either by expression in cell lines known for their glycosylation specificity (Stanley P., et al., *Glycobiology*, 6, 695-9 (1996); Weikert S., et al., *Nature Biotechnology*, 17, 1116-1121 (1999); Andresen DC and Krummen L., *Current*

Opinion in Biotechnology, 13, 117-123 (2002)) or by enrichment or depletion on specific lectins or by enzymatic treatment (Hirabayashi et al., *J Chromatogr B Analyt Technol Biomed Life Sci*, 771, 67-87 (2002); Robertson and Kennedy, *Bioseparation*, 6, 1-15 (1996)). It is known in the art that quality and extent of antibody glycosylation will differ depending on the cell type and culture condition employed. (For example, Patel et al., *Biochem J*, 285, 839-845 (1992)) have reported that the content of sialic acid in antibody linked sugar side chains differs significantly if antibodies were produced as ascites or in serum-free or serum containing culture media. Moreover, Kunkel et al., Biotechnol Prog, 16, 462-470 (2000) have shown that the use of different bioreactors for cell growth and the amount of dissolved oxygen in the medium influenced the amount of galactose and sialic acid in antibody linked sugar moieties. These studies, however, did not address how varying levels of sialic acid residues influence antibody activity in vivo.

Host Expression Systems

The polypeptide of the present invention can be expressed in a host expression systems, i.e., host cells, capable of N-linked glycosylation. Typically, such host expression systems may comprise bacterial, fungal, plant, vertebrate or invertebrate expression systems. In one embodiment the host cell is a mammalian cell, such as a Chinese hamster ovary (CHO) cell line, (e.g. CHO-K1; ATCC CCL-61), Green Monkey cell line (COS) (e.g. COS 1 (ATCC CRL-1650), COS 7 (ATCC CRL-1651)); mouse cell (e.g. NS/0), Baby Hamster Kidney (BHK) cell line (e.g. ATCC CRL-1632 or ATCC CCL-10), or human cell (e.g. HEK 293 (ATCC CRL-1573) or 293T (ATCC CRL-11268)), or any other suitable cell line, e.g., available from public depositories such as the American Type

Culture Collection, Rockville, Md. Further, an insect cell line, such as a Lepidoptora cell line, e.g. Sf9, a plant cell line, a fungal cell line, e.g., yeast such as, for example, Saccharomyces cerevisiae, Pichia pastoris, Hansenula spp., or a bacterial expression system based on Bacillus, such as B. subtilis, or Eschericiae coli can be used. It will be appreciated by one of ordinary skill in the art that in some cases modifications to host cells may be required to insure that N-linked glycosylation and glycan maturation occur to result in a complex, biantennary sugar as typically found on the Fc domain of human IgG.

Therapeutic Formulations

Therapeutic formulations comprising the polypeptides containing at least one IgG Fc region can be prepared for storage by mixing the polypeptides of the present invention having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (see, e.g., Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenyl, 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) polypeptide; 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).

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

The active ingredients may also be entrapped in a 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).

In preferred embodiments, the formulations to be used for *in vivo* administration are sterile. The formulations of the instant invention can be easily sterilized, for example, by filtration through sterile filtration membranes.

Sustained-release preparations may also be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers

containing the modified antibody, 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 (see, e.g., U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and y ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S--S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

Creation of sialylated polypeptides containing at least one IgG Fc region.

The polypeptides of the present invention can be further purified or modified so that they have an increased amount of sialic acid compared to unmodified and/or unpurified antibodies. Multiple methods exist to reach this

objective. In one method, the source of unpurified polypeptides, such as, for example, IVIG, is passed through the column having lectin, which is known to bind sialic acid. A person of the ordinary skill in the art will appreciate that different lectins display different affinities for α2,6 versus α2,3 linkages between galactose and sialic acid. Thus, selecting a specific lectin will allow enrichment of antibodies with the desired type of linkage between the sialic acid and the galactose. In one embodiment, the lectin is isolated from *Sambuccus nigra*. A person of the ordinary skill in the art will appreciate that the *Sambuccus nigra* agglutinin (SNA) is specific for sialic acids linked to galactose or *N*-acetylgalactosamine by α(2-6) linkages. Shibuya et al, *J. Biol. Chem.*, 262: 1596-1601 (1987). In contrast, the *Maakia amurensis* ("MAA") lectin binds to sialic acid linked to galactose by α(2-3) linkages. Wang et al, *J Biol Chem.*, **263**: 4576-4585 (1988).

Thus, a fraction of the polypeptides containing at least one IgG Fc region having a desired linkage between the galactose and the sialic acid will be retained in the column while a fraction lacking such linkage will pass through. The sialylated fraction of the polypeptides containing at least one IgG Fc region can be eluted by another wash with a different stringency conditions. Thus, it is possible to obtain a preparation of the polypeptide of the present invention wherein the content of sialic acid is increased compared to the normal content. Further, one may employ an enzymatic reaction with a sialyltransferase and a donor of sialic acid as described, for example, in the U.S. Pat. No. 20060030521.

Suitable non-limiting examples of sialyltransferase enzymes useful in the claimed methods are ST3Gal III, which

is also referred to as α-(2,3)sialyltransferase (EC 2.4.99.6), and α-(2,6)sialyltransferase (EC 2.4.99.1).

Alpha-(2,3)sialyltransferase catalyzes the transfer of sialic acid to the Gal of a Gal-β-1,3GlcNAc or Gal-β-1,4GlcNAc glycoside (see, e.g., Wen et al., J. Biol. Chem. 267: 21011 (1992); Van den Eijnden et al., J. Biol. Chem. 256: 3159 (1991)) and is responsible for sialylation of asparagine-linked oligosaccharides in glycopeptides. The sialic acid is linked to a Gal with the formation of an α-linkage between the two saccharides. Bonding (linkage) between the saccharides is between the 2-position of NeuAc and the 3-position of Gal. This particular enzyme can be isolated from rat liver (Weinstein et al., J. Biol. Chem. 257: 13845 (1982)); the human cDNA (Sasaki et al. (1993) J. Biol. Chem. 268: 22782-22787; Kitagawa & Paulson (1994) J. Biol. Chem. 269: 1394-1401) and genomic (Kitagawa et al. (1996) J. Biol. Chem. 271: 931-938) DNA sequences are known, facilitating production of this enzyme by recombinant expression.

Activity of α-(2,6)sialyltransferase results in 6-sialylated oligosaccharides, including 6-sialylated galactose. The name "α-(2,6)sialyltransferase" refers to the family of sialyltransferases attaching sialic acid to the sixth atom of the acceptor polysaccharide. Different forms of a-(2,6)sialyltransferase can be isolated from different tissues. For example, one specific form of this enzyme, ST6Gal II, can be isolated from brain and fetal tissues. Krzewinski-Recchi et al., *Eur. J. Biochem.* 270, 950 (2003).

In addition, a person of average skill in the art will appreciate that cell culture conditions can be manipulated to change the sialylation rate. For example, to increase

the sialic acid content, production rate is decreased and osmolality is generally maintained within a lower margin suitable for the particular host cell being cultured. Osmolality in the range from about 250 mOsm to about 450 mOsm is appropriate for increased sialic acid content. This and other suitable cell culture conditions are described in, e.g., U.S. Patent No. 6,656,466. Patel et al., *Biochem J*, 285, 839-845 (1992) have reported that the content of sialic acid in antibody linked sugar side chains differs significantly if antibodies were produced as ascites or in serum-free or serum containing culture media. Moreover, Kunkel et al., *Biotechnol. Prog.*, 16, 462-470 (2000) have shown that the use of different bioreactors for cell growth and the amount of dissolved oxygen in the medium influenced the amount of galactose and sialic acid in antibody linked sugar moieties.

In another embodiment, host cells, such as, for example, immortalized human embryonic retina cells, may be modified by introducing a nucleic acid encoding a sialyltransferase such as, for example, an α-2,3-sialyltransferase or an α-2,6-sialyltransferase, operably linked to a promoter, such as, for example, a CMV promoter. The α-2,3-sialyltransferase may be the human α-2,3-sialyltransferase, known as SIAT4C or STZ (GenBank accession number L23767), and described, for example, in the U.S. Pat. No. 20050181359.

The nucleic acid encoding the sialyltransferase may be introduced into the host cell by any method known to a person of ordinary skill in the art. Suitable methods of introducing exogenous nucleic acid sequences are also described in Sambrook and Russel, *Molecular Cloning: A Laboratory Manual* (3rd Edition), Cold Spring Harbor Press,

NY, 2000. These methods include, without limitation, physical transfer techniques, such as, for example, microinjection or electroporation; transfections, such as, for example, calcium phosphate transfections; membrane fusion transfer, using, for example, liposomes; and viral transfer, such as, for example, the transfer using DNA or retroviral vectors.

The polypeptide containing at least one IgG Fc region may be recovered from the culture supernatant and can be subjected to one or more purification steps, such as, for example, ion-exchange or affinity chromatography, if desired. Suitable methods of purification will be apparent to a person of ordinary skill in the art.

