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<p>(54) Title: PREPARATION OF GLYCOSYLATED ANTIBODIES</p> <p>(57) Abstract</p> <p>An antibody preparation in which an N-glycosylation site of the Fc domain of the antibody is substituted with a biantennary oligosaccharide, characterised in that at least 20 % of the preparation comprises antibody molecules having an oligosaccharide that contains at least one galactose residue.</p>		

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## PREPARATION OF GLYCOSYLATED ANTIBODIES

The present invention relates to glycosylated antibodies and in particular to antibody preparations.

Secreted polypeptides, including antibodies, of higher organisms are subject to a variety of post-translational modifications involving carbohydrate moieties. One of the most common is glycosylation which is the attachment of oligosaccharides (carbohydrates containing from about two up to about eight simple sugars linked together) to a polypeptide. The oligosaccharide side chains are linked to the backbone of the polypeptide through either N- or O-linkages. N-linkages form when an acetylglucosamine residue (a sugar residue) attaches an oligosaccharide chain to an asparagine residue on the polypeptide backbone via an N-glycosidic linkage. In contrast O-linked oligosaccharides have an O-glycosidic link to serine, threonine, hydroxy-lysine or hydroxy-proline. N-linked oligosaccharides have stable core structures consisting of two N-acetyl glucosamines (GN). One is attached to the asparagine residue on the polypeptide whilst the other is attached to a mannose residue (M) from which two more mannose residues (M) branch to give a biantennary core structure (P. Knight, *Biotechnology*, Volume 7/1, January 1989 : pp35-40).

Extension of the core oligosaccharide chains commonly results from the use of galactose--N-acetylglucosamine (G--GN) sequence repeats, which in turn are substituted with various terminal sugars such as sialic acid (S), fucose (F), galactose (G), N-acetylgalactosamine or N-acetylglucosamine (GN) residues attached either singly or in combination to one or more hydroxyl groups of the extension or core structure. (J.C. Paulson, *Tibs* 14, July 1989, pp272-278).

Glycosylation is controlled by the glycosyltransferase enzymes of the cell such as N-acetyl glucosaminyltransferase, mannosidase, fucosyltransferase, galactosyltransferase and sialyltransferase (C.F. Goochee et al, *Biotechnology*, Vol. 8, May 1990).

Antibodies typically comprise two heavy chains linked together by disulphide bonds and two light chains. Each light chain is linked to a respective heavy chain by disulphide bonds. Each heavy chain has at one end a variable domain followed by a number of constant domains. Each light chain has a variable

domain at one end and a constant domain at its other end. The light chain variable domain is aligned with the variable domain of the heavy chain. The variable domains of each pair of heavy and light chains form the antigen binding site (Fab region) (see, for Example, Figure 1). The light chain constant domain is aligned with the first constant domain of the heavy chain. The constant domains of the light and heavy chains are cross-linked by interchain disulphide bonds (Fc domain) and are responsible for the biological function of the antibody such as complement mediated cell lysis and antibody dependent cellular cytotoxicity (ADCC).

The two globular domains in the Fc portion of the antibody are referred to as the CH2 and CH3 domains. The heavy chains interact closely at the CH3 domain, whilst in the CH2 domain, oligosaccharides are attached to N-glycosylation sites which lie within a cleft between the two heavy chains, resulting in a separation of the two polypeptides. Each heavy chain in the CH2 domain has at least one such N-glycosylation site, normally at asparagine residue Asn297. This Fc glycosylation of the antibodies has been reported to be essential for biological activity, particularly for complement mediated cell lysis and antibody dependent cellular cytotoxicity in IgG and IgM antibodies (Naoyuki Tsuchiya et. al., 1989, *The Journal of Rheumatology*, 16, 3; Leatherbarrow et. al., 1985, *Mol. Immuno.* 22, 407; Duncal et. al., 1988, *Nature*, 332, 738).

Antibodies have an average of 2.8 N-linked oligosaccharides per antibody molecule, two of which are in the Fc region with the remaining 0.8 being in the Fab region (Rademacher et. al., 1983, *Prog. Immun.*, 5, 95). In the Fc region the oligosaccharides comprise mixtures of biantennary structures. (Parekh et. al., 1985 *Nature* 316, 452-457; Rademacher et. al., 1983, *Prog. Immun.*, 5, 95 ; Rademacher et. al., 1983, *Prog. Immun.*, 5, 95).

Numerous authors have attempted to elucidate the role of glycosylation of antibodies. Studies have involved depletion of IgG of its oligosaccharides either enzymatically with glycosidases (Winkelhake et.al., 1980, *J. Biol. Chem.*, 255, 2822; Koide et.al., 1977, *Biochem. Biophys. Res. Comm.*, 75(4), 838) or metabolically by treating IgG secreting cells with tunicamycin (Leatherbarrow et. al., 1985, *Molec. Immuno.*, 22, 407; Nose and Wigzell, 1983, *PNAS.*, 80, 6623; Walker et. al., 1989, *Biochem. J.*, 259, 347). In these studies the oligosaccharides of IgG were found not to influence the assembly or secretion of IgG from various cells lines (Leatherbarrow et. al., 1985, *Molec. Immuno.*, 22,

407; Hickman et. al., 1978, J. Immuno., 121, 990; Leatherbarrow and Dwek, 1983, FEBS lett., 164, 227; Weitzman, 1976, J. Molec. Biol., 102, 237).

Thus deglycosylation of IgG was found not to significantly alter the antigen-binding properties of the immunoglobulin, since deglycosylated IgG secreted from tunicamycin treated hybridoma were found to maintain the same antigen binding affinity as the glycosylated antibody (Nose and Wigzell, 1983, PNAS., 80, 6623; Leatherbarrow and Dwek, 1983, FEBS lett., 164, 227). Also, deglycosylated IgG has consistently been found to retain protein A binding at the CH2-CH3 interface (Deisenhofer, 1981, Biochemistry, 20, 2361). In contrast however, depletion of oligosaccharides significantly reduce ADCC activity by macrophages as well as complement mediated lysis of target cells sensitised with carbohydrate deficient IgG. Such studies have highlighted the importance of glycosylation of IgG for complement lysis and ADCC.

It has now been found that the addition of galactose residue(s) to the N-linked oligosaccharide(s) in the Fc domain of the antibody increases the lytic potency of the antibody, in particular the resulting antibody increases both ADCC and/or complement lysis. The benefit of this can be obtained with antibody preparations in which the relative amount of antibody molecules having an additional galactose residue is increased.

Accordingly, the present invention provides an antibody preparation in which a N-glycosylation site of the Fc domain of the antibody is substituted with a biantennary oligosaccharide, characterised in that at least 20% of the preparation comprises antibody molecules having an oligosaccharide that contains at least one galactose residue. Preferably each antibody molecule has two galactose residues. The galactose residues may or may not be terminal.

An increase in the overall galactose content by increasing monogalactosyl (G1) and/or digalactosyl (G2) of the antibody preparation increases complement lysis. By increasing G2 as opposed to both G1 and G2 an even greater increase (of about 50% or more) in complement lysis is achieved. The increase in complement lysis is particularly pronounced with antibody preparations in which at least 60% of the antibody molecules have an oligosaccharide that contains at least one galactose. Therefore an antibody preparation wherein at least 60% or even 70% comprises antibody molecules having an oligosaccharide that contains at least one galactose is of particular importance. Even more preferred are antibody preparations wherein at least 80% or 90% of

the preparation comprises antibody molecules having an oligosaccharide that contains at least one galactose.

Similarly, an antibody preparation wherein at least 40% or 60%, preferably 70% or 80% and most preferably 90% or 95% comprises antibody molecules having an oligosaccharide that contains two galactose residues is of particular importance.

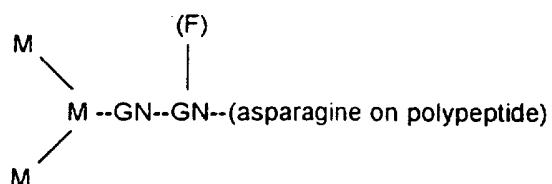
An increase in ADCC activity with the increase of galactose is more dramatic once at least 50% of the antibody preparation comprises antibody molecules having an oligosaccharide containing at least one galactose. An antibody preparation wherein at least 50% or 60%, preferably 70%, 80% or even 90% comprises antibody molecules having an oligosaccharide that contains at least one galactose, preferably two galactose residues (G2).

It is particularly preferred that at least 20% or 40, 70, 80, 90 or even 95% of the antibody preparation of the present invention comprises antibody molecules which have an oligosaccharide that contains at least one terminal sialic acid residue, preferably a single terminal sialic acid residue(S1).

As far as ADCC activity is concerned there is a small but steady increase of activity with overall increase in sialic acid, which is more pronounced with S1. This increase in ADCC activity with an increase in S1 is more pronounced when at least 70% of the antibody molecules have an oligosaccharide containing at least one sialic acid, preferably a single terminal sialic acid residue.

It should be noted that the term N-glycosylation site encompasses any site within the Fc region, comprising an asparagine residue to which an oligosaccharide can be attached. Preferably the asparagine residue is in the CH2 domain of the Fc region of the antibody, and most preferably close to or at the Asn 297 position of the Fc region. The asparagine residue may either be a naturally occurring residue or may also be a residue which has been engineered into the Fc region of the antibody.

The term biantennary oligosaccharide encompasses any structure which has a stable core structure consisting of two N-acetyl glucosamine (GN). One is attached to the asparagine residue on the antibody whilst the other is attached to a mannose residue (M) from which two more mannoses (M) branch to give a biantennary core structure as shown below :



This core is extended by two N-acetyl glucosamines (GN) which in turn may be substituted with one or two galactoses (G), each of the galactoses in turn being optionally substituted with sialic acid.

The present invention encompasses all antibodies including antibody fragments comprising the Fc region, but preferred antibodies are monoclonal antibodies, in particular humanised monoclonal antibodies.

Antibodies of use in the invention may be prepared by any method well known in the art or more particularly as described in GB9022547.5. Purification may be carried out as described in EP-A-91917891.

The present invention also provides a method of preparing an antibody preparation according to the present invention.

