



THE EFFECT OF THE REMOVAL OF SIALIC ACID, GALACTOSE AND TOTAL CARBOHYDRATE ON THE FUNCTIONAL ACTIVITY OF CAMPATH-1H

P. N. BOYD,* A. C. LINES and A. K. PATEL

Biotechnology Analytical Laboratories, Wellcome Foundation Ltd, Langley Court, South Eden Park Road, Beckenham, Kent BR3 3BS, U.K.

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Abstract—A monoclonal human IgG1, Campath-1H, was digested with glycosidases to assess the effect of carbohydrate on the functional activities of an IgG1. Removal of the complete carbohydrate moiety abolished complement lysis activity and antibody-dependent cell-mediated cytotoxicity, but left antigen binding activity and protein A binding activity intact. Removal of terminal sialic acid residues through glycopeptidase F digestion was not found to effect any of the tested IgG activities. Removal of the majority of the galactose residues from desialylated Campath-1H was found to reduce but not abolish complement lysis activity. Other activities were not affected by degalactosylation. This indicates a rare separation of complement lysis activity and antibody-dependent cell-mediated cytotoxicity of IgG in the way they behave under controlled conditions. This paper underlines the overall importance of carbohydrate in IgG function and stresses the relative contributions of some of the carbohydrate residues.

Key words: structure–function, IgG1, carbohydrate, sialic acid, galactose, glycosidase.

INTRODUCTION

The carbohydrate component of a glycoprotein is important in influencing its own diverse physical and biological functions. In many cases these properties are thought to be a composite of those of the individual glycoforms present in each glycoprotein. Such variety is highlighted by structural analysis of recombinant proteins such as prorenin (Aeed *et al.*, 1992) and OKT3 (Krotkiewski *et al.*, 1990) and is primarily the result of the activity of host cell glycosyltransferases (Nishiura *et al.*, 1990) but also from environmental influences. Not all structural variations are known to affect the physical or biological activities and general structure–function relationships cannot be predicted at present. One possible exception is terminal desialylation. In tissue plasminogen activator, erythropoietin, renin and other recombinant proteins, terminal desialylation is known to reduce plasma half-life through binding to the hepatic asialo-glycoprotein receptor.

Human IgG is a glycoprotein with an average of 2.8 N-linked oligosaccharides per IgG. The CH₂ domain in the Fc region contains 2.0 of these and the hypervariable region of the Fab contains 0.8. The frequency of the latter depends on the availability of an N-glycosylation site on the hypervariable regions of the heavy and light chains, and are thought to be involved in aggregation of IgG via sialic acid residues (Rademacher *et al.*, 1988). The Fc N-glycosylation is of the complex biantennary type and is characterized by the absence of disialylated structures, a low incidence of monosialylation (about 10%), low levels

of cores carrying a “bisecting” N-acetylglucosamine (GlcNAc) and the absence of galactose in the alpha(1–3) arm from at least one oligosaccharide chain (Rademacher *et al.*, 1988). This structure and the structure of a complete branch are illustrated in Fig. 1.

Human IgG has been found to contain about 30

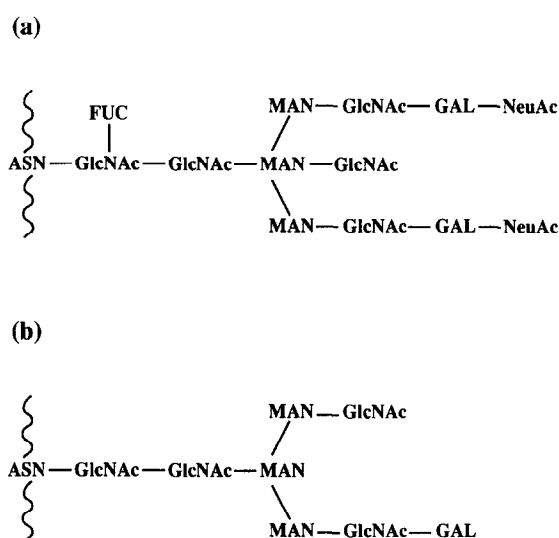


Fig. 1. Carbohydrate sequence of the glycan present on Human IgG1. (a) Full sequence that could theoretically be present. (b) Most common sequence in human blood. Abbreviations: ASN = asparagine 297 of the heavy chain; GlcNAc = N-acetylglucosamine; MAN = mannose; GAL = galactose; NeuAc = sialic acid; FUC = fucose.

