Biochem. J. (1992) 285, 839–845 (Printed in Great Britain)

Different culture methods lead to differences in glycosylation of a murine IgG monoclonal antibody

Thakor P. PATEL,* Raj B. PAREKH,*§ Bill J. MOELLERING† and Christopher P. PRIOR‡

*Oxford GlycoSystems Ltd, Hitching Court, Blacklands Way, Abingdon, OX14 1RG, Oxon, U.K.,
†Amgen, 1900 Oak Terrace Lane, Thousand Oaks, CA 91320, U.S.A., and
‡Rhone-Poulenc-Rorer, 680 Allendale Road, King of Prussia, PA 19406, U.S.A.

A monoclonal IgG-1 was produced by culture of a murine hybridoma (3.8.6) by three different methods, namely culture in ascites, in serum-free media and in serum-supplemented media. IgG-1 was purified to homogeneity (as judged by SDS/PAGE under reducing conditions) from each medium by ion-exchange chromatography and h.p.l.c. Protein A chromatography. Oligosaccharides were released from each IgG-1 preparation by hydrazinolysis and radiolabelled by reduction with alkaline sodium borotritide, and 'profile' analysis of the radiolabelled oligosaccharide alditols was performed by a combination of paper electrophoresis and gel-filtration chromatography. This analysis indicated clear and reproducible differences in the glycosylation patterns of the three IgG-1 preparations. Sequential exoglycosidase analysis of individual oligosaccharides derived from each IgG-1 preparation was used to define these differences. Ascites-derived material differed from serum-free-culture-derived material only with respect to the content of sialic acid. IgG-1 derived from culture in serum-containing media had an intermediate sialic acid content and a lower incidence of outer-arm galactosylation than the other two preparations. These differences in glycosylation could not be induced in any IgG-1 preparation by incubating purified IgG-1 with ascites or culture medium. It is concluded that the glycosylation pattern of a secreted monoclonal IgG is dependent on the culture method employed to obtain it.

INTRODUCTION

All antibodies, whether monoclonal or serum-derived, are glycoproteins. In the case of IgG antibodies, glycosylation involves the attachment of N-glycans to the conserved Asn-297 residue in the Fc region of the heavy chains (for a review, see Rademacher *et al.*, 1988). Additional N-glycans may be attached to N-glycosylation sites (Asn-Xaa-Ser/Thr) in the hypervariable regions (Spiegelberg *et al.*, 1970; Sox *et al.*, 1970). O-Glycans have not been reported on either human or murine IgG molecules.

Glycosylation of IgG influences both its biological function and its physicochemical properties (Winkelhake, 1978; Nose & Wigzell, 1983; Leatherbarrow *et al.*, 1985; Malaise *et al.*, 1987). These include resistance to proteases, binding to monocyte Fc receptors, interaction with complement component CIq, feedback immunosuppression of IgG synthesis, and circulatory lifetime *in vivo*. Changes in the sequence of N-glycans attached to IgG have been clearly shown to be associated with disease activity in rheumatoid arthritis (Parekh *et al.*, 1985). It has further been suggested that such changes contribute to the pathology of the disease by predisposing the IgG to self-associate into immune complexes, and by activating macrophages by binding to the GlcNAc receptor on the surface of these cells (Rademacher *et al.*, 1988).

Monoclonal antibodies are finding increasing use as specific *in vitro* and *in vivo* diagnostic reagents, as therapeutic agents, and as ligands for affinity purification of both macromolecules and smaller-molecular-mass substances. Murine monoclonal antibodies are generally obtained in one of three ways, namely after cell culture in ascites, serum-containing media or serum-free media. It can be the case that production methods for a given antibody can vary through a process development program, usually involving initial isolation from ascites to large-scale production from a variety of *in vitro* culture techniques. Given the importance of glycosylation to the functional properties of IgG, and the preference to obtain a structurally defined, or at least biochemically consistent, product for *in vivo* administration, we have compared the glycosylation of a murine IgG monoclonal antibody derived after expression in ascites, serum-containing media and serum-free media. In each case, the IgG was purified from each culture fluid using the same protocol. The results clearly indicate that IgG glycosylation is significantly influenced by culture method.

EXPERIMENTAL

Materials

Cell line. Murine hybridoma (3.8.6) producing IgG-1 subclass was provided by Invitron Corporation (St. Louis, MO, U.S.A.).

Cell culture medium components. Materials were obtained from the sources indicated: bovine transferrin, BSA and human insulin (Miles Inc., Kankakee, IL, U.S.A.); trypsin (Sigma, St. Louis, MO, U.S.A.); Iscove's basal medium and foetal calf serum (Hazleton Biologics, Lenexa, KA, U.S.A.); ascites fluid generated from myelomas (non-IgG producers) in Balb/c mice (Invitron Corp., Redwood City, CA, U.S.A.); ascites fluid containing IgG-1 (Charles River Biotechnology Services, Wilmington, MA, U.S.A.); 0.2 μm-pore-size Sartobran capsule filter and mini-Ultrasort (20 kDa cut-off) (Sartorius, New York, NY, U.S.A.).