Since glycosylation is controlled by glycosyltransferase enzymes such as galactosyltransferase and /or sialyltransferase methods of increasing the number of galactose and sialic acid residues of oligosaccharides may be achieved by boosting levels of the enzymes. This can be done by adding the enzymes to the antibody in vitro or by increasing the endogenous levels of galactosyltransferase and/or sialyltransferase within the cell.

The oligosaccharides may be manipulated in vitro with the aid of galactosyltransferase (from for Example, bovine milk) and/or sialyltransferase (from for Example, rat liver). For the addition of galactose to the oligosaccharides of the antibody the enzyme galactosyltransferase is added to the antibody together with a substrate such as, for Example, uridine - 5' - diphosgalactose in appropriate buffer and incubated.

Similarly, for the addition of sialic acid the enzyme sialyltransferase is added to the antibody together with a substrate such as cytidine - 5' monophospho-N-acetyl neuraminic acid in appropriate buffer and incubated.

High concentrations of the enzymes may be produced by recombinant DNA technology, whereby genes encoding the enzymes galactosyltransferase or sialyltransferase (Behringwerke Artiengesellschaft, Patent. Document No:AU-A-

83760/91) may be manipulated and transfected into cell lines to produce the enzymes either as soluble proteins or fusion proteins. Alternatively, cell lines secreting the antibody may be co-transfected with genes expressing the enzymes sialyltransferase (Behringwerke, Artengellschaft, Patent. Document No: AU-A-83760/91) and/or galactosyltransferase.

This can be carried out in a variety of ways. By way of example, the DNA sequence coding for sialyltransferase or galactosyltransferase is inserted together with a marker gene and then the DNA sequence coding for the glycoprotein to be sialylated (galactosylated) is inserted together with another marker gene into the eukaryotic cell. Alternatively, the DNA sequence for the glycoprotein to be sialylated (galactosylated) is inserted together with a marker gene and then the DNA sequence coding for the sialyltransferase (galactosyltransferase) is inserted with another marker gene into the eukaryotic cell. As a further alternative, The DNA sequence coding for the sialyltransferase (galactosyltransferase) and the DNA sequence coding for the glycoprotein to be sialylated (galactosylated) are inserted at the same time together with a marker gene into the eukaryotic cell.

Cell lines for transfection with DNA sequences specifically for antibody production or cell lines secreting the antibody may be selected through cloning for its endogenously high levels of galactosyltransferase and/or sialyltransferase. Similarly, cell lines for transfection or cell lines secreting the antibody may be selected for its endogenously low levels of sialidase or  $\beta$ -galactosidase (Gramer et. al., 1995, Biotechnology, 13, 692). Sialidase and  $\beta$ -galactosidase are enzymes which are involved in the removal of galactose and sialic acid residues from oligosaccharide structures so that low levels of these enzymes indirectly boost the levels of galactose and sialic acid containing structures. Specific inhibitors and activators of the enzymes galactosyltransferase,  $\beta$ -galactosidase, sialyltransferase or sialidase (Grammer et. al., 1995, Biotechnology, 13, 692) may thus be used to manipulate the amounts of galactose and sialic acid incorporated into oligosaccharide structures. The growth media for cells secreting the antibody can also be manipulated for production of high level of galactose and sialic acid containing oligosaccharide structures.

Another method of obtaining an antibody preparation with a high level of galactose and/or sialic acid in the oligosaccharides is to select for such antibodies, for Example, as part of an antibody purification procedure. Thus, for



Example, specific lectins may be immobilised on a column (Dobre et. al., 1983, J. Immunol. Methods, 59(3), 339) which specifically bind to structures containing galactose or sialic acid containing oligosaccharides (Osawa and Tsuji, 1987, Ann Rev. Biochem. 56, 21). In this way antibodies with no galactose and/or sialic acid can be removed as they do not stick to the column, thereby increasing the levels of galactose/sialic acid containing antibodies on the column. The column can then be eluted to give an antibody preparation wherein most of the antibody molecules have an oligosaccharide containing galactose and/or sialic acid.

The present invention also includes a method of increasing the lytic activity of an antibody preparation, specifically complement lysis activity and ADCC activity. As such lytic antibodies are of particular relevance to this invention such as for Example, IgGM, IgG1, IgG2, IgG3 and IgG4. Complement lysis activity is increased by increasing the % of biantennary oligosaccharides at the N-glycosylation site of the Fc domain of the antibody having more than 20% galactose residues. This activity can be further increased by increasing the % of terminal sialic acid residues to more than 20%. The invention provides an antibody with an increase in complement lysis activity of more than 30%. The invention also provides an antibody with an increase in ADCC activity of more than 30%.

According to another aspect of the present invention there is provided the use of an antibody composition of the invention in therapy and diagnosis. In particular there is provided use of antibodies according to the invention in the treatment of cancers such as lymphomas and leukaemias and in particular small cell and non-small cell lung cancer, prostatic cancer, colorectal cancer, prostatic cancer and ovarian cancer. They may also be used, for Example as immunosuppressives and more particularly for the treatment of T-cell mediated disorders including severe vasculitis, rheumatoid arthritis, systemic lupis, also autoimmune disorders such as multiple sclerosis, graft vs host disease, psoriasis, juvenile onset diabetes, Sjogrens' disease, thyroid disease, myasthenia gravis, transplant rejection and asthma.

The invention also provides the use of an antibody composition described above in the manufacture of a medicament for the treatment of any of the aforementioned disorders.

According to another aspect of the present invention there is provided a method of treatment of conditions amenable to therapy and diagnosis with an antibody

composition according to the invention comprising administering a therapeutically efficacious amount of antibody composition to a mammal requiring such treatment. In particular there is provided a method of treatment of cancers such as lymphomas and leukaemias and in particular small cell and non-small cell lung cancer, prostatic cancer as well as colorectal cancer and ovarian cancer and associated metastases. They may also be used in a method of treatment of T-cell mediated disorders including severe vasculitis, rheumatoid arthritis, systemic lupis and also autoimmune disorders such as multiple sclerosis, graft vs host disease, psoriasis, juvenile onset diabetes, Sjogren's disease, thyroid disease, myasthenia gravis, transplant rejection and asthma.

There is also provided in the present invention a pharmaceutically acceptable composition containing conjugated antibodies according to the present invention which comprise an antibody composition of the present invention and one or more pharmaceutically acceptable excipients.

Such compositions include, in addition to an antibody composition according to the present invention a physiologically acceptable diluent or carrier possibly in a mixture with other agents such as other antibodies or an antibiotic. Suitable carriers include but are not limited to physiological saline, phosphate buffered saline, phosphate buffered saline glucose and buffered saline. Routes of administration are routinely parenteral including intravenous, intramuscular, subcutaneous and intraperitoneal injection or delivery.

The antibodies according to the present invention are preferably monoclonal antibodies. Antibodies according to the present invention can be from any species. The antibodies may be chimaeric antibodies that have variable regions from one antibody and constant regions from another, such as a human antibody. Thus, chimaeric antibodies may be species/species chimaeras or class/class chimaeras. Such chimaeric antibodies may have one or more further modifications to improve antigen binding ability or to alter effector functions. Another form of altered antibody is a humanised antibody including a composite antibody, wherein the constant regions and the hypervariable regions other than the CDRs constitute the human framework. Additional amino acids in the framework or constant regions of such antibodies may be altered if required to restore binding. Thus the antibodies of use in the present invention include any altered antibodies in which the amino acid sequence is not one which exists in nature. However, CDR-grafted antibodies are most preferred. Antibodies of the present invention include IgG and IgM of different isotypes such as. for

Example, G1, G2 G3 and G4. Whilst IgM ,IgG1,IgG2 and IgG3 are known to mediate complement lysis, IgG1,IgG2,IgG3 and IgG4 are expected to bind to Fc receptors and would therefore be expected to mediated ADCC.

Examples of antigens to which antibodies of the present invention may bind include the 40KD antigen (CO/17.1.A) as disclosed in J.Cell Bio., 125(2), 437-446, April 1994 and in Proc. Natl.Acad.Sci., 87, 3542-3546, May 1990, the anti-folate antigen as disclosed in A. Tomasetti et.al., Federation of European Biochemical Societies Vol 317, 143 -146, Feb 1993, anti-CEA, anti mucin, anti-20/200KD, anti-ganglioside, anti-digoxin, anti-CD4, anti-CD23, anti-CD52 and more specifically, Campath-1H which is a humanised anti-CD52 antibody. The antibody chain DNA sequences including the CDRs of Campath-1H are set out in EPO328404, the disclosure of which is hereby incorporated by reference. (Page, M.J. and Sydenham, M.A., 1991, High level expression of the humanised monoclonal antibody Campath-1H in Chinese Hamster Ovary cells , Biotech. 9: 64-68).

#### Description of Figures

**Figure 1 Structure of Human IgG.**

**Figure 2 A typical carbohydrate profile of Campath-1H.**

A carbohydrate map for Campath-1H was obtained by releasing the glycan from the antibody with the aid of Glycopeptidase F. The glycan was derivatised with 1-phenyl-3-methyl-2-pyrazolin-5-one (PMP) for detection and analysed by reverse phase HPLC. The map showing that a majority of the structures are non-sialylated biantennary structures and a small proportion consists of sialylated biantennary structures.

**Figure 3 Typical carbohydrate profiles of Campath-1H after sialidase and  $\beta$ -galactosidase treatment.**

Typical carbohydrate profiles of oligosaccharides released from (a) Campath-1H (control); (b) Campath-1H digested with sialidase; and (c) Campath-1H digested with sialidase and  $\beta$ -galactosidase. (i) represents monosialylated biantennary structures; (ii) asialo, galactosylated biantennary structures; (iii) and (iv) asialo, monogalactosylated, biantennary structures; (v) and (vi) asialo, agalactosylated, biantennary structures.

**Figure 4 Oligosaccharide structures of Campath-1H.**

Typical biantennary structures showing agalactosyl (G0), monogalactosyl (G1), digalactosyl (G2), monosialyl (S1) and disialyl (S2) structures associated with human IgG and detected by reverse phase HPLC. Symbols: S - sialic acid; G - galactose; GN - acetylglucosamine, M - mannose and F - fucose.