A person of ordinary skill in the art will appreciate that different combinations of sialylation methods, disclosed above, can lead to production of the polypeptides containing at least one IgG Fc region with an extremely high level of sialylation. For example, one can express the polypeptide containing at least one IgG Fc region in the host cells overexpressing sialyltransferase, as described above, and then further enrich the sialylated fraction of these polypeptides by, for example, sialylating these polypeptides in an enzymatic reaction followed by an affinity chromatography using lectin-containing columns. Similarly, an enzymatic reaction followed by affinity chromatography may be used for IVIG source of the polypeptides containing at least one IgG Fc region.

To examine the extent of glycosylation on the polypeptides containing at least one IgG Fc region, these polypeptides can be purified and analyzed in SDS-PAGE under reducing conditions. The glycosylzation can be determined by reacting the isolated polypeptides with specific lectins,

or, alternatively as would be appreciated by one of ordinary skill in the art, one can use HPLC followed by mass spectrometry to identify the glycoforms. (Wormald, MR et al., Biochem 36:1370 (1997).

To describe the instant invention in more details, several non-limiting illustrative examples are given below.

EXAMPLES

EXAMPLE 1. IVIG WITH INCREASED SIALIC ACID CONTENT EXHIBITS DECREASED CYTOTOXICITY

To determine if specific glycoforms of IgG are involved in modulating the effector functions of antibodies the role of specific, Asn^{297} - linked carbohydrates in mediating the cytotoxicity of defined IgG monoclonal antibodies was explored. The anti-platelet antibodies, derived from the 6A6 hybridoma, expressed as either an IgG1, 2a or 2b switch variant in 293 cells as previously described (6), were analyzed by mass spectroscopy to determine their specific carbohydrate composition and structure. These antibodies contain minimal sialic acid residues. Enrichment of the sialic acid containing species by *Sambucus nigra* lectin affinity chromatography yielded antibodies enriched 60-80 fold in sialic acid content. Comparison of the ability of sialylated and asialylated 6A6-IgG1 and 2b antibodies to mediate platelet clearance revealed an inverse correlation between sialylation and in vivo activity. Sialylation of 6A6 IgG antibodies resulted in a 40-80% reduction in biological activity.

To determine the mechanism of this reduction in activity surface plasmon resonance binding was performed on these antibodies for each of the mouse FcYRs and to its cognate antigen.

Surface plasmon resonance analysis was performed as described in Nimmerjahn and Ravetch, *Science* 310, 1510 (2005). Briefly, 6A6 antibody variants containing high or low levels of sialic acid residues in their sugar side chains were immobilized on the surface of CM5 sensor chips. Soluble Fcy-receptors were injected at different concentrations through flow cells at room temperature in HBS-EP running buffer (10mM Hepes, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, and 0.005% surfactant P20) at a flow rate of 30 uI/min. Soluble Fc-receptors were injected for 3 minutes and dissociation of bound molecules was observed for 7 minutes. Background binding to control flow cells was subtracted automatically. Control experiments were performed to exclude mass transport limitations. Affinity constants were derived from sensorgram data using simultaneous fitting to the association and dissociation phases and global fitting to all curves in the set. A 1:1 Langmuir binding model closely fitted the observed sensorgram data and was used in all experiments.

A 5-10 fold reduction in binding affinity was observed for the sialylated forms of these antibodies to their respective activating FcyRs as compared to their asialylated counterparts, while no differences in binding affinity for the antigen were observed. Since IgG2b binds with a higher affinity to its activation receptor, FcyRIV, when compared to IgG1 binding to its activation receptor FcyRIII, the effect of sialylation was to generate a binding affinity for IgG2b for its activation receptor FcyRIV that was comparable to that of asialylated IgG1 binding to its activation receptor FcYRlII. This effect of this quantitative difference in activation receptor binding resulted in sialylated IgG2b displaying an in vivo activity comparable

to that of asialylated IgG1. Similarly, sialylation of IgG1 reduces its already low binding affinity for its activation receptor FcyRlll by a factor of 7 thereby generating a physiologically inactive antibody. Thus, sialylation of the Asn^{297} linked glycan structure of IgG resulted in reduced binding affinities to the subclass-restricted activation FcyRs and thus reduced their in vivo cytotoxicity.

To determine the generality of the observation that sialylation of the N-linked glycan of IgG was involved in modulating its in vivo inflammatory activity, we next examined the role of N-linked glycans on the anti-inflammatory activity of IVIG. This purified IgG fraction obtained from the pooled serum of 5-10,000 donors, when administered intravenously at high doses (1-2 g/kg), is a widely used therapeutic for the treatment of inflammatory diseases. Dwyer, *N. Engl. J. Med.* 326, 107 (1992). This anti-inflammatory activity is a property of the Fc fragment and is protective in murine models of ITP, RA and nephrotoxic nephritis. Imbach *et al.*, *Lancet* 1, 1228 (1981), Samuelsson *et al.*, *Science* 291, 484 (2001), Bruhns *et al.*, *Immunity* 18, 573 (2003), Kaneko *et al.*, *J. Exp. Med.* 203(3):789-97 (2006).

A common mechanism for this anti-inflammatory activity was proposed involving the induction of surface expression of the inhibitory FcyRIIB molecule on effector macrophages, thereby raising the threshold required for cytotoxic IgG antibodies or immune complexes to induce effector cell responses by activation FcyR triggering. Nimmerjahn and Ravetch, *Immunity* 24, 19 (2006).

EXAMPLE 2. ASIALYLATION OF IVIG DECREASES THE ANTI-INFLAMMATORY EFFECT OF IVIG IN MOUSE ARTHRITIS MODEL

Mice

C57BL/6 and NOD mice were purchased from the Jackson Laboratory (Bar Harbor, ME). FcγRIIB$^{-/-}$ mice were generated in the inventors' laboratory and backcrossed for 12 generations to the C57BL/6 background. KRN TCR transgenic mice on a C57BU6 background (K/B) were gifts from D. Mathis and C. Benoist (Harvard Medical School, Boston, MA) and were bred to NOD mice to generate K/BxN mice. Female mice at 6-10 weeks of age were used for all experiments and maintained at the Rockefeller University animal facility.

Serum was prepared as described previously (Bruhns, et al., *Immunity* 18, 573 (2003)). Briefly, serum is separated from blood collected from the K/BxN mice (6-12 weeks old). Several weeks of serum collection were pooled together and frozen in aliquots to be used in all the experiments described here. One intravenous injection of 1.5X diluted K/BxN serum (4µl of pooled K/BxN serum per gram of mouse) induced arthritis. Arthritis was scored by clinical examination. Indices of all four paws are added: 0 [unaffected], 1 [swelling of one joint], 2 [swelling of more than one joint], and 3 [severe swelling of the entire paw]. IVIG is injected 1 hr before K/BxN serum injection. Some mice received 5µg of platelet depleting 6A6-IgG2b antibody, and platelet counts were determined at 0, 4, and 24 hours post treatment using an Advia 120 haematology system (Bayer). All experiments were done in compliance with federal laws and institutional guidelines and have been approved by the Rockefeller University (New York, NY).

Antibodies and soluble Fc receptors

6A6 antibody switch variants were produced by transient transfection of 293T cells followed by purification via protein G as described. Nimmerjahn and Ravetch, *Science* 310, 1510 (2005). Sialic acid rich antibody variants were isolated from these antibody preparations by lectin affinity chromatography with Sambucus nigra agglutinin (SNA) agarose (Vector Laboratories, Burlingame, CA). Enrichment for sialic acid content was verified by lectin blotting (see below). Human intravenous immune globulin (IVIG, 5% in 10% maltose, chromatography purified) was purchased from Octapharma (Hemdon, VA). Digestion of human IVIG was performed as described. Kaneko Y. et al., *Exp. Med.* 203(3):789-97 (2006). Briefly, IVIG was digested by 0.5 mg/ml papain for 1 hr at 37 °C, and stopped by the addition of 2.5 mg/ml iodoasetamide. Fab and Fc resulting fragments were separated from non-digested IVIG on a HiPrep 26/60 S-200HR column (GE Healthcare, Piscataway, NJ), followed by purification of Fc and Fab fragments with a Protein G column (GE Healthcare) and a Protein L column (Pierce, Rockford, IL). Fragment purity was checked by immunoblotting using anti-human IgG Fab or Fc-specific antibodies. (Jackson ImmunoResearch, West Grove, PA). Purity was judged to be greater than 99%. The F4/80 antibody was from Serotec (Oxford, UK). The Ly 17.2 antibody was from Caltag (Burlingame, CA). Sheep anti-glomerular basement membrane (GBM) antiserum (nephrotoxic serum, NTS) was a gift from M. P. Madaio (University of Pennsylvania, Philadelphia, PA). Soluble Fc receptors containing a C-terminal hexa-hisitidine tag were generated by transient transfection of 293T cells and purified from cell culture supernatants with Ni-NTA agarose as suggested by the manufacturer (Qiagen).

IVIG was treated with neuraminidase and the composition and structure of the resulting preparation was analyzed by mass spectroscopy. No detectable sialic acid containing glycans remained after neuraminidase treatment. These IgG preparations were then tested for their ability to protect mice from joint inflammation induced by passive transfer of KxN serum, an IgG 1 immune complex-mediated inflammatory disease model. De-sialylation with neuraminidase abrogated the protective effect of the IVIG preparation in the KXN serum induced arthritis model. This loss of activity was not the result of reduced serum half-life of the asialylated IgG preparations or the result of changes to the monomeric composition or structural integrity of the IgG. Removal of all glycans with PNGase had a similar effect and abrogated the protective effect of IVIG *in vivo*.