Additional materials were obtained from the sources indicated: S-Sepharose Fast-Flow, Q-Sepharose Fast-Flow and CNBr-activated Sepharose (Pharmacia LKB Biotechnology, Piscataway, NJ, U.S.A.); Affi-Prep Protein A analytical cartridge (Bio-Rad Laboratories, Richmond, CA, U.S.A.); kit for quantification of bovine IgG (ICN Immunobiologicals). All reagents for SDS/PAGE analysis were from Sigma. All exoglycosidases were

Abbreviations used: PNGase F, peptide *N*-glycosidase F; PBS, phosphate-buffered saline (140 mM-NaCl, 3 mM-KCl, 10 mM-Na_2HPO_4, 2 mM-KH_2PO_4, pH 7.2); TBS, Tris-buffered saline (140 mM-NaCl, 3 mM-KCl, 25 mM-Tris base, pH 8.0); DTT, dithiothreitol; TFA, trifluoroacetic acid; ABTS, 2,2-azinobis(3-ethylbenzothiazoline-6-sulphonic acid).

§ To whom correspondence should be addressed.

from Oxford GlycoSystems (Abingdon, Oxon, U.K.). Dowex AG50X 12 (H^+) and Chelex 100 (Na^+) were from Bio-Rad (Watford, Herts., U.K.).

Ascites culture

The origin of the hybridoma used in this study is as follows. Mice (Balb/c) were injected intraperitoneally on day 0 with a mixture (1:1, v/v) of protein antigen (U.S. patent No 4; 631509113082) (50 mg·ml^{-1}) in Tris-buffered saline (TBS)/complete Freund's adjuvant. On day 14 and then on day 38, the mice were injected with a mixture (1:1, v/v) of protein antigen (50 mg·ml^{-1}) in TBS/incomplete Freund's adjuvant. On day 42, spleens were surgically removed and cells fused with the myeloma partner to form hybridomas as described by Brown *et al.* (1980). Positive hybridoma clones were identified using a radioimmunoassay, and positive clones were sub-cloned at least twice. After karyotypic analysis, stable sub-clones were then injected into the intraperitoneal cavities of Balb/c mice which had been pretreated intraperitoneally with 0.5 ml of Pristane 5 days prior to injection of hybridoma cells. Hybridoma cells were injected (5×10^6 cells/mouse) in 0.5 ml of Dulbecco's modified Eagle's medium (without foetal bovine serum). Ascites fluid was collected (in heparin), and pooled from several mice for subsequent isolation of the monoclonal IgG. The same hybridoma was used to derive the ascitic IgG as was used to derive IgG from cell culture.

Serum culture

Murine hybridomas were inoculated into a 3 litre spinner at a seeding density of around 2×10^5 cells/ml and were harvested following 3.5 days of growth at 1×10^6 cells/ml. The medium contained 2% foetal calf serum, 2 μg of insulin/ml, 10 μg of bovine transferrin/ml and Iscove's basal medium. Cell viability was measured by Trypan Blue staining and phase-contrast light microscopy using a described procedure (Adams, 1990). Cells were harvested at a viability of > 95%. In addition to microscopic examination, assay of the cell-free supernatant for lactate dehydrogenase activity also indicated negligible cell lysis.

Serum-free culture

The conditions were essentially the same as described above, except that 0.1% BSA was used in place of 2% foetal calf serum. Cells were harvested at a viability of > 95%, as described above.

Recovery of IgG-containing culture medium

Culture fluids were initially filtered to remove cells and debris using a 0.2 μm-pore-size Sartobran capsule filter. The clarified fluid was then concentrated approx. 15-fold using a mini-Ultrasort with a 20 kDa molecular mass cut-off.

Purification of IgG

IgG was purified from each culture medium using the following protocol. Concentrated medium was diluted 10-fold (with 10 mM-sodium acetate, pH 5.9) and loaded on to an S-Sepharose column (dimensions 6 cm × 30 cm; pre-equilibrated with 20 mM-sodium acetate, pH 5.9, containing 10 mM-NaCl). IgG was then eluted (20 mM-dibasic sodium phosphate, pH 5.9, containing 85 mM-NaCl); the pH of the eluate was adjusted to 6.9, and then the eluate was loaded on to a Q-Sepharose column (3 cm × 30 cm; pre-equilibrated in 20 mM-sodium phosphate, pH 5.9, containing 85 mM-NaCl). The IgG was not bound to Q-Sepharose under these conditions, whereas most contaminating proteins were. The unbound IgG fraction so obtained was diluted 3-fold (with 20 mM-sodium acetate, pH 5.5) and loaded on to the S-Sepharose column (equilibrated with 20 mM-sodium acetate, pH 5.5, containing 30 mM-NaCl). The IgG bound to the S-Sepharose, and after washing (5 column vol. of equilibration buffer) it was eluted (with 20 mM-sodium acetate, pH 5.5, containing 200 mM-NaCl). After this initial purification, samples were ultra-purified by h.p.l.c. Protein A chromatography. The IgG sample was dialysed into sodium phosphate buffer, pH 8.9, containing 3 M-NaCl and 1.5 M-glycine, an aliquot was loaded on to an Affi-Prep Protein. A cartridge, and the IgG was eluted using a pH 4.0 solution of 0.1 M-citric acid. The final ultra-purified IgG preparation was shown to be pure and homogeneous by SDS/PAGE analysis (according to Laemmli, 1970) under reducing conditions (see Fig. 1).