**Figure 5 Reverse Phase HPLC profiles of oligosaccharides structures released from galactosyltransferase and sialyltransferase treated Campath-1H.**

Carbohydrate profiles of oligosaccharides structures released from (a) and (d) untreated antibody; (b) antibody treated with galactosyltransferase, pH 7.4; (e) antibody treated with galactosyltransferase, pH 8.4; (c) antibody treated with galactosyltransferase at pH 7.4 followed by sialyltransferase and (f) antibody treated with galactosyltransferase at pH 8.4 followed by sialyltransferase. Symbols: G0 - agalactosyl; G1 - monogalactosyl; G2 - digalactosyl; S1 - monosialyl and S2 - disialylated structures.

**Figure 6 Effect of galactose containing oligosaccharides on (a) complement lysis and (b) ADCC activities.**

Complement lysis and ADCC activities together with carbohydrate profiles were obtained for control antibody and antibody treated with  $\beta$ -galactosidase or galactosyltransferase (as in Examples 1, 2, and 4). Peaks showing G0, G1, G2, S1 and S2 were integrated and the data expressed as a percentage of total oligosaccharides detected (Tables 1 and 2). The graph shows (a) complement lysis, (b) ADCC activities against percent oligosaccharides containing digalactosylated structures (G2), and structures containing at least 1 galactose (G1+G2+S1+S2) or 2 galactose residue (G2+S1+S2). The graphs also include an average of percent oligosaccharide structures for control Campath-1H.

**Figure 7 Effect of sialic acid containing oligosaccharides on (a) complement lysis and (b) ADCC activities.**

Complement lysis and ADCC activities together with carbohydrate profiles were obtained for control Campath-1H and Campath-1H treated with either sialyltransferase alone or galactosyltransferase and sialyltransferase (as in Examples 3 and 4). Peaks showing G0, G1, G2, S1 and S2 were integrated and the data expressed as a percentage of total oligosaccharides (Tables 1, 2 and 4). The graph shows (a) complement lysis, (b) ADCC activities against percent oligosaccharides containing monosialylated (S1), disialylated (S2) and structures containing at least one sialic acid (S1+S2). The graphs also include an average of percent oligosaccharide structures for control Campath-1H.

**Table 1** Complement lysis, ADCC activities and relative percent composition of G0, G1, G2, S1 and S2 structures found in Campath-1H and  $\beta$ -galactosidase treated Campath-1H.

$\beta$ -Galactosidase digested Campath-1H was compared against control Campath-1H. Peaks showing G0, G1, G2, S1 and S2 were integrated and the data expressed as a percentage of the total oligosaccharide. Complement lysis and ADCC activities were determined and expressed as described in Example 1.

**Table 2** Relative percent composition of G0, G1, G2, S1 and S2 structures in control and galactosyltransferase / sialyltransferase treated Campath-1H.

Reverse phase HPLC carbohydrate profiles were obtained for control Campath-1H (Figure 5a and 5d), Campath-1H treated with either galactosyltransferase (Figure 5b and 5e) or galactosyltransferase and sialyltransferase (Figure 5c and 5f). Peaks showing G0, G1, G2, S1 and S2 were integrated and the data expressed as a percentage of total oligosaccharide present.

**Table 3** Relative complement lysis and ADCC activities of Campath-1H treated with galactosyltransferase and sialyltransferase.

Campath-1H was initially treated with galactosyltransferase at either pH 7.4 or pH 8.4. The antibodies were then treated with sialyltransferase at pH 6.0. Galactosyltransferase (Example 2) or galactosyltransferase followed by sialyltransferase (Example 4)

treated antibodies are compared against control Campath-1H for activities. Activities (eg. ADCC or complement lysis) were determined and expressed as described in Example 1.

**Table 4 Percent composition of G0, G1, G2, S1 and S2 structures in control and sialyltransferase treated Campath-1H.**

Reverse phase HPLC carbohydrate profiles were obtained for untreated Campath-1H and sialyltransferase treated antibody (Example 3). Peaks showing G0, G1, G2, S1 and S2 were integrated and the data expressed as a percent of total carbohydrate.

**Table 5 Relative complement lysis and ADCC activities of control and Campath-1H treatment with sialyltransferase.**

Complement lysis and ADCC activities of untreated Campath-1H and Campath-1H treated with the sialyltransferase at pH 6.0 (Example 3), was determined and calculated according to the method described in Example 1.

The following Examples are illustrative of the present invention and not intended to constitute any limitation thereof.

### **Campath-1H**

Campath-1H is a purified human IgG monoclonal antibody, which mediates lysis of target cells (Wein 133 cells) through either complement or ADCC. Campath-1H has only two N-linked oligosaccharides and no O-linked sugars per antibody were detected. Importantly the amino acid sequence shows no sites for N-linked oligosaccharide structures in the Fab regions of the antibody and protease digestion of the antibody followed by amino acid sequencing of the resultant peptides containing N-linked sugars, demonstrated that the sugars were only restricted to the Fc region of the antibody. Hence, N-linked oligosaccharide structures are restricted only to the Fc region of the antibody.

It should, however be remembered, that the Fc region of all antibodies is relatively conserved so that studies using Campath-1H as an Example are therefore relevant to other antibodies (Wright et al, 1994 J. Exp. Med. Vol 180, pp1087).

Example 1. Enzymatic removal of sialic acid and galactose from Campath-1H by treatment with Sialidase and  $\beta$ -Galactosidase.

Purified Campath-1H in PBS was dialysed overnight against 2 changes of 100 mM sodium acetate buffer, pH 6.0. The concentration of the antibody was then determined spectrophotometrically at 280 nm. The antibody was initially digested with 2 units of Sialidase (from *Salmonella typhimurium*, Oxford Glycosystems) for 18 hr at 37°C, followed by further incubated (18 hr) in the presence of 60 mUnits of  $\beta$ -Galactosidase (from *streptococcus pneumoniae*, Oxford Glycosystems). Controls consist of the same antibody treated as above but in the absence of Sialidase and  $\beta$ -Galactosidase. The samples were divided into 2 portions and stored at 5°C.

The first portion is used for carbohydrate detection and mapping. The second is retained for activity assays. For carbohydrate detection and mapping (Figure 3), intact oligosaccharides chains are released with glycopeptidase F (*Flavobacterium meningosepticum*, Boehringer Mannheim) and derivatized with 1-phenyl-3-methyl-2-pyrazolin-5-one (PMP) to facilitate UV detection and analysed by reverse phase HPLC on a Spherisorb S30DS C18 column (particle size 3 $\mu$ , 15 cm x 4.6 mm ID from phase Separations Ltd). The composition of agalactosyl (G0), monogalactosyl (G1), digalactosyl (G2), monosialyl (S1) and disialyl (S2) structures (Figure 4) of Campath 1-H is calculated and is shown in Table 1. The results in the first experiment (Table 1) shows a decrease mainly in the G1 structures whilst the levels of G2 remained unchanged compared to the control. In the second experiment, there was a decrease in both G1 and G2 structures compared to the control.

The antibody is analysed for complement lysis and ADCC activities using methods described previously (Blumberg et.al., 1986, J. Immunol. Methods, 92, 117; Patel et.al., 1995, J. Immunol. Methods, 184, 29). Wein 133 cells (non-Hodgkins's B cell lymphoma) are used as target cells. The cells ( $2 \times 10^7$ ) are harvested and washed in 50mM HEPES, 93mM NaCl, 5mM KCl and 2mM MgCl<sub>2</sub> adjusted to pH 7.4 by NaOH (buffer A). The cells are suspended in 1 ml buffer A containing 600  $\mu$ l EuCl<sub>3</sub>, 3mM diethylene triaminepentaacetate and 25 mg/ml dextran sulphate and incubated for 20 minutes at 4°C. Incubation is continued for a further 5 minutes after the addition of an equal volume of Buffer B (buffer A containing 2mM CaCl<sub>2</sub> and 10mM L-glucose). Target cells are then washed 3 times in buffer B and twice in Iscoves media supplemented with 2% FCS. The cell density is adjusted to  $1 \times 10^6$  cells/ml in the same media and kept on ice.

For complement lysis and ADCC activities, Campath-1H standard, positive control and samples are diluted in Iscoves media containing 2% FCS. The samples (at 3 different dilutions) and the standard are pipetted (50 $\mu$ l) into "U"-bottomed 96 well plates and equilibrated to 4°C for 10 minutes. Europium labelled target cells (150 $\mu$ l, 5x10<sup>4</sup> cells) containing either normal human serum (for complement lysis activity) or freshly purified peripheral blood mononuclear cells as effector cells (for ADCC activity) are added to the microtitre plate. The plates are incubated at 4°C for 30 minutes. Plates scheduled for estimation of ADCC activities are centrifuged at 1800 rpm (5 min) to pellet effector and target cells, prior to incubation at 37°C. The plates are incubated at 37°C for either 90 minutes (complement lysis) or 180 min (ADCC), followed by centrifugation at 1800 rpm for 5 minutes to pellet intact cells. An aliquot (20 $\mu$ l) of the supernatant is taken from each well of the microtitre plate. Lysis of W133 cells is estimated by quantifying fluorescence of europium released into the supernatant after mixing with enhancement solution (200 $\mu$ l). Complement lysis or ADCC activities (units/ml) of samples is estimated from a standard curve obtained using the Campath-1H standard. Relative complement lysis or ADCC activities is derived using the equation below:

$$\text{Relative activity} = \frac{\text{Complement lysis or ADCC activity of samples (units/ml)}}{\text{Complement lysis or ADCC activity of control (units/ml)}}$$

In the first experiment (Table 1), a decrease only in the G1 structures caused a decrease in complement lysis activity from 1.0 to 0.58. The results also shows that a loss of G1 structures only had little influence on ADCC activity. In contrast, the second experiment shows a simultaneous loss of G1 and G2 structures, causing a decrease in complement lysis activity from 0.97 to 0.48. A greater loss in complement lysis activity in the second experiment compared to the first was found to be due to a loss of G2 structures. In the same experiment, a loss in G2 structures also caused a decrease in ADCC activity from 0.99 to 0.81.