different carbohydrate structures (Parekh *et al.*, 1985). This heterogeneity is not just seen in polyclonal populations, but also in myeloma and hybridoma-derived IgG (Mizuuchi *et al.*, 1982). Much of this heterogeneity comes from incomplete carbohydrate synthesis onto the manose core in the Golgi. In human-mouse hybridomas, processing of ASN-linked sugar chains of IgG is less complete than in human or mouse cells. The mouse glycosylation machinery is dominant in hybrid cells (Tandai *et al.*, 1991). Differential glycosylation of human IgG has been associated with a number of diseases (Parekh *et al.*, 1985), and up to 1% of all circulating human serum antibodies are directed against the largely non-human determinant galactose $\alpha(1-3)$ galactose (Galili *et al.*, 1987). The carbohydrate content and its affect on functional activity is important in the understanding of how glycoproteins work.

Campath-1H is a CHO cell-derived human IgG1. It recognises and mediates lysis of CDw52-bearing cells. Carbohydrate analysis has shown the absence of N-acetylgalactosamine and hence O-linked carbohydrate structures. The principle carbohydrate is the N-linked biantennary complex type. Both fucose and sialic acid have been detected. The HPLC analysis of cleaved carbohydrate chains indicates heterogeneity (Lines, 1995). Heterogeneity of desialylated Campath-1H has been detected by laser desorption mass spectrometry (M. R. Lifely, personal communication). This indicates particular heterogeneity in the occurrence of galactose and fucose residues. This paper considers the structure-function relationships of the carbohydrate in Campath-1H. Campath-1H was treated with neuraminidase and glycopeptidase F to cleave off sialic acids and carbohydrate chains, respectively. The effect of the treatments on the Campath-1H activities of complement-mediated cell lysis (CMCL), antibody-dependent cell-mediated cytotoxicity (ADCC), protein A binding and antigen binding were followed.

METHODS

Sources of Campath-1H

The CHO-derived Campath-1H was produced in the Biotechnology Development Laboratories. The batches used were 25.6, 29.6, 32B.6 and 41A.6, and routine product characterization of these batches is available. Myeloma-derived Campath-1H from NSO cells was supplied by Dr N. Sharp from Wellcome Research. All work was performed at the Wellcome Foundation Laboratories, Beckenham.

Preparation of Campath-1H digests

Intact oligosaccharide chains were released from Campath-1H enzymatically using glycopeptidase-F (GPF) from Boehringer Mannheim (No. 913-782, *Flavobacterium meningosepticum*). In order to create the optimum conditions for GPF, the samples were concentrated using ultracentrifugation and the buffer was exchanged with 0.2 M pH 8.5 sodium phosphate. The

samples were digested for 40 hr at 37°C, and then stored at 4°C.

The sialic acid residues were selectively removed using neuraminidase (Boehringer Mannheim, from *Arthrobacter ureafaciens*, No. 611). The samples were ultracentrifuged and exchanged into 0.2 M sodium acetate buffer pH 5. Samples were digested for 18 hr at 37°C, and then stored at 4°C.

Galactose residues were selectively removed using a β -galactosidase (Oxford GlycoSystems, from *Streptococcus pneumoniae*, No. X-5014) from desialylated Campath-1H. The antibody in PBS was diluted into 100 mM sodium acetate buffer, pH 6.0. The β -galactosidase (60 mU) was added and the antibody incubated for 18 hr at 37°C, and then stored at 4°C. Control samples were similarly treated without β -galactosidase but incubated at 37°C.

Detection of released carbohydrates

After the carbohydrates had been released from the Campath-1H, the samples were divided into two portions. The first was retained for activity assays and the second for carbohydrate detection and mapping. After digestion with GPF, the protein was dot-blotted onto Trans-Blot nitrocellulose membrane (0.4 μ m) along with serial dilutions of a control sample of Campath-1H. The remaining glycosylated material was estimated by oxidation and digoxigen labelling using a Glycan Detection Kit from Boehringer Mannheim (No. 1142-372).