EXAMPLE 3. IVIG FRACTION WITH ENRICHED SIALIC ACID CONTENT DECREASES INFLAMMATION IN MOUSE ARTHRITIS MODEL

Preparation of IVIG with an increased content of sialic acid

Since sialic acid appeared to be required for the anti-inflammatory activity of IVIG, the basis for the high dose requirement (1 g/kg) for this anti-inflammatory activity could be the limiting concentration of sialylated IgG in the total IVIG preparation. The IVIG was fractionated on an SNA-lectin affinity column to obtain IgG molecules enriched for sialic acid modified glycan structures.

These sialic acid enriched fractions were tested for protective effects in the KxN serum transfer arthritis model as compared to unfractionated IVIG. A 10 fold enhancement in protection was observed for the SNA-binding fraction, such that equivalent protection was obtained at 0.1 g/kg of SNA-enriched IVIG as compared to 1 g/kg of unfractionated IVIG.

The serum half-life and IgG subclass distribution of the SNA enriched fraction was equivalent to that of unfractionated IVIG. The effect of sialylation was specific to IgG; sialylated N-linked glycoproteins such as fetuin or transferrin with similar bi-antennary, complex carbohydrate structures had no statistically significant anti-inflammatory activity at equivalent molar concentrations of IgG. Finally, the mechanism of protection of the sialylated IVIG preparation was similar to unfractionated IVIG in that it was dependent on FcγRIIB expression and resulted in the increased expression of this inhibitory receptor on effector macrophages.

EXAMPLE 4. THE INCREASED ANTI-INFLAMMATORY RESPONSE OF IVIG WITH INCREASED SIALIC ACID CONTENT IS MEDIATED BY SIALYLATION OF THE N-LINKED GLYCAN ON THE FC DOMAIN

Since the polyclonal IgG in IVIG may also contain O and N linked glycans on the light chains or heavy chain variable domains that can be sialylated, we confirmed that the increase in anti-inflammatory activity of the SNA-enriched IgG preparation resulted from increased sialylation of the N-linked glycosylation site on the Fc. Fc fragments were generated from unfractionated and SNA fractionated IVIG and tested for their in vivo activity. As observed for intact IgG, SNA-purified Fc fragments were enhanced for their protective effect in vivo when compared to Fc fragments generated from unfractionated IVIG. In contrast, Fab fragments displayed no anti-inflammatory activity in this in vivo assay. Thus, the high dose requirement for the anti-inflammatory activity of IVIG can be attributed to the minor contributions of sialylated IgG present in the total preparation. Enrichment of these fractions by sialic acid

binding lectin chromatography consequently increased the anti-inflammatory activity.

These results using passive immunization of IgG antibodies indicated that the ability of IgG to switch from a pro-inflammatory to an anti-inflammatory species is influenced by the degree of sialylation of the N-linked glycan on the Fc domain.

EXAMPLE 5. INCREASE OF ANTI-INFLAMMATORY ACTIVITY, MEDIATED BY SIALYLATION OF IgG, OCCURS DURING AN ACTIVE IMMUNE RESPONSE

Murine model for Goodpasture's Disease

In this model, mice are first sensitized with sheep IgG together with adjuvant and four days later injected with a sheep anti-mouse glomerular basement membrane preparation (nephrotoxic serum, NTS). Briefly, mice were pre-immunized intraperitoneally with 200 μg of sheep IgG (Serotec) in CFA, followed by intravenous injection of 2.5 μl of NTS serum per gram of body weight four days later. Blood was collected from non-treated control mice four days after the anti-GBM anti-serum injection, and serum IgG was purified by Protein G (GE Healthcare, Princeton, NJ) and sepharose-bound sheep IgG column, generated by covalently coupling sheep IgG on NHS-activated sepharose column (GE Healthcare, Princeton, NJ), affinity chromatography.

Pre-sensitization followed by treatment with NTS induces mouse IgG2b anti-sheep IgG antibodies (NTN immunized). Kaneko Y. et al., *Exp. Med.*, 203:789 (2006). Mouse IgG2b antibodies are deposited in the glomerulus together with the NTS antibodies and result in an acute and fulminant inflammatory response by the IgG2b mediated activation of FcγRIV on infiltrating macrophages. In the absence of pre-sensitization inflammation is not observed,

indicating that the mouse IgG2b anti-sheep IgG antibodies are the mediators of the inflammatory response.

To determine if active immunization resulting in pro-inflammatory IgG is associated with a change in sialylation, serum IgG and IgM from preimmune and NTS immunized mice were characterized for sialic acid content by SNA lectin binding. Total IgG sialylation was reduced on average by 40% in immunized mice as compared to the unimmunized controls. The effect was specific for IgG; sialylation of IgM was equivalent pre and post immunization. This difference in sialylation was more pronounced when the sheep specific IgG fraction from mouse serum was analyzed, showing a 50-60% reduction in sialylation compared to preimmune IgG.

These results were confirmed by MALDI-TOF-MS analysis. Monosaccharide composition analysis was performed by UCSD Glycotechnology Core Resource (San Diego, CA). Glycoprotein samples were denatured with SDS and 2-mercaptoethanol, and digested with PNGase F. The released mixed N-glycans were purified by reversed-phase HPLC and solid-phase extraction, and then exposed hydroxyl groups of the N-glycans were methylated. The resulting derivatized saccharides were purified again by reversed-phase HPLC and subject to MALDI-TOF-MS.

The analysis of the pre and post immunization IgGs confirmed that the changes in the N-glycan structure were specific to the terminal sialic acids moieties. The mouse IgG2b anti-sheep antibodies that were deposited in the glomeruli, previously shown to be responsible for engagement of the FcγRIV bearing, infiltrating macrophages displayed reduced sialic acid content as compared to the pre-immunized controls.

EXAMPLE 6. ANALYSIS OF LINKAGES BETWEEN SIALIC ACID AND GALACTOSE IN IVIG

Sequential Maldi-Tof analysis of SNA^{+} (*Sambuccus Nigra* Agglutinin) IVIG Fc linkages was performed to determine the structure of the sialylated IgG Fc fraction that was protective in the ITP, RA and nephrotoxic nephritis models described above. Glycan peaks generated in Maldi-TOF were isolated, further fractionated, and reanalyzed until galactose-sialic acid structures were obtained. The footprint histogram of the enriched galactose-sialic acid structures with in vivo anti-inflammatory activity (Figure 1A) were compared to histograms from sialic acid linkage standards, α2-3 sialyllactose (Figure 1B) and α2-6 sialyllactose (Figure 1C). The signature peaks of the standards are identified by arrows, shown by arrows for α2-3 (Figure 1B) or α2-6 (Figures 1A and 1C), respectively, and compared to the peaks obtained from the sample.

EXAMPLE 7. ENRICHMENT OF IVIG FC FRAGMENTS IN α2,6 LINKAGES BY IN VITRO GLYCOSYLATION IMPROVES ANTI-INFLAMMATORY PROPERTIES OF IVIG.

As shown in Figure 2A, glycan Maldi-Tof MS analysis of IVIG Fc fragments showed structures ending in no galactose (peak G0), one galactose (peak G1), two galactose (peak G2), or in sialic acid (indicated by a bracket entitled "Terminal sialic acid"). To determine the in vivo activity of 2,3 or 2,6 sialylated IgG Fc, samples were treated with sialidase, followed by galactose transferase to convert the G0 (no galactose) and G1 (single___galactose) to G2 (fully galactosylated) to increase potential sialylation sites. As shown in Figure 2B hypergalactosylation was verified by comparing relative band intensity ratios of terminal galactose as measured by ECL and coomassie loading controls.

In vitro sialylation was performed (Figure 2C) using either α 2-6 sialyltransferase ("ST6Gal") or α 2-3 sialyltransferase ("ST3Gal") and confirmed by lectin blotting for α 2-6 linkages with SNA (top) or α2-3 linkages with ECL (middle) and coomassie (bottom). To evaluate the ability of in vitro sialylated Fc to inhibit inflammation (Figure 2D) mice received either 0.66 mg of α 2-6 sialylated Fcs (black triangles) or 0.66 mg α 2-3 sialylated Fcs (red triangles). 1 hour later, 0.2 ml of K/BxN sera was administered, and the swelling of footpads (clinical score) was monitored over the next seven days. Anti-inflammatory activity was observed for the 2,6 sialylated IgG Fc fragments but not for the 2,3 sialylated molecules. These results are consistent with the data shown above and indicate that a preferential linkage of 2,6 sialic acid-galactose is involved in the anti-inflammatory activity of sialylated IgG.