Thus studies have been done involving specific cleavage of N-linked sugar in the Fc region of the antibody using the enzyme glycopeptidase F (GPF). The resulted deglycosylated antibody retained antigen binding and protein A binding activity (Boyd et.al., 1995, Mol. Immuno, in press). However, no complement lysis or ADCC activity was detected. Using Campath-1H, we have succeeded in



removing terminal sugar residues by specific enzymatic cleavage using sialidase,  $\beta$ -galactosidase and  $\beta$ -N-acetylhexosamidase. We have shown that removal of terminal sialic acid residues does not influence antigen and Protein A binding, complement lysis or the ADCC activities. Following removal of sialic acid, the exposed terminal galactose residue was removed using the enzyme  $\beta$ -galactosidase.  $\beta$ After  $\beta$ -galactosidase treatment, G1 (monogalactosyl) was removed completely whilst the composition of G2 (digalactosyl) remained almost unchanged. It was therefore with some surprise and contrary to expectation that removal of the galactose from the antibody resulted in a 42% decline in complement lysis activity and no effect on the ADCC activity. Therefore some loss in complement lysis activity was due to the removal of G1 structures whilst the remaining 58% activity was still likely to be associated with G2 or G0 structures. The data further shows that conversion of G1 structures to G0 has no effect on ADCC activity.

Example 2. Enzymatic addition of galactose to the antibody. Campath-1H at either pH 7.4 or pH 8.4.

The purified antibody, Campath-1H is dialysed overnight against 2 changes of either 10mM sodium phosphate buffer containing 0.15M NaCl and 5  $\mu$ M MnCl<sub>2</sub>, pH 7.4 or 100 mM glycine / NaOH buffer containing 5 $\mu$ M MnCl<sub>2</sub>, pH 8.4. The concentration of the antibody is determined spectrophotometrically at 280nm. The lyophilised enzyme galactosyltransferase, is suspended in 500 $\mu$ l of distilled water, giving an activity of 10 units/ml. The lyophilised substrate, uridine-5'-diphosgalactose is suspended into either 10mM sodium phosphate buffer containing 0.15M NaCl and 5  $\mu$ M MnCl<sub>2</sub>, pH 7.4 or 100 mM Glycine/NaOH buffer containing 5 $\mu$ M MnCl<sub>2</sub>, pH 8.4. To a solution of Campath-1H at pH 7.4 or 8.4, add 0.5 units of galactosyltransferase and 160  $\mu$ M uridine - 5' - diphosgalactose in the appropriate buffers. Controls consist of an absence of enzyme or enzyme and substrate. The reaction mixture is then incubated for 18 hrs at 37°C and stored at 5°C.

The antibodies generated in this Example are then divided into three portions. The first portion is used for carbohydrate detection and mapping (Figure 5), whilst the second portion was retained for activity assays as described in Example 1. The third is reserved for subsequent treatment with the enzyme sialyltransferase (see Example 4). The percentage composition of G0, G1, G2, S1 and S2 structures of Campath 1-H is calculated (Table 2) and compared against relative composition of the same structures found in serum IgG (Parekh

et.al., 1985, Nature, 316, 452). The results (Table 2) demonstrate an overall increase from 69% to 100% in structures containing at least one galactose (G1, G2, S1 and S2). More specifically, the proportion of mainly G2 structures are found to increase with respect to the starting material (control Campath-1H), whilst levels of G0 and G1 structures declined. The levels of G2 structures after galactosyltransferase treatment are almost three to four times that found in serum IgG of control patients (Parekh et. al., Nature, 1985, 316, 452) and five to seven times higher than that found in the starting material (control Campath-1H).

The results demonstrate a 60 - 160% increase in complement lysis activity of Campath-1H compared to the control after treatment with galactosyltransferase (Table 3). Whilst the increase in complement lysis activity is related to an overall increase in the galactose composition (G1 and G2) of the antibody, the relative increase in activity is greater with the G2 structures compared to the G1 structures. Hence, a higher G2 composition (92%) obtained by treatment of the antibody with the enzyme at pH 7.4 resulted in an increase of 160% complement lysis activity. In contrast, a lower G2 composition (68%) obtained at pH 8.4 only resulted in an increase in activity by 60%. This confirms our findings in Example 1, whereby although the total galactose containing structures were important, complement lysis activity was influenced more specifically by G2 structures. In Example 2, we have also found that only the G2 structures are important for ADCC activity, whereby a five fold and seven fold increase in G2 structure was associated with a 20% and 100% increase in activity, respectively. We have also shown (Example 1) that following  $\beta$ -galactosidase treatment of Campath-1H, a small decrease in G2 structures caused a loss in ADCC activity, whilst a decrease in mainly G1 structures did not change ADCC activity.

The results highlight the importance of G2 structures for both ADCC and complement mediated cell lysis. However, only the overall galactose composition (G1 and G2) was found to influence complement mediated cell lysis. Thus, manipulation of the G0, G1 and G2 structures of the antibody can lead to an unexpected but separate enhancement of either complement lysis or ADCC activities. This is particularly important for increasing the potency of antibodies likely to be used therapeutically for removal of tumour cells *in vivo* through a choice of cytolytic mechanisms involving either complement lysis and ADCC.

Example 3. Enzymatic addition of sialic acid to the antibody, Campath-1H.

The lyophilised substrate cytidine-5'-monophospho-N-acetylneuraminic acid is dissolved in 50mM Citrate / 50 $\mu$ M EDTA buffer, pH 6.0. To the solution of Campath-1H in 50 mM Citrate buffer containing 50 $\mu$ M EDTA pH 6.0, add 0.04 units of sialyltransferase (Sigma) and 2mM substrate cytidine-5'-monophospho-N-acetylneuraminic acid. Controls consist of an absence of enzyme or enzyme and substrate. Incubate at 37°C for 16 hrs and store at 5°C.

The antibodies generated in this Example are divided into two portions. The first portion is used for carbohydrate detection and mapping (as above) and the second is reserved for determination of complement lysis and ADCC activities (as described previously).

Analysis of oligosaccharide composition (Table 4) shows an increase in S1 and S2 structures and a corresponding decrease in G1 and G2 structures after treatment with sialyltransferase. No change in the relative composition of G0 structure is detected which is not surprising since a terminal galactose is required for addition of sialic acid. Further increases in S1 and S2 structures of Campath-1-H would require pretreatment of the antibody with the enzyme galactosyltransferase in order to increase the terminal galactose content. The antibody is then treated with sialyltransferase to maximise the S1 and S2 oligosaccharide composition of the antibody (Example 4).

The relative complement lysis and ADCC activities of sialyltransferase treated Campath-1H compared to the control is shown in Table 5. The data shows that following sialyltransferase treatment of the antibody, complement lysis and ADCC activities compared to the control increased by 28% and 20%, respectively. Such an increase in the cytolytic activity of the antibody is related to an increase in S1 and S2 oligosaccharide structures on Campath-1H. In Examples 1 and 2, we have highlighted that the overall galactose content (important for complement lysis) and more specifically the G2 structures are important for increasing both complement lysis and ADCC activities. It was therefore possible that further increases in the cytolytic activities of the antibody may be achieved by bringing together those components of galactose and sialic acid responsible for enhancing complement lysis and ADCC activities. It is also likely that further increases in S1 and S2 oligosaccharides may increase the cytolytic activities of the antibody.

Example 4. Enzymatic addition of sialic acid to galactosyltransferase treated Campath-1H

Campath-1H previously treated with galactosyltransferase (Example 2) is dialysed against 50mM Citrate buffer containing 50 $\mu$ M EDTA, pH 6.0. The lyophilised substrate cytidine-5'-monophospho-N-acetylneuraminic acid is also dissolved in 50mM Citrate / 50 $\mu$ M EDTA buffer, pH 6.0. To a solution of galactosyltransferase treated Campath-1H, add 0.05 units of sialyltransferase (Sigma) and 1.64mM substrate cytidine-5'-monophospho-N-acetylneuraminic acid. Controls consist of an absence of enzyme or enzyme and substrate. The antibodies are incubate at 37°C for 16 hrs and store at 5°C.

The antibodies generated in this Example is divided into two portions. The first portion is used for carbohydrate detection and mapping (Figure 5) and the second is reserved for determination of complement lysis and ADCC activity (as described previously).

An analysis of the oligosaccharides composition (Table 2) show a 17-44% S2 structures after treatment with sialyltransferase compared to undetectable levels of the same structures in control preparation of the antibody. In contrast, only 6.5% S2 structures are detected in serum IgG (Parekh et al., Nature 316, 452). Levels of S1 structures also increased from 6% (control antibody) to 46-80% after treatment with sialyltransferase compared to 21% detected in serum IgG. Only 3-10% of the oligosaccharides structures are found not to be sialylated (G2, G1 and G0) after incubation of the antibody with sialyltransferase compared to 96% non-sialylated structures in the control.

Relative complement lysis and ADCC activities for Campath-1H is shown in Table 3. The results show further increases in complement lysis activities are detected after treatment with sialyltransferase compared to that obtained with the addition of galactose alone. A higher increase in complement lysis activity (from 160% to 210%) was associated with a larger increase in S1 structures (from 4.6% to 80%) after treatment with sialyltransferase. Consequently, a small increase in complement lysis activity from 60% to 90% was due to a smaller increase in S1 structures from 4.6% to 46%. In contrast, increases in S2 structures did not result in a proportion increases in complement lysis activities. The data therefore suggests that whilst increases in the overall sialylation of the antibody is important for complement lysis activity, an increases in S1 structures is preferred. Following sialylation, increasing in ADCC activities are not as great as that detected for complement lysis activities. Increases in ADCC activities with respect to the control antibody was mainly due to the S1 structures. Compared to the control antibody, ADCC activities following

galactosyltransferase and sialyltransferase treatment increased by 50 - 90%, whilst complement lysis activities increased by 90 - 210%.

Digalactosylated (G2) structures together with oligosaccharides with at least one galactose (G1, G2, S1 and S2) or two galactose residues (G2, S1 or S2) determined in Examples 1,2,3 and 4 are shown against complement lysis and ADCC activities (Figure 6). The data confirms that an increase in structures containing at least 1 galactose (to levels beyond 20%) caused an increase in complement lysis activity. The data also confirms that G2 and structures containing at least 2 galactose residues also caused an increase in both complement lysis and ADCC activities. This was particularly more evident when structures with at least 2 galactose residues exceeded 20%. Figure 7 shows the effect of S1 and S2 structures together with oligosaccharides containing at least 1 sialic acid (from Examples 1, 2, 3 and 4) on complement lysis and ADCC activities. The data shows that S2 structures alone have little influence on either complement lysis or ADCC activities. In contrast, structures containing at least 1 sialic acid (at levels exceeding 7%) caused a proportion increase in both complement lysis and ADCC activities. The data confirm that increases in activities following sialylation are mainly due to S1 structures.