The isolated carbohydrates were derivatized with 1-phenyl-3-methyl-2-pyrazolin-5-1 (PMP) to facilitate UV detection by reverse phase HPLC. The oligosaccharides with sialic acids removed were also analysed by this method. The PMP derivatives were separated on a Spherisorb S30DS 2 reverse-phase C18 column (particle size 3 μ m, 15 cm \times 4.6 mm ID from Phase Separations Ltd).

Labelling of target cells by europium

This has been described by Blomberg *et al.* (1986). Briefly, 2×10^7 W133 cells were harvested and washed once in 50 mM HEPES, 93 mM NaCl, 5 mM KCl and 2 mM MgCl₂ adjusted to pH 7.4 by NaOH (buffer A). The cells were suspended in 1 ml buffer A containing 600 μ l EuCl₃, 3 mM diethylene triaminepentaacetate and 25 mg/ml dextran sulphate and incubated for 20 min at 4°C. Incubation was continued for 5 min after the addition of an equal volume of buffer B (buffer A containing 2 mM CaCl₂ and 10 mM L-glucose). Target cells were then washed 3 \times in buffer B and twice in Iscoves media supplemented with 2% FCS. The cell density was adjusted to 1×10^6 cells/ml in the same media and kept on ice.

Complement lysis assay

Standard Campath-1H, positive control, negative control (irrelevant antibody) and neuraminidase, GPF or β -galactosidase-treated Campath were diluted in Iscoves media containing 2% FCS. The samples (at three different dilutions) and the standard (50 μ l) were pipetted into

"U"-bottomed 96-well plates and equilibrated to 4°C for 10 min. Europium-labelled cells (150 μ l, 5×10^4 cells) containing 10% normal human serum were added and the plates allowed to incubate at 4°C for 30 min. The plates were then incubated at 37°C for 60 min for complement-mediated cell lysis, followed by centrifugation at 1800 rpm for 5 min to pellet intact cells. An aliquot of supernatant was taken (20 μ l). Lysis of W133 cells was estimated by quantifying fluorescence of europium released into the supernatant after mixing with enhancement solution (200 μ l). Complement lysis activity (units/ml) of samples was estimated from a standard curve obtained using standard Campath-1H. Specific complement lysis activity (units/ μ g) was derived using the equation below:

Specific complement lysis activity (units/ μ g)

$$= \frac{\text{Complement lysis activity (units/ml)}}{\text{Antibody concentration } (\mu\text{g/ml})}$$

whereby antibody concentrations were determined spectrophotometrically at 280 nm prior to the addition of enzymes.

Antibody-dependent cell-mediated cytotoxicity

Blood (100 ml) was obtained from normal healthy volunteers and defibrinated over glass beads. The blood was then diluted with an equal volume of phosphate-buffered saline and layered over 15 ml lymphocyte separation media and centrifuged at 1200 rpm for 30 min. Peripheral blood mononuclear cells (effector cells) at the interface were harvested and washed twice in Iscoves media containing 2% FCS. The cells were resuspended in Iscoves media containing 10% FCS at a cell density of 8×10^6 cells/ml and kept on ice.

Standard, positive control, negative control (irrelevant antibody) and digested Campath were diluted in Iscoves media containing 2% FCS. The samples (at three different dilutions) and the standard (50 μ l) were pipetted into "U"-bottomed 96-well plates. Europium-labelled W133 cells (50 μ l, 2×10^4 cells/well) were added and the plates incubated at 4°C for 30 min. Effector cells (100 μ l, 8×10^5 cells/well) were added and the cells pelleted by centrifugation at 1800 rpm for 5 min. The cells were then incubated at 37°C in a CO₂ incubator for 3.5 hr for cell lysis by antibody-dependent cellular cytotoxicity. After incubation, the cells were centrifuged again at 1800 rpm for 5 min. Lysis of target cells by ADCC was quantified by estimating the fluorescence of released europium into the supernatant (20 μ l) after added of enhancement solution (200 μ l). The ADCC activity (units/ml) of samples was estimated from a standard curve obtained using standard Campath-1H. Specific ADCC activity (units/ μ g) was derived using the equation below:

Specific ADCC activity (units/ μ g)

$$= \frac{\text{ADCC activity (units/ml)}}{\text{Antibody concentration } (\mu\text{g/ml})}$$

Antigen and protein A binding

Campath binding to HUT78 T cells, coated onto microtitre plates, were the basis of both these assays. The bound antibody was detected with either iodinated protein A (for protein A binding) or with iodinated sheep anti-human IgG (for antigen binding). Specific antibody-binding activity of samples was derived using the equation below:

Specific binding activity (units/ μ g)

$$= \frac{\text{Binding activity (units/ml)}}{\text{Antibody concentration } (\mu\text{g/ml})}$$

RESULTS

Digestion of CHO-derived Campath

Deglycosylated Campath from GPF digestion was immobilized onto nitrocellulose and stained with Ponceau-S to show binding of the protein to the membrane. Deglycosylated Campath was also oxidized and labelled with digoxigen using the Glycan Detection Kit. On comparison with undigested Campath, it was estimated that 85% of the Campath had been deglycosylated.

The intact glycans which had been released from GPF-treated samples, or from GPF-treated samples that had first been treated with neuraminidase or neuraminidase and β -galactosidase, were derivatized as described in the Methods section. Figure 2(a) is a typical carbohydrate map for non-digested Campath showing the majority of structures as non-sialylated biantennary structures [peaks (ii)–(vi)] and a small proportion of sialylated biantennary structures [peak (i)]. The carbohydrate maps resulting from digests are shown in Fig. 2(b) and (c) for the CHO-derived material. The absence of peak (i) in Fig. 2(b) shows that the removal of sialic acid after treatment with neuraminidase had occurred. When desialylated material was treated with β -galactosidase, the carbohydrate profile changed dramatically [Fig. 2(c)]. The monogalactosylated structures have disappeared [peaks (iii) and (iv)] and there is an increase in the peak of agalactosylated material [peak (v)]. Asialylated and β -galactosidase-treated preparations of Campath were used in activity experiments.

Functional activity of CHO-derived Campath after GPF digestion

Complement lysis activity of Campath was removed by GPF treatment in all four batches tested [Fig. 3(c)]. This reduction was shown to be due to GPF rather than the incubation conditions by the inclusion of the controls in the absence of GPF. The same samples were also analysed for ADCC activity. The GPF treatment removed ADCC activity in all batches [Fig. 3(d)]. The controls indicate that the reduction in activity was due to the GPF. These data indicate that the carbohydrate moiety of Campath is critical for supporting complement lysis and ADCC activities.