EXAMPLE 8. REMOVAL OF α 2-6 BUT NOT 2,3 SIALIC ACID LINKAGES ABROGATES THE IMMUNOSUPPRESSIVE PROPERTIES OF IVIG

IVIG was treated with linkage specific sialidases (SAs), and the digestion verified by lectin blotting (Figure 3A). The top panel shows positive *Sambucus nigra* lectin (SNA) staining for α 2-6 linkages in IVIG (left lane), and α 2-3 SA tx IVIG (center lane), but not in α 2-3,6 SA tx IVIG (right lane). The middle panel is a dot blot for α2-3 sialic acid linkages (MAL I), displaying positive staining for the fetuin positive control only; 100 µg protein are loaded per dot. The bottom panel shows coomassie loading control. 10 µg/lane are shown in the blot and gel. To examine the effect of specific removal of sialic acid moieties, mice were given 1g/kg of IVIG preparations prior to 200 µl of K/BxN sera. As shown in Figure 3B, footpad swelling was observed in mice

administered K/BxN sera (white circles) over the course of a week, as measured by clinical scoring. IVIG treated mice showed minimal swelling (black triangles), as did mice treated with α2-3 SA tx IVIG (white triangles), while mice receiving α2-3,6 SA tx IVIG (squares) were not protected from footpad swelling.

EXAMPLE 9. REDUCED CYTOTOXICITY DOES NOT DEPEND ON THE NATURE OF LINKAGE BETWEEN SIALIC ACID AND GALACTOSE.

The inventors have previously demonstrated that sialylation of the N-linked glycan associated with the Fc domain of IgG resulted in reduced FcR binding, leading to a reduction in the A/I ratio (Kaneko, et al., *Science* 313, 670 (2006)), a value derived from the affinity constants for an IgG Fc binding to individual activating (A) or inhibitory (I) IgG Fc receptors. This ratio has been shown to be predictive of the *in vivo* cytotoxicity for a specific IgG Fc (F. Nimmerjahn, J. V. Ravetch, *Science* 310, 1510 (2005)). Fc sialylation thus reduced the cytotoxicity of IgG antibodies in the induced thrombocytopenia model as well as in *in vitro* models of ADCC (Kaneko, et al., *Science* 313, 670 (2006), Scallon, et al., *Mol. Immunol* 44, 1524 (2007)). The inventors, therefore, set out to determine if this reduction in FcR binding and cytotoxicity was influenced by the sialic acid-galactose linkage. A monoclonal anti-platelet IgG2b antibody previously shown to lead to platelet consumption was sialylated *in vitro* as described above and tested for *in vivo* activity. Both terminal 2,3 and 2,6 *in vitro* sialylated IgG Fc reduced the cytotoxicity of this anti-platelet antibody, 6A6-IgG2b, in an *in vivo* model of thrombocytopenia (Figure 4), consistent with previous studies (Kaneko, et al., *Science* 313, 670 (2006), Scallon, et al., *Mol. Immunol* 44, 1524 (2007)). Thus, the effect of

Fc sialylation on the cytotoxicity of an IgG antibody is not dependent on the specificity of the linkage to the penultimate galactose.

In contrast, the anti-inflammatory activity of the sialylated IgG Fc fragment (a property which the inventors have shown to be independent of the canonical IgG Fc receptors(F. Nimmerjahn, J. V. Ravetch, *Science* 310, 1510 (2005); F. Nimmerjahn, J. V. Ravetch, *J Exp Med* 204, 11 (2007)) displayed a clear preference for the 2,6 sialic acid-galactose linkage, as seen in Figure 3B.

These results further support the inventors' previous observations that the anti-inflammatory property of IVIG is mediated through a distinct pathway that does not involve binding to canonical FcγRs, which is in sharp contrast to previously accepted models (Park-Min *et al.*, *Immunity* **26**, 67 (2007); Siragam *et al.*, *Nat Med* 12, 688 (2006)).

EXAMPLE 10: *IN VIVO* ANTI-INFLAMMATORY ACTIVITY OF THE 2,6 SIALYLATED IGG FC IS SOLELY A PROPERTY OF THE IGG FC GLYCAN

To fully demonstrate that the *in vivo* anti-inflammatory activity of the 2,6 sialylated IgG Fc is solely a property of the IgG Fc glycan and not the result of other components that might be found in the heterogeneous, IVIG Fc preparations, the anti-inflammatory activity of sialylated IVIG Fc was recapitulated using a homogeneous, recombinant human IgG1 Fc substrate (rFc), derived from a cDNA (SEQ ID NO. 1) expressed in 293T cells. The purified recombinant human IgG1 Fc fragment was glycan engineered *in vitro*, as described above, by β1,4 galactosylation, followed by 2,6 sialylation (Figure 5A). The preparation was purified and characterized by lectin blotting and MALDI-TOF analysis (Figure 5A) before *in vivo* analysis. Glycosylation was confirmed by lectin blotting for terminal galactose with ECL

(top panel), α2,6 sialic acid with SNA (middle panel), and coomassie loading controls are shown in the bottom panel.

Mice were administered IVIG, SNA+ IVIG Fcs, or sialylated rFc (2,6ST rFc) 1 hour prior to K/BxN sera, and footpad swelling was monitored over the next several days. As seen in Figure 5B, the 2,6 sialylated recombinant human IgG1 Fc fragment demonstrated comparable anti-inflammatory activity to that obtained with either IVIG-derived sialic-enriched Fc fragments (SNA+ IVIG Fc) or *in vitro* 2,6 sialylated IVIG-derived Fc fragments (2,6ST IVIG Fc). Mean and standard deviation of clinical scores of 4-5 mice per group are plotted; *denotes $p<0.05$ as determined by Kruskal-Wallis Anova followed by Dunn's post hoc.

Each of these preparations was active at 30mg/kg, as compared to the 1,000-2,000 mg/kg required for native IVIG (Table 1).

Table 1. Different dosages of Fc fragment containing preparations result in the same extent of inflammation suppression in arthritis model.

IVIG prep	IVIG	IVIG Fc	SNA$^+$ IVIG	SNA$^+$ IVIG Fc	2,3ST IVIG Fc	2,6ST IVIG Fc	2,6ST rFc
Dose	1g/kg	0.33g/kg	0.1g/kg	0.033g/kg	0.033g/kg	0.033g/kg	0.033g/kg
Amount/ mouse injection	20mg	6.66mg	2mg	0.66mg	0.66mg	0.66mg	0.66mg

All patent and non-patent publications cited in this disclosure are incorporated herein in to the extent as if each of those patent and non-patent publications was incorporated herein by reference in its entirety. Further, even though the invention herein has been described with reference to particular examples and embodiments, it is to be understood that these examples and embodiments are merely illustrative of the principles and applications of the

present invention. It is therefore to be understood that numerous modifications may be made to the illustrative embodiments and that other arrangements may be devised without departing from the spirit and scope of the present invention as defined by the following claims.

WHAT IS CLAIMED IS:

1. An isolated polypeptide containing at least one IgG Fc region, having altered properties compared to an unpurified antibody preparation, wherein sialylation of the isolated polypeptide is higher than the sialylation of the unpurified antibody preparation.

2. The isolated polypeptide of claim 1, wherein said at least one IgG Fc region is glycosylated with at least one galactose moiety connected to a respective terminal sialic acid moiety by a α 2,6 linkage, and wherein said polypeptide having a higher anti-inflammatory activity as compared to an unpurified antibody preparation.

3. The isolated polypeptide of claim 1, wherein said at least one IgG Fc region is glycosylated with at least one galactose moiety connected to a respective terminal sialic acid moiety by a α 2,6 linkage, and wherein said polypeptide having a reduced binding to an Fc activating receptor selected from the group consisting of FcγRIIA, FcγRIIC and FcγRIIIA, as compared to an unpurified antibody preparation.

4. The isolated polypeptide of claim 1 comprising a human IgG1, IgG2, IgG3 or IgG4 Fc region, said polypeptide having a higher content of the at least one galactose moiety connected to the respective terminal sialic acid moiety by a α 2,6 linkage as compared to an unpurified antibody.

5. The isolated polypeptide of claim 1, derived either from a naturally occurring antibody source or a recombinant antibody source.

6. The isolated polypeptide of claim 1, wherein said unmodified antibody comprises IVIG.

7. The isolated polypeptide of claim 1 produced from a recombinant source and lacking Fab region, wherein said at least one IgG Fc region is glycolylated with two galactose moieties.

8. The isolated polypeptide of claim 1 encoded by a nucleic acid sequence comprising SEQ ID NO: 1.

9. The isolated polypeptide of claim 1, derived from a cell line having an enhanced activity of creating α2,6 linkages between at least one galactose moiety and a respective terminal sialic acid in a protein's polysaccharide chain.

10. The isolated polypeptide of claim 1, modified by treatment with α2-6 sialyltransferase.

11. A method of modulating properties of a polypeptide comprising an Fc region comprising altering the sialylation of the polysaccharide chain of the Fc region.

12. A method of claim 11, wherein said properties comprise a higher anti-inflammatory activity than an unpurified antibody.

13. The method of claim 11, wherein the step of altering sialylation comprises:

providing an unpurified source of the polypeptide containing at least one Fc region, said unpurified source of the polypeptide containing at least one Fc region comprising a plurality of the polypeptides containing at least one Fc region having a polysaccharide chain comprising a terminal sialic acid connected to a galactose moiety through a α 2,6 linkage, and a plurality of the polypeptides containing at least one Fc region lacking a polysaccharide chain comprising a terminal sialic acid connected to a galactose moiety through the α 2,6 linkage; and

increasing the ratio of the plurality of the polypeptides containing at least one Fc region having the polysaccharide chain comprising the terminal sialic acid connected to the galactose moiety through the α 2,6 linkage to the plurality of the polypeptide containing at least one Fc region lacking the polysaccharide chain comprising the terminal sialic acid connected to the galactose moiety through the α 2,6 linkage.