The results highlight the importance of galactosylated and sialylated oligosaccharide structures for increasing the potency of antibody molecules for inducing cell lysis through either complement lysis or ADCC.

**Table 1**

**Relative composition of G0, G1, G2, S1 and S2 in control and galactosyltransferase treated Campath-1H (compared to relative composition in serum IgG).**

% oligosaccharide

Oligosaccharide structures	Control C1H	Galactosylated C1H at pH 7.4	Galactosylated C1H at pH 8.4	Serum IgG *
G0	30.8	0.0	0.0	16.5
G1	49.4	3.1	27.6	29.7
G2	13.6	92.3	67.8	26.4
S1	6.2	4.6	4.6	21.0
S2	0.0	0.0	0.0	6.5

\* data extracted from control group ( Parekh et al, 1985, Nature, 316, 452)

**Table 2.**

Relative percent composition of G0, G1, G2, S1 and S2 structures in control and sialyltransferase treated Campath-1H.

## % oligosaccharide structures

	G0	G1	G2	S1	S2
Control					
Campath-1H	27.5	53.2	15.7	3.6	0.0
Sialyltransferase treated Campath-1H	27.2	24.4	1.1	37.9	9.5

**Table 3**

**Relative complement lysis and ADCC activities of control and Campath-1H treatment with sialyltransferase.**

	<b>Control Campath-1H</b>	<b>Sialyltransferase treated Campath-1H</b>
<b>Complement lysis activity</b>	<b>1.00</b>	<b>1.28</b>
<b>ADCC activity</b>	<b>1.00</b>	<b>1.20</b>



**Table 4**

Relative percent composition of G0, G1, G2, S1 and S2 structures in control and galactosyltransferase / sialyltransferase treated Campath-1H.

## % oligosaccharide

	Control C1H	Galactosylated C1H at pH 7.4	Galactosylated C1H at pH 8.4	Sialylated C1H at (pH 7.4)	Sialylated C1H at (pH 8.4)	Serum IgG *
<b>G0</b>	30.8	0.0	0.0	0.0	0.0	16.5
<b>G1</b>	49.4	3.1	27.6	0.5	8.9	29.7
<b>G2</b>	13.6	92.3	67.8	2.9	1.0	26.4
<b>S1</b>	6.2	4.6	4.6	80.1	46.0	21.0
<b>S2</b>	0.0	0.0	0.0	16.5	44.0	6.5

\* data extracted from control group ( Parekh et al, 1985, Nature, 316, 452)

**Table 5**

Relative complement lysis and ADCC activities of Campath-1H treated with galactosyltransferase and Sialyltransferase.

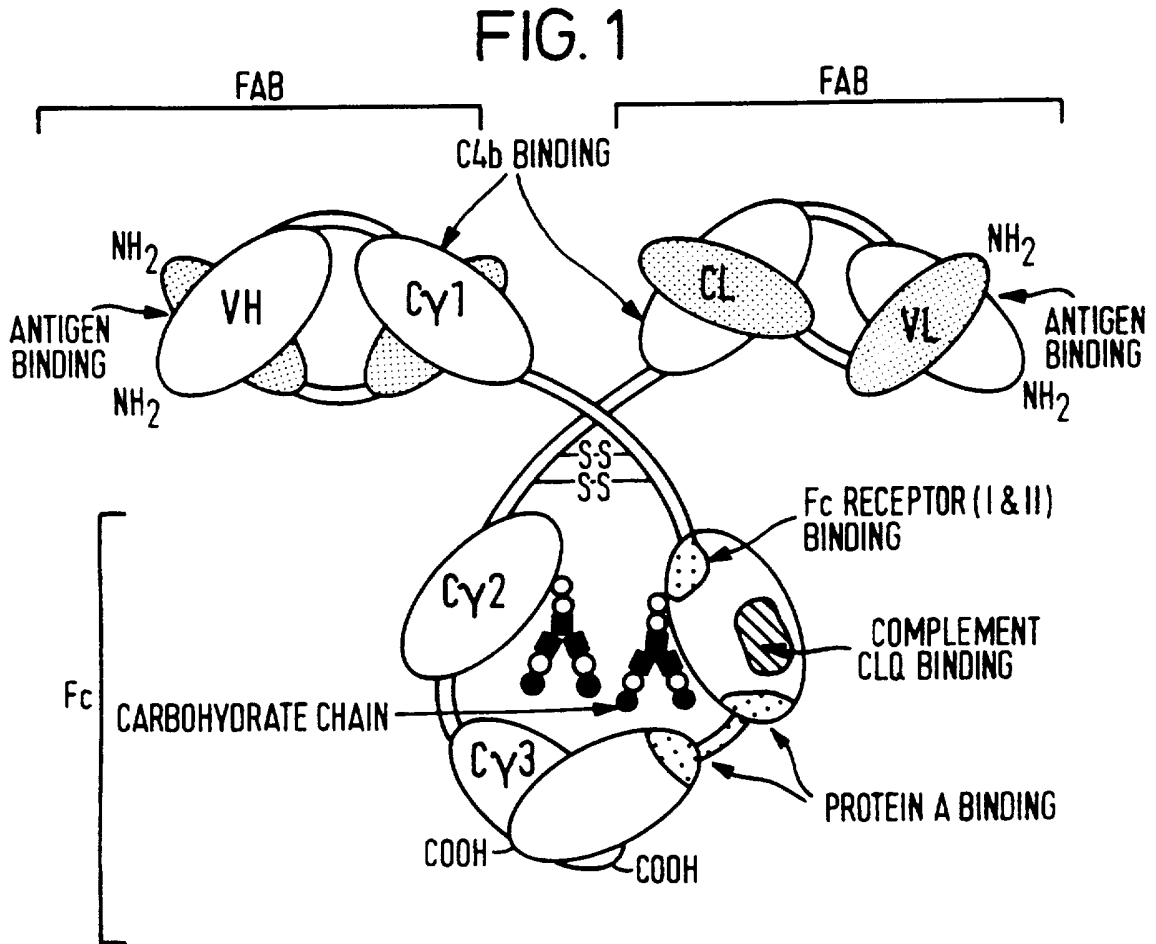
	Control C1H	Galactosylated C1H at pH 7.4	Sialylated C1H at pH 7.4	Control C1H	Galactosylated C1H at pH 8.4	Sialylated C1H at pH 8.4
Complement lysis activity	1.0	2.6	3.1	1.0	1.6	1.9
ADCC activity	1.0	2.0	1.9	1.0	1.2	1.5

$$\text{Relative complement lysis activity} = \frac{\text{Complement lysis activity of sample (u/ml)}}{\text{Complement lysis activity of control (u/ml)}}$$

$$\text{Relative ADCC activity} = \frac{\text{ADCC activity of sample (u/ml)}}{\text{ADCC activity of control (u/ml)}}$$

**Claims**

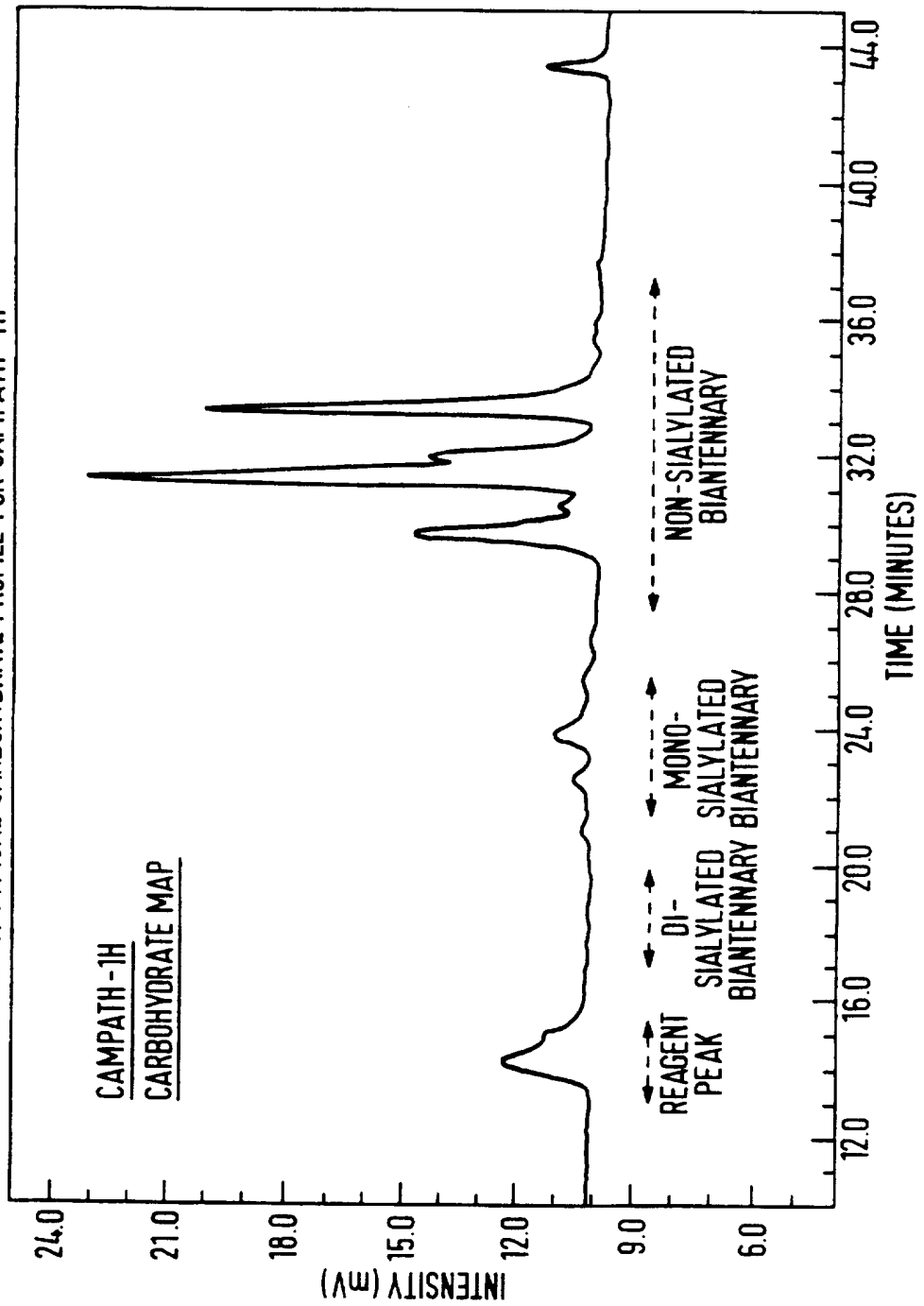
- 5 1. An antibody preparation in which a N-glycosylation site of the Fc domain of the antibody is substituted with a biantennary oligosaccharide, characterised in that at least 20% of the preparation comprises antibody molecules having an oligosaccharide that contains at least one galactose residue.