The loss of complement lysis and ADCC activities

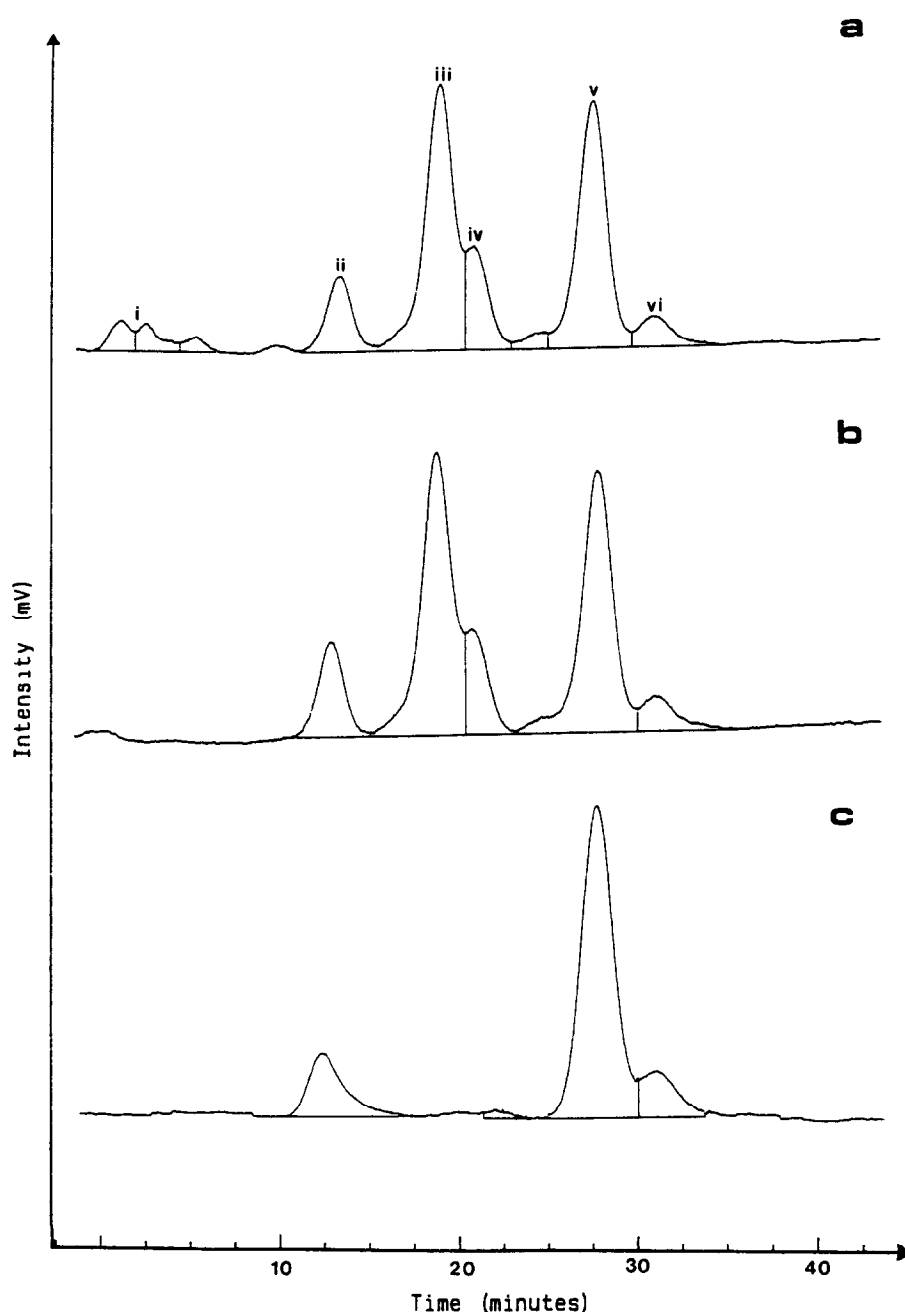


Fig. 2. Typical carbohydrate profile of released oligosaccharides from (a) CHO-derived Campath-1H (control); (b) CHO-derived Campath-1H digested with neuraminidase; and (c) CHO-derived Campath-1H digested with neuraminidase and β -galactosidase. (i) Monosialylated biantennary structures; (ii) asialo, galactosylated biantennary structures; (iii) and (iv) asialo, monogalactosylated, biantennary structures; (v) and (vi) asialo, agalactosylated, biantennary structures.

raised the question of whether the GPF-digested Campath retained antigen binding. This was determined using HUT78-coated microtitre plates and the results are shown in Table 1. The GPF treatment was found to have no effect on the protein A or antigen-binding activities of Campath. The loss of complement lysis and ADCC activities occurred despite the antigen and protein A binding regions being intact.

Functional activity of CHO-derived Campath after neuraminidase digestion

Three batches of Campath were analysed. Neuraminidase treatment resulted in no loss of complement

lysis or ADCC activity in all the batches tested [Fig. 3(a) and (b)]. This indicates that the sialic acid residues are not critical for either complement lysis or ADCC.

Functional activity of CHO-derived Campath after β -galactosidase digestion

Three batches of Campath were analysed for complement lysis activity [Fig. 3(e)]. The β -galactosidase treatment was found to reduce complement lysis activity by 35–50%. Whether complement lysis activity would have been completely abolished if all of the galactose residues were removed is unclear. However, the percentages of