14. The method of claim 11, wherein the unpurified source of the polypeptide containing at least one Fc region is provided from expressing a vector comprising a nucleic acid sequence in an expression system, wherein said nucleic acid sequence is translated into an IgG antibody.

15. The method of claim 11, wherein the step of increasing the ratio of the plurality of the polypeptides containing at least one Fc region having the polysaccharide chain comprising the terminal sialic acid connected to the galactose moiety through the α 2,6 linkage to the plurality of the polypeptide containing at least one Fc region lacking the polysaccharide chain comprising the terminal sialic acid

connected to the galactose moiety through the α 2,6 linkage is achieved through a removal of the polypeptides containing at least one Fc region lacking the polysaccharide chain comprising the terminal sialic acid connected to the galactose moiety through the α 2,6 linkage.

16. The method of claim 15 wherein said removal is achieved by a method selected from the group consisting of HPLC, lectin affinity chromatography, high pH anion exchange chromatography, and any combination thereof.

17. The method of claim 16, wherein the lectin affinity chromatography is performed using a lectin having a lower affinity to α2,6 linkages than to α2,3 linkages between the galactose moiety and the terminal sialic acid.

18. The method of claim 15, wherein the step of increasing the ratio of the plurality of the polypeptides containing at least one Fc region having the polysaccharide chain comprising the terminal sialic acid connected to the galactose moiety through the α 2,6 linkage to the plurality of the polypeptide containing at least one Fc region lacking the polysaccharide chain comprising the terminal sialic acid connected to the galactose moiety through the α 2,6 linkage is achieved through an enrichment of said unpurified source of the polypeptide containing at least one Fc region having the polysaccharide chain comprising the terminal sialic acid connected to the galactose moiety through the α 2,6 linkage.

19. The method of claim 18 wherein said enrichment is achieved by a method selected from the group consisting of

HPLC, lectin affinity chromatography, high pH anion exchange chromatography, and any combination thereof.

20. The method of claim 19, wherein the lectin affinity chromatography is performed using a lectin having a higher affinity to α2,6 linkages than to α2,3 linkages between the galactose moiety and the terminal sialic acid.

21. The method of claim 18, wherein said enrichment is achieved by a chemical reaction with an enzyme creating α 2,6 linkages between the carbohydrate attached to the polypeptide containing least one Fc region and a terminal sialic acid.

22. A method of treating an inflammatory disease selected from the group consisting of arthritis, thrombocytopenia, and nephritis comprising administering to a patient a therapeutically effective dose of the polypeptide of claim 1.

23. A method of treating an inflammatory disease comprising administering to a subject in need thereof a therapeutic composition comprising a plurality of isolated polypeptides, each containing at least one IgG Fc region, wherein

a first portion of the respective Fc regions comprises respective carbohydrate chains having galactose moieties connected to respective terminal sialic acid moieties by 2,6 linkage;

a dose of the therapeutic composition is smaller than a dose of a second composition which comprises a plurality of isolated polypeptides, each containing at least one IgG

Fc region, having a second portion of the respective Fc regions comprising respective carbohydrate chains having galactose moieties connected to respective terminal sialic acid moieties by 2,6 linkage; and either

the first portion is greater than the second portion, whereby the dose of the therapeutic composition and the dose of the second composition suppress inflammation to substantially the same extent, or

the first portion is greater than the second portion, whereby the therapeutic composition suppresses inflammation to substantially a greater extent than an equal dose of the second composition.

24. A composition comprising glycoproteins containing an Fc region wherein the composition has been formulated to contain sialylated glycoproteins in an amount sufficient to achieve an immunosuppressive activity in a mammal.

25. The composition of claim 24, wherein the composition comprises sialylated glycoproteins in an amount of about 5% or more.

26. The composition of claim 24, wherein the composition comprises sialylated glycoproteins in an amount of about 10% or more.

27. The composition of claim 24, wherein the composition comprises sialylated glycoproteins in an amount of about 30% or more.

28. The composition of claim 24, wherein the composition comprises sialylated glycoproteins in an amount of about 5% to about 30%.

29. The composition of claim 24, wherein the sialylated glycoproteins comprise one or more terminal sialic acid residues or analogues thereof.

30. The composition of claim 29, wherein the terminal sialic acid residue(s) is linked to the glycoprotein by an alpha 2,6 linkage.

31. An IVIG derived composition formulated to contain sialylated Fc containing glycoproteins in an amount of about 5% to about 30% and wherein the sialylated glycoproteins comprise one or more terminal sialic acid residues linked to the glycoprotein by an alpha 2,6 linkage.

32. A recombinant Fc glycoprotein, or fragment thereof, comprising at least one terminal sialic acid residue(s), or analogue(s) thereof, linked to the glycoprotein by an alpha 2,6 linkage.

33. A recombinant Fc glycoprotein comprising an N-linked carbohydrate at Asn 297 wherein in the carbohydrate has a biantennary GlnNac2, Man3, GlcNAc2, Gal2 structure having one or more terminal sialic acid residue(s) linked by an alpha 2,6 linkage.

34. An Fc containing glycoprotein of any of the above claims wherein the Fc region is IgG or a subclass thereof.

35. A pharmaceutical preparation comprising the glycoproteins of claim 24.

36. A method of treating an inflammatory disorder in a subject using the pharmaceutical preparation of claim 35.

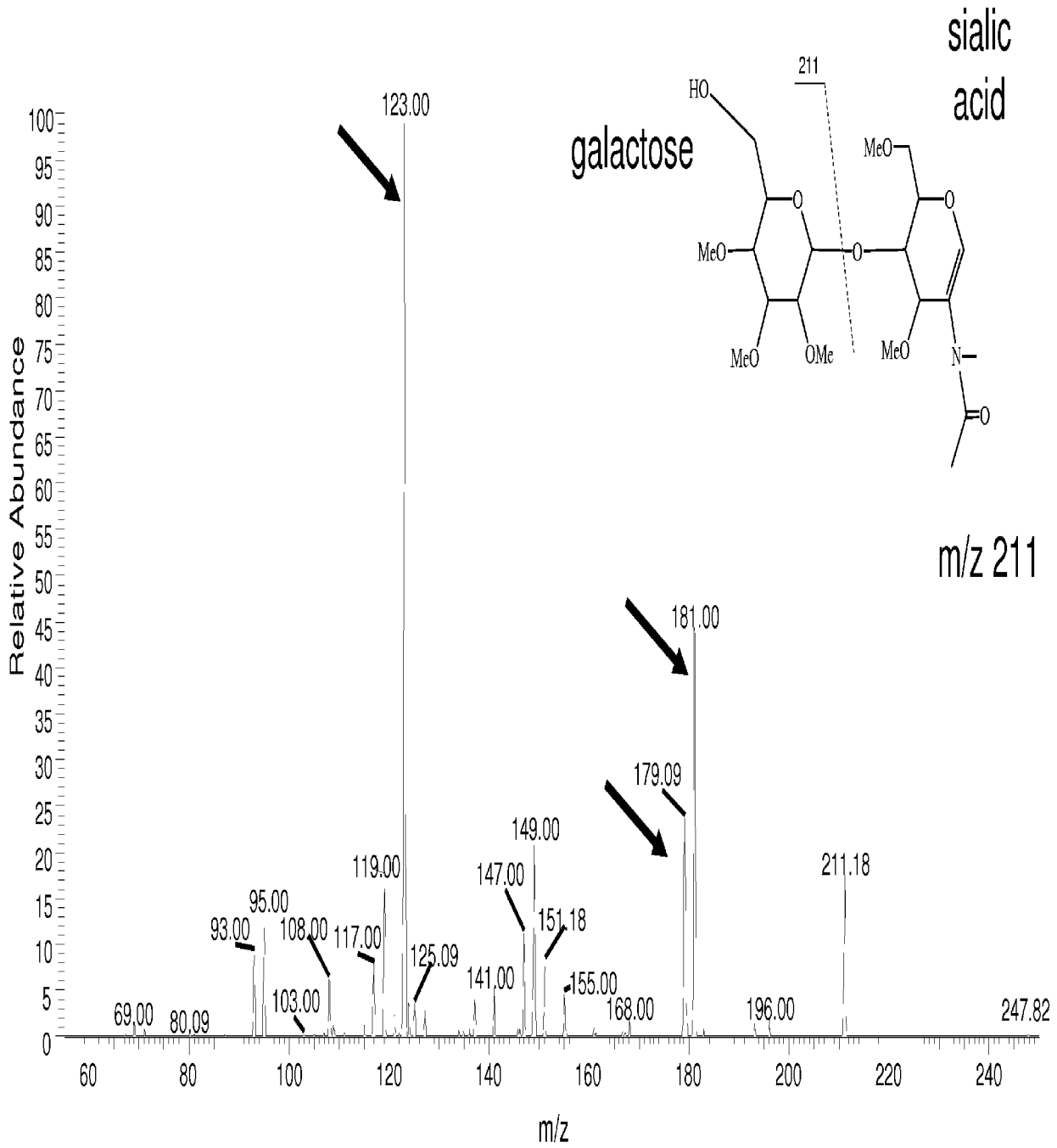

sialic acid
galactose
211
HO
MeO
MeO
MeO
OMe
MeO
N
O
m/z 211
Relative Abundance
m/z
123.00
181.00
179.09
149.00
119.00
147.00
211.18
95.00
93.00
108.00
117.00
151.18
125.09
141.00
155.00
103.00
69.00
80.09
168.00
196.00
247.82