**STRUCTURE OF HUMAN IgG1**

THE STRUCTURE OF HUMAN IgG1 SHOWING HEAVY (H OR  $\gamma$ ) AND LIGHT (L) CHAINS, BINDING SITES AND THE POSITION OF THE CARBOHYDRATE. THE SUGARS ARE: ○ = N-ACETYLGLUCOSAMINE, ■ = MANNOSE AND ● = GALACTOSE. NO SIALIC ACID IS PICTURED ON THE TERMINAL GALACTOSE, ALTHOUGH SMALL AMOUNTS ARE PRESENT IN A POPULATION OF ANTIBODIES. DISULPHIDE BRIDGES ARE ONLY SHOWN IN THE HINGE REGION.

**FIG. 2**  
A TYPICAL CARBOHYDRATE PROFILE FOR CAMPATH-1H



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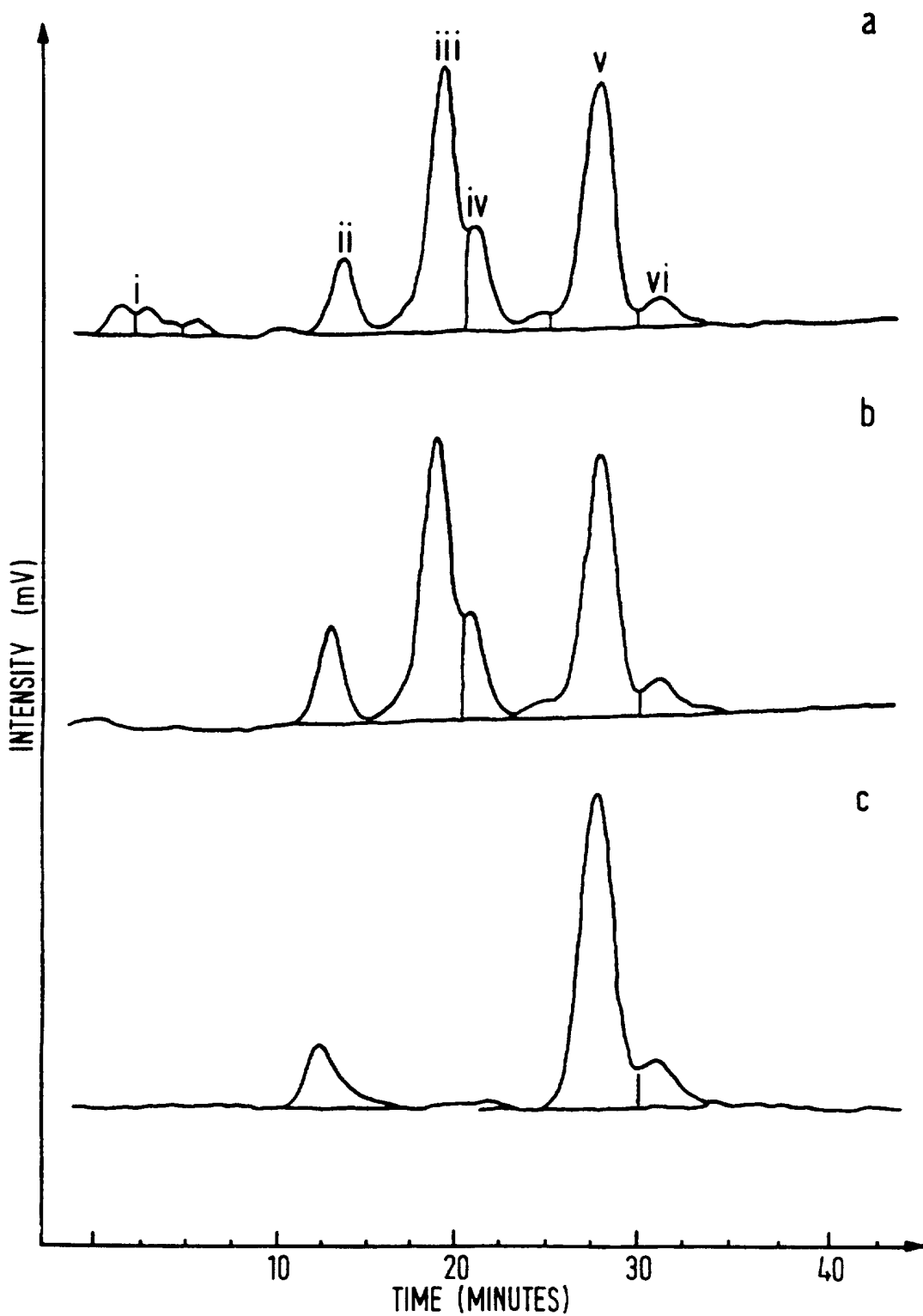
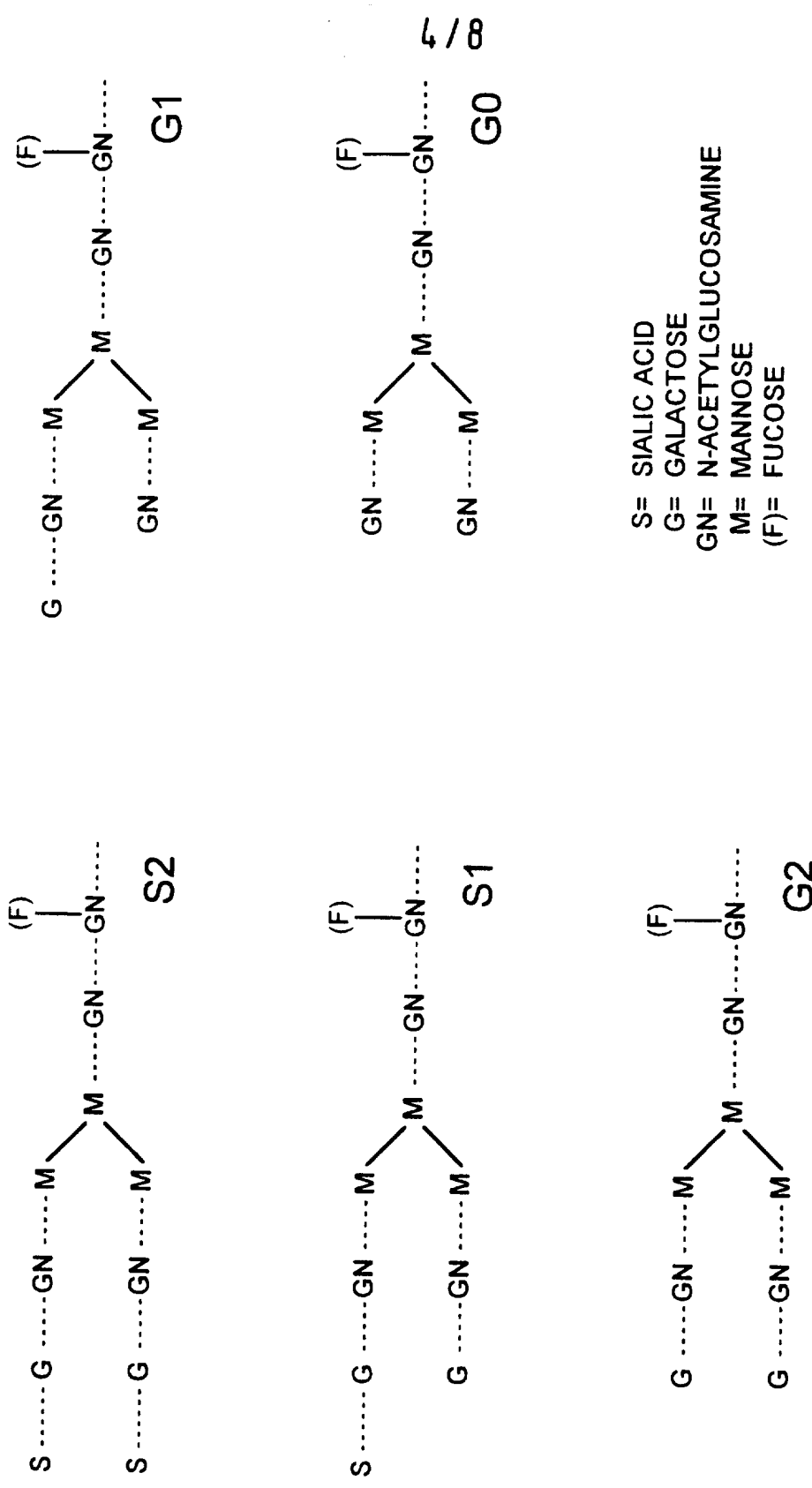


FIG. 3



S= SIALIC ACID  
 G= GALACTOSE  
 GN= N-ACETYLGLUCOSAMINE  
 M= MANNOSE  
 (F)= FUCOSE

**FIG. 4**

OLIGOSACCHARIDES STRUCTURES OF CAMPATH-1H

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REVERSE PHASE HPLC PROFILES OF OLIGOSACCHARIDE STRUCTURES RELEASED FROM GALACTOSYLTRANSFERASE AND SIALYLTRANSFERASE TREATED CAMPATH-1H