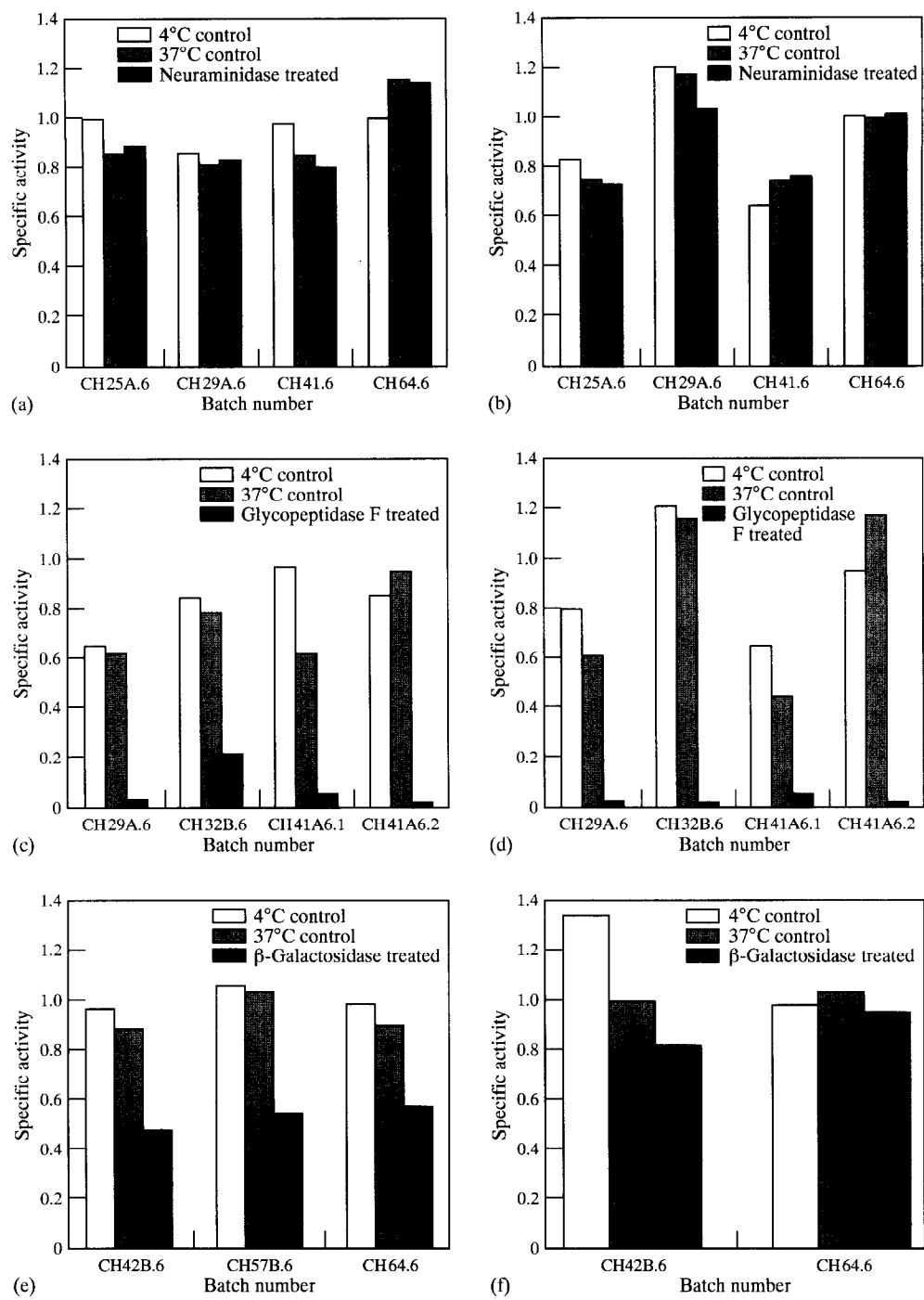


Fig. 3. Complement lysis and ADCC activity of Campath-1H, which was digested with neuraminidase [(a) and (b)], glycopeptidase F [(c) and (d)] and β -galactosidase [(e) and (f)]. The antibody was analysed for complement mediated cell lysis [(a), (c) and (e)] and ADCC activity [(b), (d) and (f)] according to the protocol described in Methods. Data were expressed as specific activities. Controls consisted of antibody incubated without enzymes at either 4°C or 37°C.