FIG. 1A

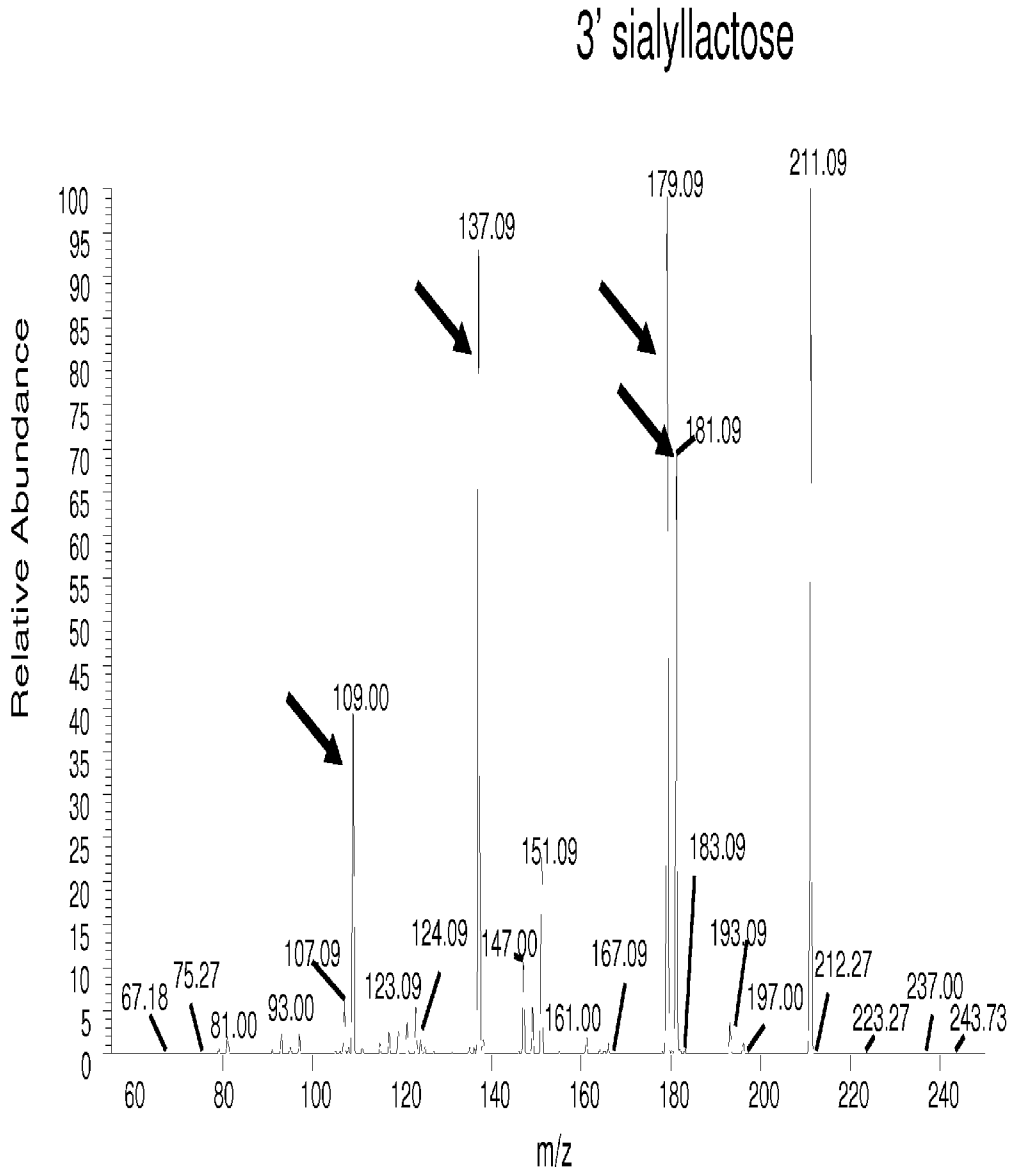

3' sialyllactose
Relative Abundance
m/z
211.09
179.09
137.09
181.09
109.00
151.09
183.09
124.09
147.00
193.09
107.09
167.09
212.27
75.27
123.09
197.00
237.00
67.18
93.00
161.00
223.27
243.73
81.00

FIG. 1B

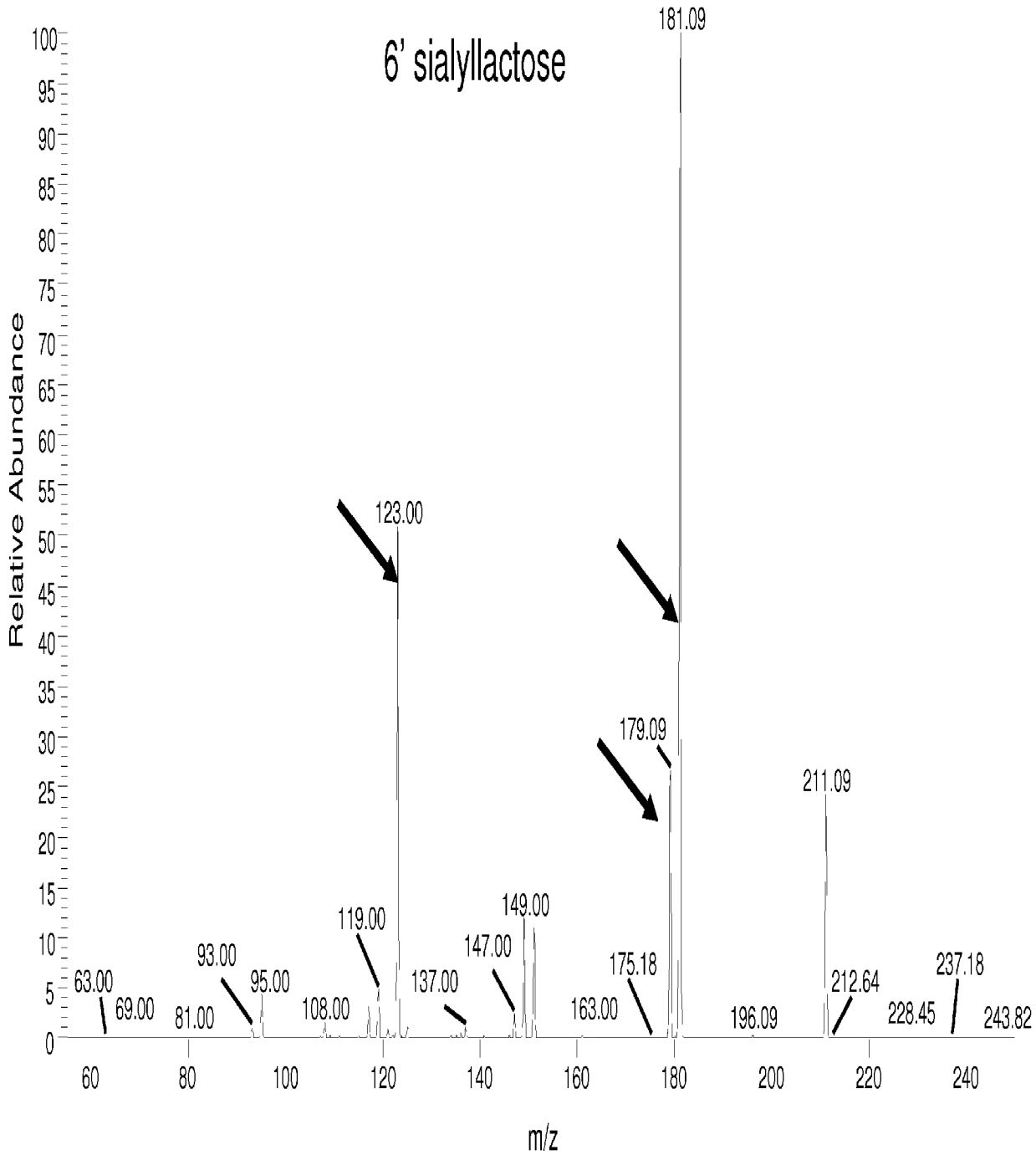
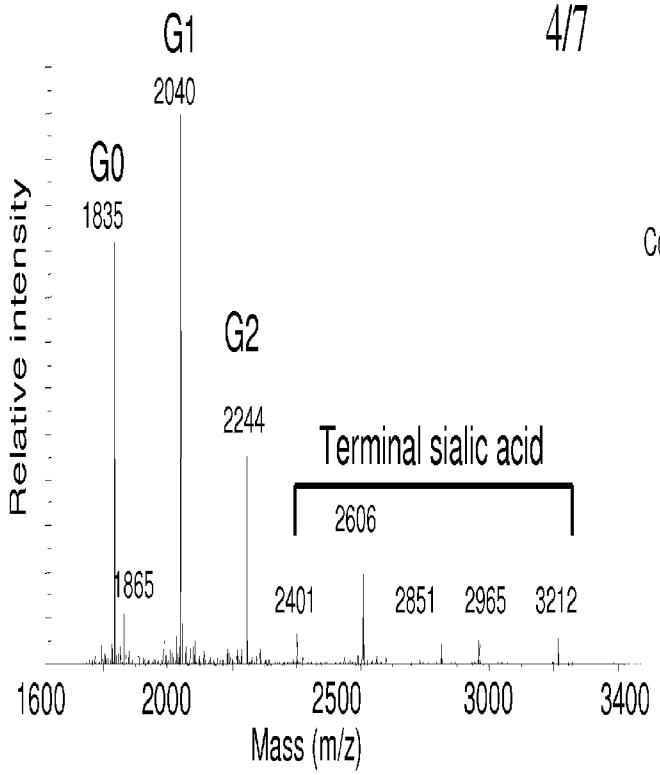
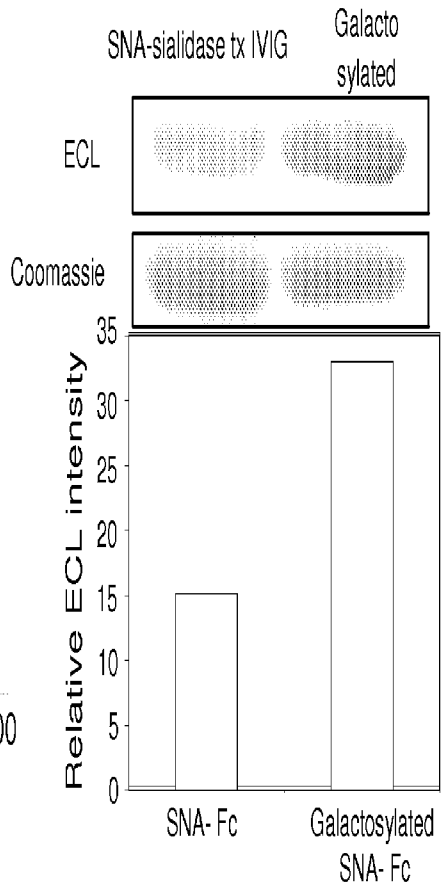
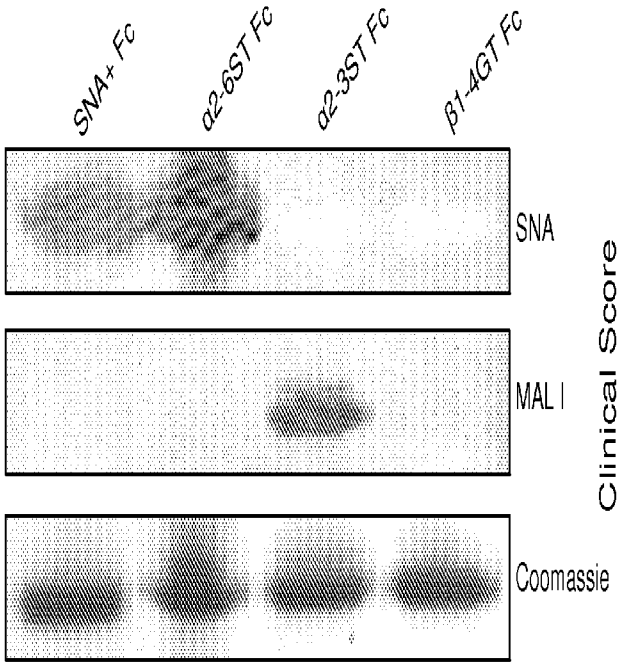