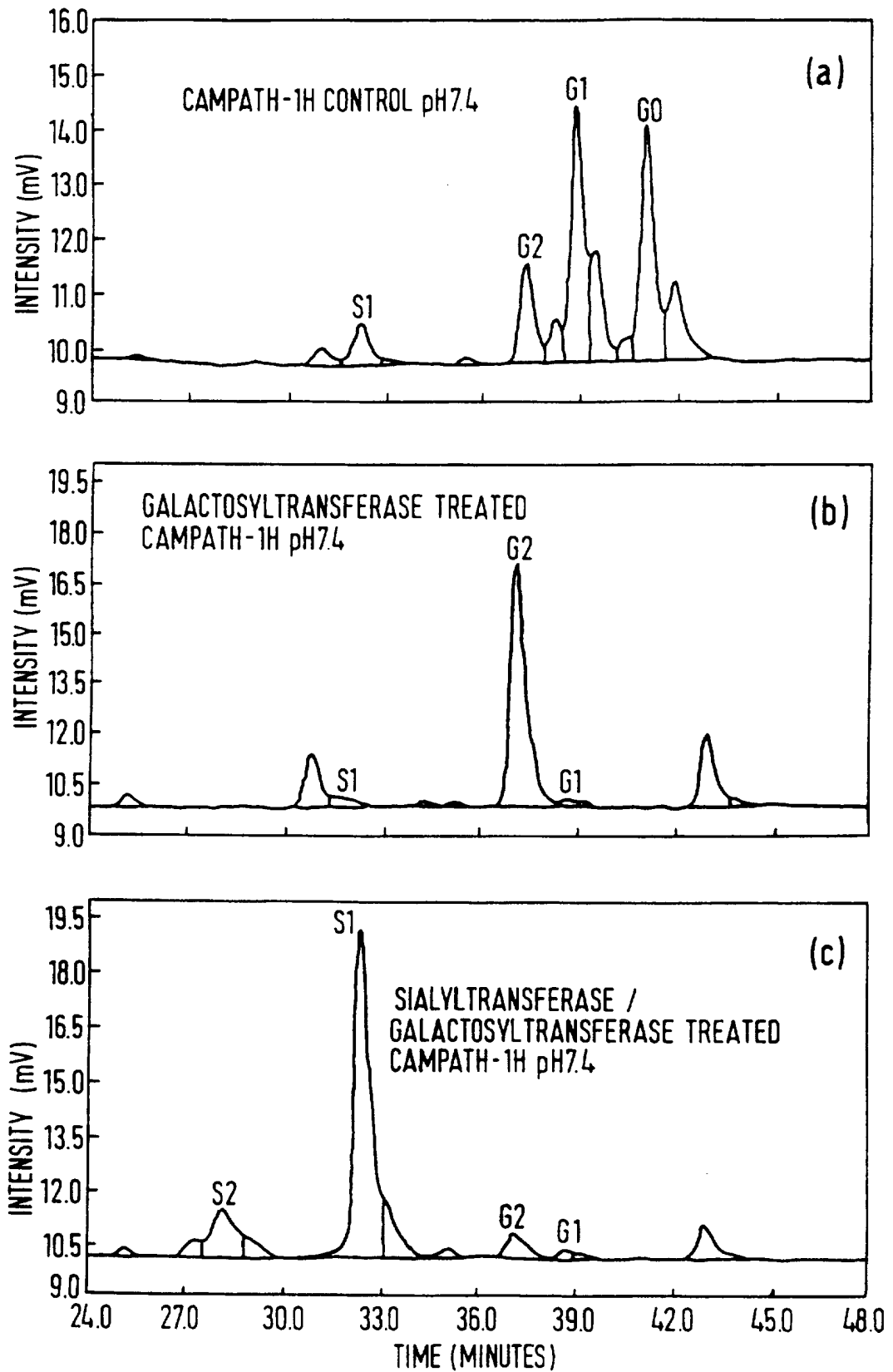


FIG. 5



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REVERSE PHASE HPLC PROFILES OF OLIGOSACCHARIDE STRUCTURES RELEASED FROM GALACTOSYLTRANSFERASE AND SIALYLTRANSFERASE TREATED CAMPATH-1H

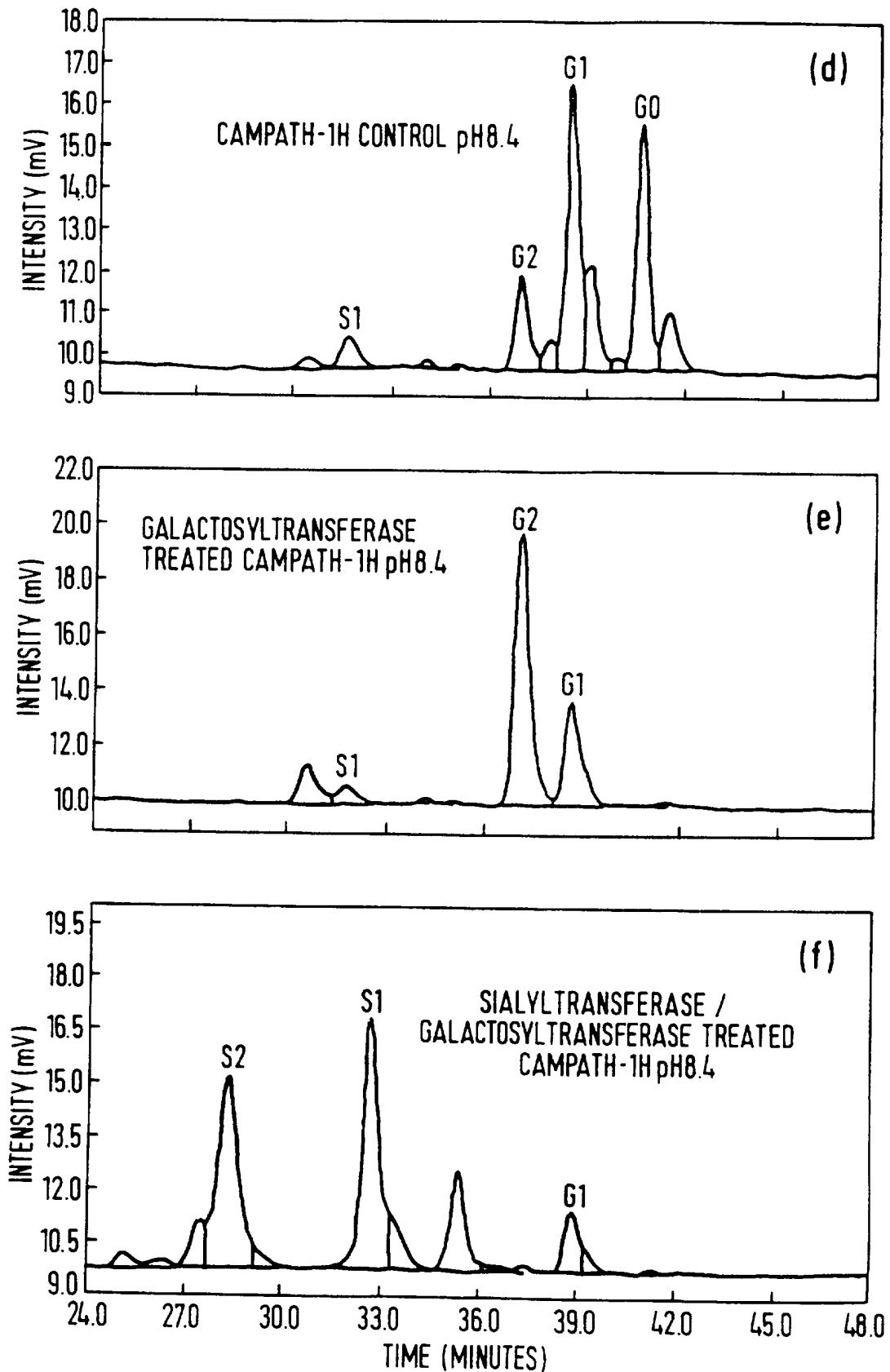
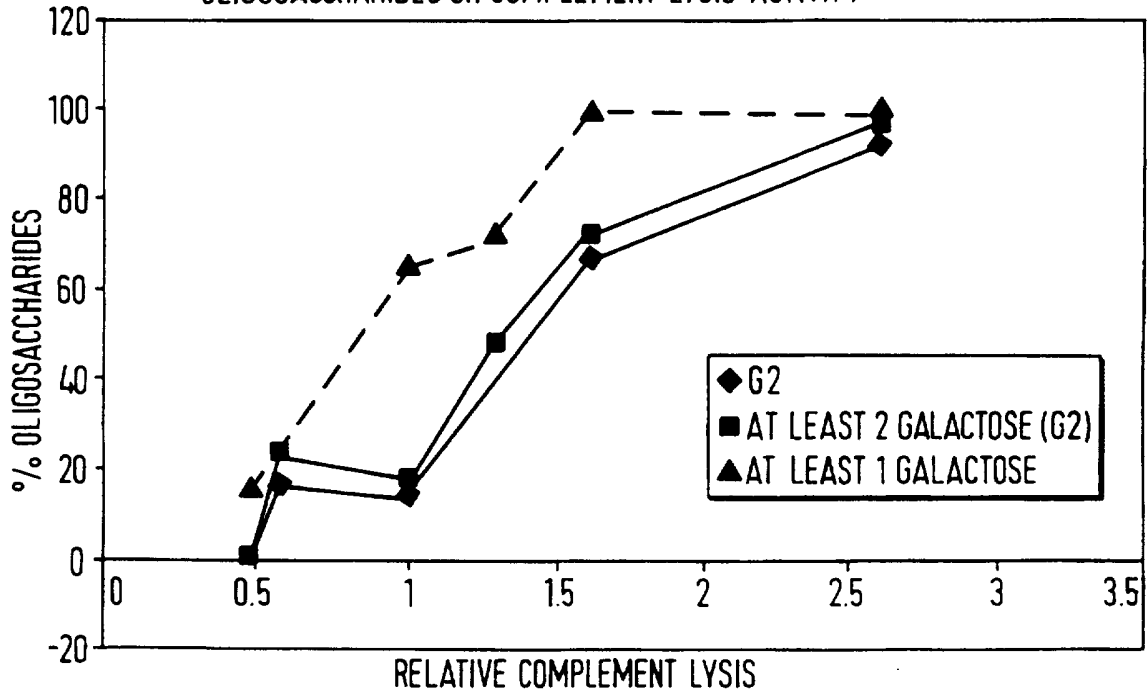


FIG. 5 CONT'D  
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(a) EFFECT OF GALACTOSE CONTAINING OLIGOSACCHARIDES ON COMPLEMENT LYSIS ACTIVITY