Table 1. The affect of GPF treatment on antigen and protein Abinding of CHO-derived Campath

Sample	Protein A binding specific activity	Antigen binding specific activity
GPF treated, 37°C	1.10	0.80
Control, 37°C	1.05	1.00
Control, 4°C	0.93	0.90

activities remaining following digestion and the percentages of galactosyl peaks remaining suggest that activity is unlikely to have been fully abolished if all the galactose was removed. In contrast, when ADCC activities of the antibodies were looked at, there was no obvious decline in activity after digestion with β -galactosidase.

Digestion of the myeloma-derived Campath

Figure 4 shows the effect of the digestion of myeloma-derived Campath with GPF or neuraminidase, the parallel digests to those in Fig. 3. The carbohydrate map is slightly different from the CHO-derived Campath. There is a higher proportion of sialylated biantennary structures and a different ratio of non-sialylated biantennaries in the myeloma-derived material. Reverse-phase HPLC analysis demonstrated that the glycan was released by the GPF digestion, and sialic acids were removed by neuraminidase (data not shown).

Functional activity of myeloma-derived Campath after neuraminidase and GPF digestion

Myeloma-derived Campath was digested with neuraminidase and analysed for CMCL and ADCC [Fig.

4(a)]. No difference in CMCL was detected between the neuraminidase digest and the undigested controls. Although ADCC activity of the control sample was lower than previous preparations of Campath, neuraminidase digestion did not affect ADCC activity. These results are similar to CHO-derived Campath.

Myeloma-derived Campath was digested with GPF and analysed for functional activity [Fig. 4(b)]. Controls showed no change in either CMCL or ADCC. In contrast, GPF abolished both CMCL and ADCC activity. Consequently, the functional activity of myeloma-derived Campath was similar to CHO-derived Campath following GPF digestion.

Neuraminidase- and GPF-treated samples were tested for protein A and antigen binding. Similar to CHO-derived Campath, myeloma Campath experienced no reduction in either activities on digestion.

DISCUSSION

The role of the carbohydrate moiety in IgG has been the subject of interest for many years (Table 2). Its entire role has still to be determined, but it is vital to certain important activities. The central role of the carbohydrate is shown by the removal of the structures by GPF. This abolished CMCL and ADCC activities (Fig. 3), but left Campath with the ability to bind antigen and protein A (Table 1). These results agree with those found in the literature (Table 2). The ADCC activity, and to a lesser extent complement lysis activity, are thought to be important in *in vivo* function (Dyer *et al.*, 1990), indicating that the carbohydrate may be essential to Campath-1H efficacy. Carbohydrate is thought to be important in determining the conformation of the molecule. Protein A is used as the basis of Campath purification and the results indicate that Campath deficient in carbohydrate (if present) would co-purify with its glycoform. In addition, assays based solely on antigen and protein A binding would not be sensitive to changes in the carbohydrate content, whereas CMCL and ADCC assays would be sensitive to the more major alterations.

Given that the carbohydrate moiety is essential to the functional activity of Campath, the question arises as to which sugar residues are critical for activity. In a preliminary attempt to answer this, the terminal sialic acid residues were removed by neuraminidase digestion. This was found to have no affect on the activities of CMCL, ADCC (Fig. 3), antigen binding or protein A binding (Table 1). It appears that sialic acid residues have little affect on Campath function. There is a proviso in this statement in that there was little sialic acid present in the first place, so that its removal would only be predicted to result in a small change in activity. Given the proviso, it follows that heterogeneity in the content of sialic acid residues, which is known to occur in Campath (R. Lively, personal communication) would not be expected to compromise the antibody function.

Galactose becomes the terminal carbohydrate following removal of the sialic acid. Treatment of asialyl Campath with β -galactosidase was found to remove all