6' sialyllactose
Relative Abundance
m/z
181.09
123.00
179.09
211.09
149.00
119.00
147.00
93.00
175.18
237.18
63.00
95.00
137.00
212.64
69.00
108.00
163.00
228.45
81.00
196.09
243.82
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
60
80
100
120
140
160
180
200
220
240

FIG. 1C


G1
2040
G0
1835
G2
2244
Terminal sialic acid
2606
1865
2401
2851
2965
3212
Relative intensity
1600
2000
2500
3000
3400
Mass (m/z)


SNA-sialidase tx IVIG
Galacto sylated
ECL
Coomassie
Relative ECL intensity
0
5
10
15
20
25
30
35
SNA- Fc
Galactosylated SNA- Fc


SNA+ Fc
α2-6ST Fc
α2-3ST Fc
β1-4GT Fc
SNA
MAL I
Coomassie

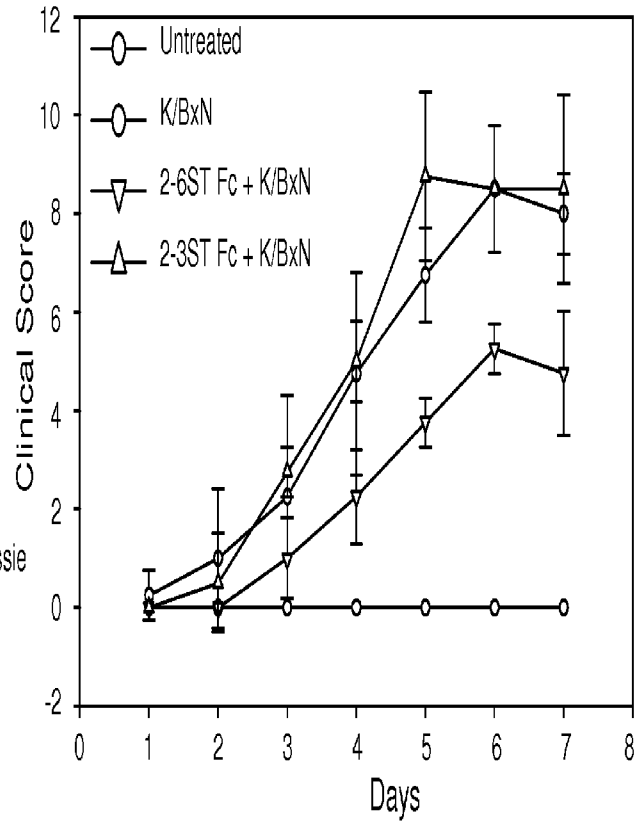

Untreated
K/BxN
2-6ST Fc + K/BxN
2-3ST Fc + K/BxN
Clinical Score
-2
0
2
4
6
8
10
12
0
1
2
3
4
5
6
7
8
Days