(b) EFFECT OF GALACTOSE CONTAINING OLIGOSACCHARIDES ON ADCC ACTIVITIES

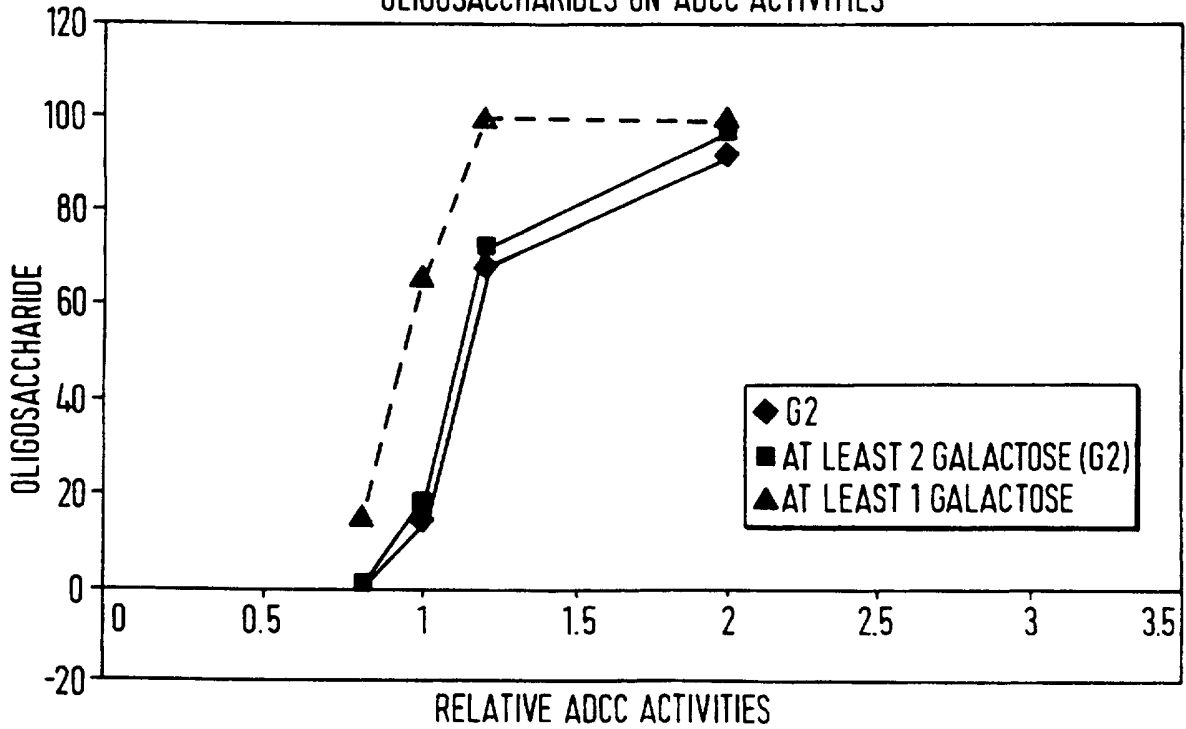
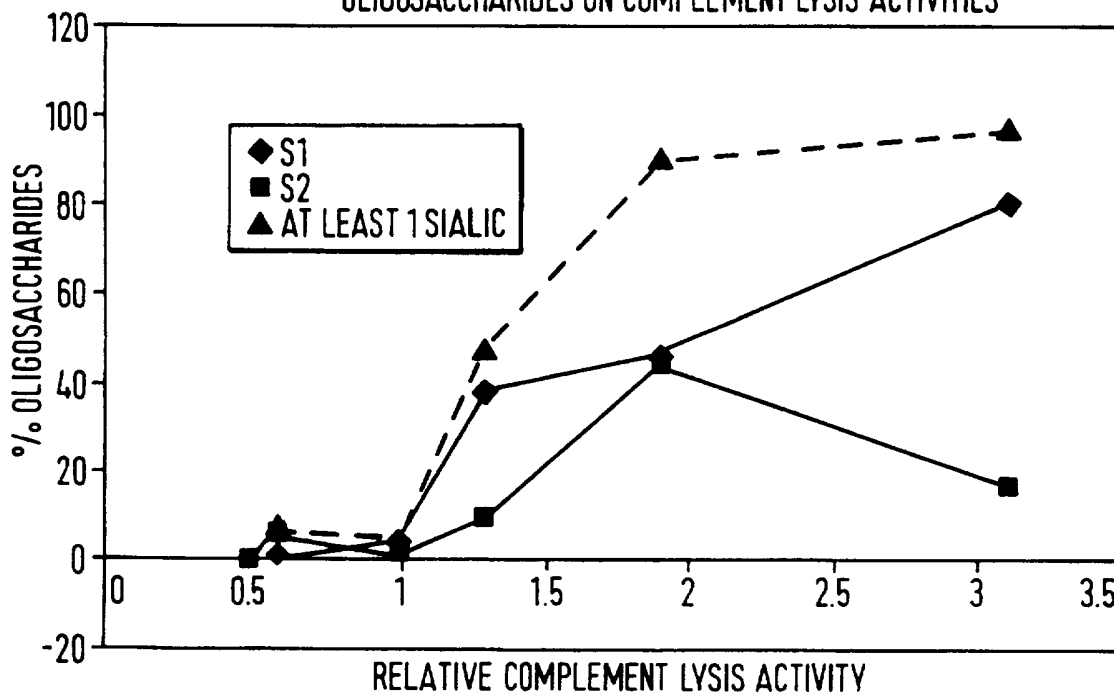


FIG. 6

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(a) EFFECT OF SIALIC ACIDS CONTAINING OLIGOSACCHARIDES ON COMPLEMENT LYSIS ACTIVITIES



(b) EFFECT OF SIALIC ACIDS CONTAINING OLIGOSACCHARIDES ON ADCC ACTIVITIES

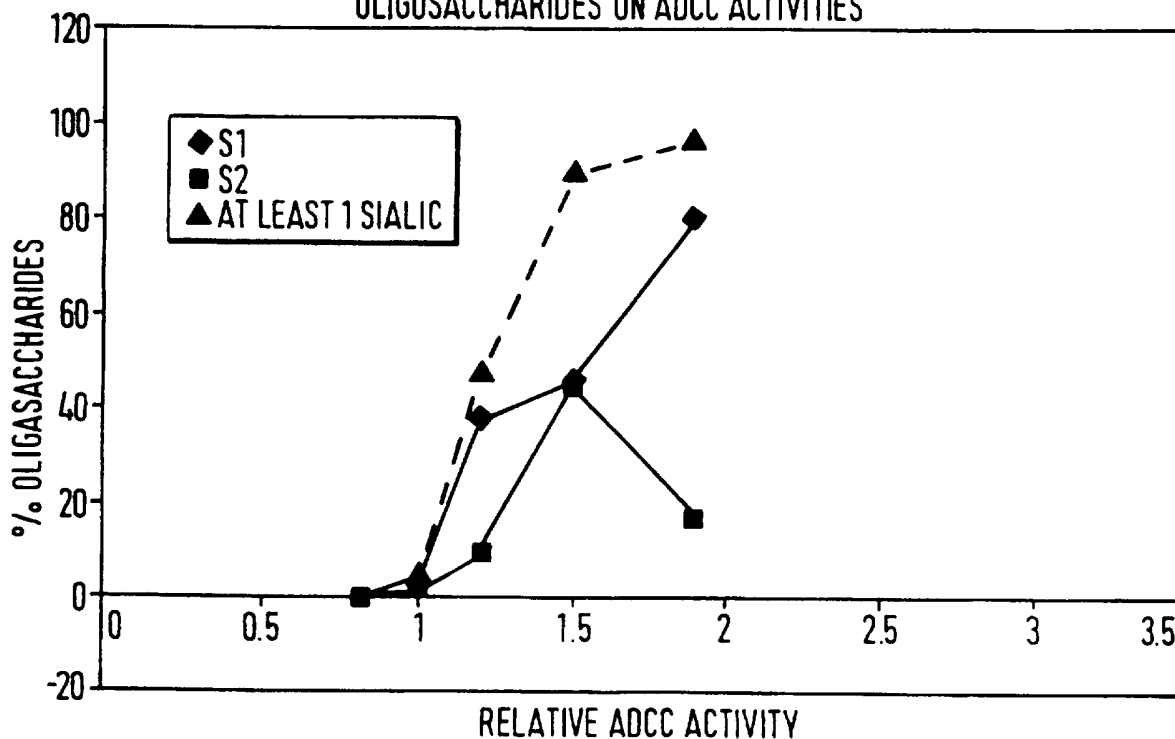


FIG. 7

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/GB 97/00423

**A. CLASSIFICATION OF SUBJECT MATTER**  
 IPC 6 C07K16/00 C07K1/107 //C12N5/10,C12N9/10

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 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MOL. IMMUNOLOGY, vol. 32, no. 17/18, 1995, pages 1311-1318, XP000676340 BOYD ET AL.: "The effect of the removal of sialic acid, galactose and total carbohydrate on the functional activity of Campath-1H" see the whole document ---	1
A	US 5 047 335 A (PAULSON JAMES ET AL) 10 September 1991 see the whole document ---	1
A	WO 90 07000 A (JOLLA CANCER RES FOUND) 28 June 1990 see the whole document ---	1
	-/--	

Further documents are listed in the continuation of box C.       Patent family members are listed in annex.

- \* Special categories of cited documents :
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| <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"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)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> | <p>"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</p> <p>"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</p> <p>"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.</p> <p>"&amp;" document member of the same patent family</p> |
|--|--|

Date of the actual completion of the international search  <p style="text-align: center;">18 June 1997</p>	Date of mailing of the international search report  <p style="text-align: center;">07.07.97</p>
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Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer  <p style="text-align: center;">Hagenmaier, S</p>
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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/GB 97/00423

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 89 09275 A (NILSSON KURT G I) 5 October 1989 see the whole document ---	1
A	BIOTECHNOLOGY, vol. 13, July 1995, pages 692-698, XP002033147 GRAMER ET AL.: "Removal of sialic acid from a glycoprotein in CHO cell culture supernatant by action of an extracellular CHO cell sialidase" cited in the application see the whole document ---	1
A	WO 92 07084 A (WELLCOME FOUND) 30 April 1992 cited in the application see the whole document ---	1
A	US 5 272 066 A (MIT) 21 December 1993 see the whole document -----	1

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International Application No

PCT/GB 97/00423

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PCT/GB 97/00423

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