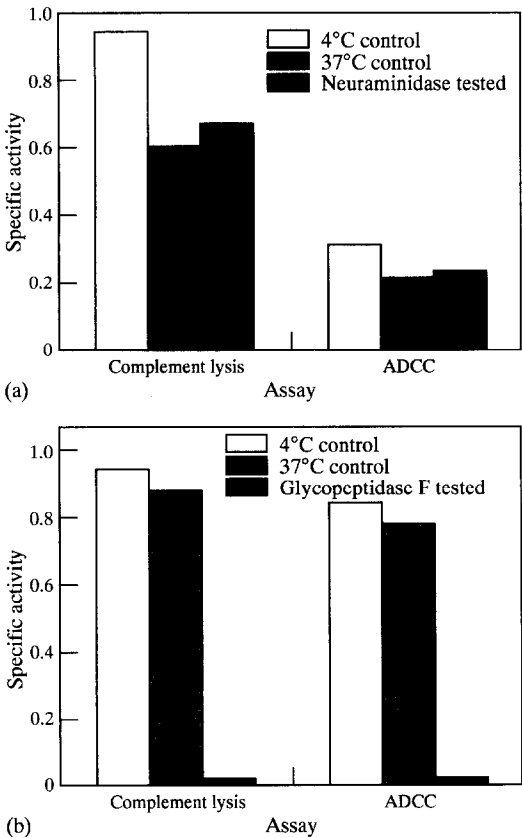


Fig. 4. Campath-1H derived from the myeloma cell line was digested with either neuraminidase (a) or glycopeptidase F (b). Digested antibody was compared in the complement lysis and ADCC assays against similarly treated control preparations of Campath-1H incubated without enzymes at either 4°C or 37°C. Data were expressed as specific activities as described in the Methods section.

Table 2. The effect of IgG carbohydrate on its functional activities

Reference	Model	Treatment	Activities independent of its carbohydrate	Activities dependent on its carbohydrate
Williams <i>et al.</i> (1973)	Rabbit IgG	Glycosidase vs <i>E. coli</i> vs <i>S. aureus</i> vs <i>Streptococ.</i> vs <i>Pneumoc.</i>		HA, complement fixation, opsonic HA, complement fixation, opsonic HA, complement fixation, opsonic Complement fixation, opsonic
Koide <i>et al.</i> (1977)	Rabbit IgG	Glycosidase	Antigen binding	ADCC, CMCL Rosette formation
Nose and Wigzell (1983)	Mouse IgG2b	Tunicamycin	Antigen binding	ADCC
			Protein A binding	Complement fixation Longer serum half-life
Heyman <i>et al.</i> (1985)	Mouse IgG2b	Tunicamycin	Antigen binding	Macrophage FcR binding
			Protein A binding	Complement fixation Longer serum half-life
Leatherbarrow <i>et al.</i> (1985)	Mouse IgG2a	Tunicamycin		Monocyte binding
Rothman <i>et al.</i> (1989)	Mouse IgG	Tunicamycin	Antigen binding	C1 activation PMN ADCC PBL or monocyte ADCC
Tao and Morrison (1989)	Mouse-human IgG1	Mutagenesis	Protein A binding	Protease sensitivity
Lund <i>et al.</i> (1990)	Mouse-human IgG3	Tunicamycin		Serum half-life ADCC
Matsuda <i>et al.</i> (1990)	Human IgG1	<i>E. coli</i> expression system	FcR1 binding Protein A binding	C1 activation
Dorai <i>et al.</i> (1991)	Mouse-human IgG1	Site-directed mutagenesis	FcR1 binding	
Pound <i>et al.</i> (1993)	Mouse-human IgG1	Tunicamycin	Serum half-life and body distribution Respiratory burst in monocytes	ADCC CMCL (slight)

but the digalactosyl glycans. Removal of the monogalactosyl glycans was found to reduce the complement lysis activity by almost one-half, but caused no reduction in ADCC activity. In our experience, it is not usual to see the two functional activities of IgG differ on treatment. However, the differential effect of galactose removal on the two functional activities can be easily explained as being due to the different binding sites that IgG has for C1q and Fc receptors. Changes in carbohydrate have greater effect on complement lysis activity, presumably because the activity is more sensitive to the conformational changes that result. It is considered that the

galactose content of IgG would be important in dictating the activity of Campath *in vivo*. The fact that, in this instant, complement lysis activity is a more sensitive indicator of functional intactness of the IgG than ADCC activity does not mean that it will always be this way.

Campath from both CHO and myeloma expression systems were analysed. No difference in the behaviour of the two Campaths to GPF or neuraminidase digestion was seen (Fig. 4). The only difference seen between the two was in the carbohydrate maps, where the asialylbiantennary peaks are present in different ratios, and the sialylated moieties are in greater proportions in the

myeloma Campath. The latter observation would not be expected to affect functional activity from what is reported here.

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