FIG. 2

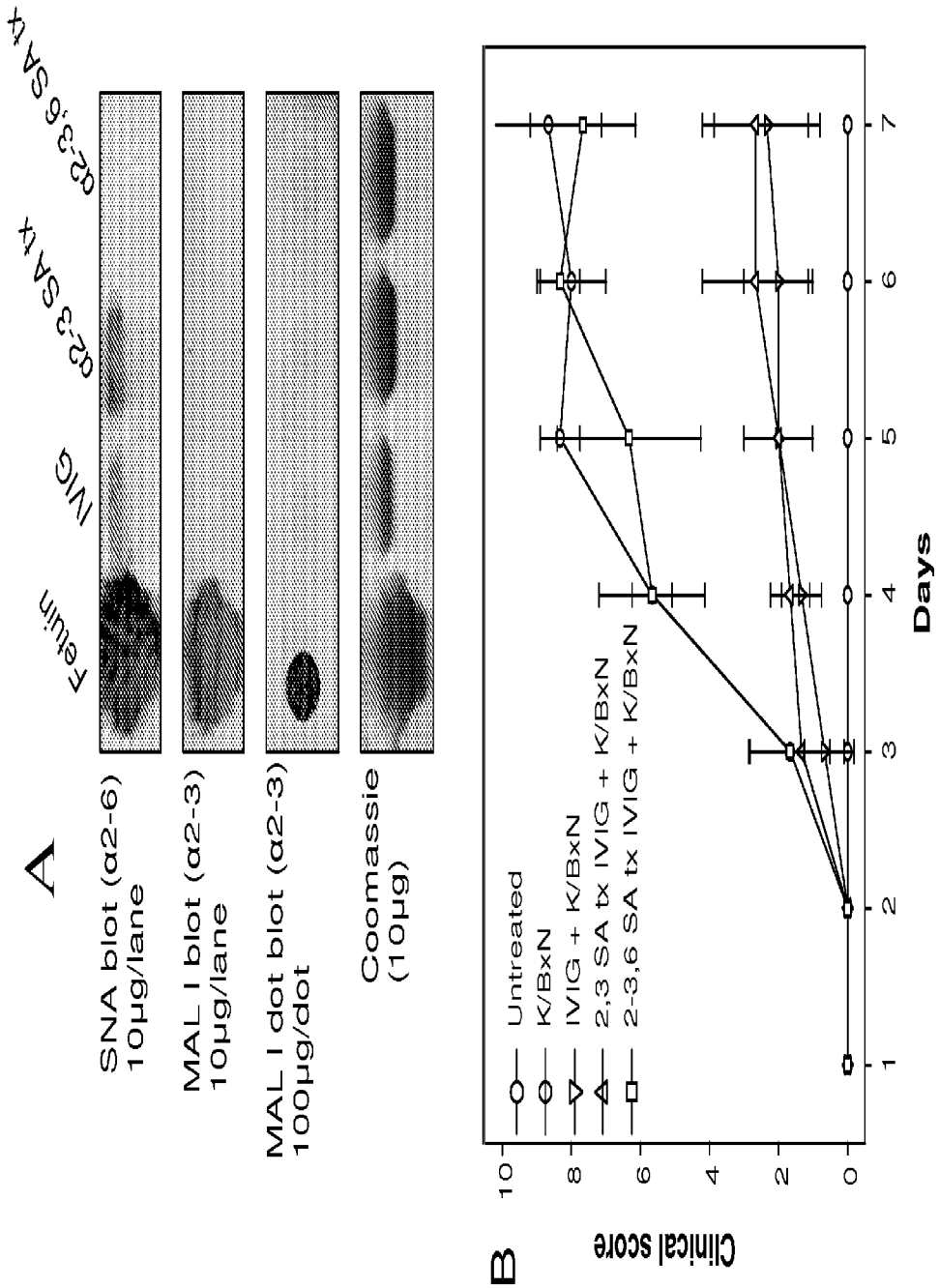

A
Fetuin
IVIG
α2,3SAx
α2,3,6SAx
SNA blot (α2-6)
10μg/lane
MAL I blot (α2-3)
10μg/lane
MAL I dot blot (α2-3)
100μg/dot
Coomassie
(10μg)
B
Clinical score
Days
Untreated
K/BxN
IVIG + K/BxN
2,3 SA tx IVIG + K/BxN
2-3,6 SA tx IVIG + K/BxN

FIG. 3

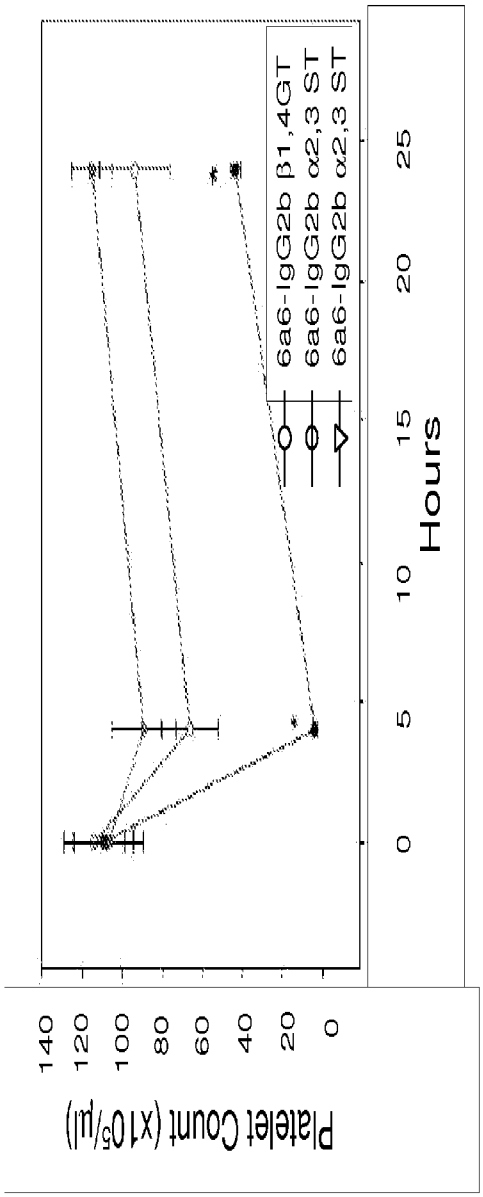

Platelet Count (x10⁵/μl)
140
120
100
80
60
40
20
0
0
5
10
15
20
25
Hours
6a6-IgG2b β1,4GT
6a6-IgG2b α2,3 ST
6a6-IgG2b α2,3 ST

FIG. 4

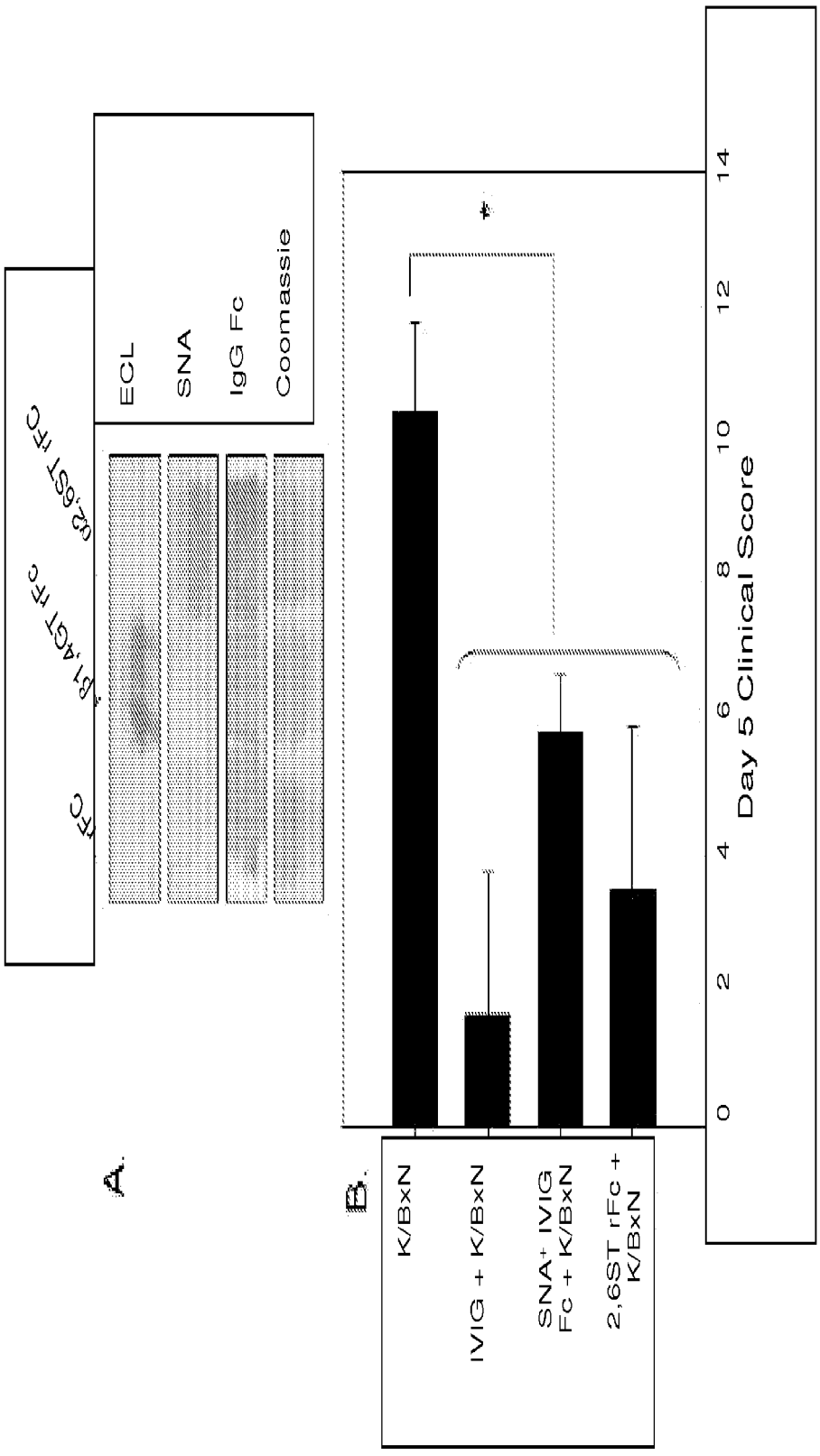

A.
ECL
SNA
IgG Fc
Coomassie
α2,6ST rFc
β1,4GT rFc
CFC
B.
K/BxN
IVIG + K/BxN
SNA+ IVIG Fc + K/BxN
2,6ST rFc + K/BxN
Day 5 Clinical Score
0
2
4
6
8
10
12
14
*

FIG. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 08/86622

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 39/395 (2009.01)
USPC - 424/130.1
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)
USPC- 424/130.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC- 424/133.1, 529, 530, 531, 532, 804; 530/386, 387.1, 388.22; 210/656, 660, 905

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Dialog Web, Google Scholar
Search Terms: Fc, antibody, polypeptide, sialyltransferase, galactose, sial?, alpha 2,6 linkage, fragment crystallizable region, IgG, sialic acid, purify, isolate, inflammation, chromatography

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- A	WO 2007/117505 A2 (RAVETCH et al.), 18 Oct. 2007 (18.10.2007), Fig. 5,pg 4, para 3; pg 8, para 2 to pg 9, para 1; pg 11, para 2; pg 14, para 3; pg 21, para 2; pg 23, para 3 to pg 24, para 1; pg 25, para 2; pg 26, para 2; pg 27, para 1; pg 29, para 2; pg 32, para 2 to pg 33, para 3; pg 35, para 3	1-7, 9-33, 35, 36 ----- 8
A	US 2007/0048740 A1 (ISOGAI et al.), 01 Mar. 2007 (01.03.2007), SEQ ID NO 762	8

☐ Further documents are listed in the continuation of Box C. ☐

* 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
03 Feb. 2009 (03.02.2009)	16 MAR 2009
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 08/86622

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.: 34
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.