Tryptic mapping of monoclonal IgG-1 preparations

To IgG samples at 1 mg/ml in phosphate-buffered saline (PBS) was added guanidine hydrochloride (final concentration of 6 M), followed by 10 μl of 500 mM-dithiothreitol (DTT). Samples were incubated (30 min) under nitrogen at 37 °C. The resultant reduced protein was carboxymethylated by addition of a volume (100 μl) of 500 mM-iodoacetate in 100 mM-Tris/HCl (pH 8.15)/6 M-guanidine HCl/2 mM-EDTA to each sample tube, followed by a 1 h incubation at 37 °C in the dark under nitrogen. The samples were then dialysed overnight against PBS. Reduced/carboxymethylated samples (500 μg) were digested overnight at 37 °C with 10 μl of trypsin at 1 mg/ml. Each sample was brought to 0.1% trifluoroacetic acid (TFA) and chromatographically separated on a 15 cm C_{18} reversed-phase resin (Waters, Milford, CT, U.S.A.) equilibrated at 45 °C. Solvent A consisted of 0.1% TFA in water while solvent B was 0.1% TFA in 60% acetonitrile. The gradient was a linear increase from 0% to 100% solvent B in solvent A over a 120 min run time at a flow rate of 1 ml/min. Detection was at 229 nm.

Amino acid composition analysis

This was performed using the Picotag method with subsequent detection of phenyl thiocyanate-derivatized amino acids (Waters; Picotag Station).

Immunoglobulin subclass identification

Duplicate columns of wells on an e.l.i.s.a. plate were coated with capture antibody to each mouse IgG subclass (IgG-1, IgG-2a, IgG-2b, IgG-3), blocked with BSA buffer (3% BSA in PBS), rinsed and incubated with aliquots of sample and three levels of the same subclass of mouse antibody (quantitative positive control). The e.l.i.s.a. plate was then rinsed and all wells were incubated with peroxidase-conjugated goat anti-mouse IgG antibody (Porstmann *et al.*, 1985). Plates were washed in phosphate-buffered Tween solution (0.1% Tween in PBS) to remove unbound conjugate and then developed by incubation with ABTS/H_2O_2 development solution. The amount of sample bound to each IgG specificity was compared to the 3-point standard curve for each IgG subclass to calculate the percentage of each subclass present.

Release, labelling and isolation of IgG-associated glycans

Three separate aliquots (approx. 1 mg) of each purified IgG preparation were exhaustively dialysed (micro-flow dialysis) against 0.1% (v/v) TFA. The dialysed solution was lyophilized and then cryogenically dried over activated charcoal at −196 °C (< 10^{11} Pa). The dried preparation was then incubated with anhydrous hydrazine, and the liberated N-glycans were re-*N*-acetylated, separated from peptide, radiolabelled and purified as follows. The samples were suspended in 200 μl of freshly distilled anhydrous hydrazine (toluene/CaO; 25 °C; 1.33 kPa) under an argon atmosphere. The temperature was raised at 17 °C/h from 30 to 85 °C, and then maintained at 85 °C for a further 12 h. The hydrazine was removed by evaporation under reduced pressure (< 10 Pa) at 25 °C followed by repeated (5×) flash evaporation

from anhydrous toluene. The hydrazinolysates were *N*-acetylated by the addition of excess (5× over amino groups) acetic anhydride (0.5 M) in saturated $NaHCO_3$ at 4 °C for 10 min. The temperature was then raised to 25 °C, a second aliquot of acetic anhydride added and the reaction allowed to proceed for a further 50 min. Following *N*-acetylation, the samples were applied to a column of Dowex AG50X 12 (H^+), eluted with water and evaporated to dryness (27 °C). The desalted samples were dissolved in water and applied to Whatman 3MM chromatography paper. Descending paper chromatography (27 °C, 60% relative humidity) was subsequently performed with butan-1-ol/ethanol/water (4:1:1, by vol.) (solvent I). After 48 h, the first 5 cm measured from the origin was eluted with water, flash-evaporated to dryness (27 °C), redissolved in a 5× molar excess of 1 mM-copper(II) acetate, and incubated at room temperature for 45 min. After passage through a tandem column of Chelex 100 (Na^+)/Dowex AG50X 12 (H^+), the oligosaccharides were flash-evaporated to dryness (27 °C) and reduced with a 5× molar excess of 6 mM-NaB^3H_4 (12 Ci/mmol; Amersham) in 50 mM-NaOH adjusted to pH 11 with saturated boric acid (30 °C, 4 h). The mixture was then acidified (pH 4–5) with 1 M-acetic acid and applied to a column of Dowex AG50X 12 (H^+), eluted with water, evaporated to dryness (27 °C) and flash-evaporated (27 °C) from methanol (5×). The samples were then applied to Whatman 3MM chromatography paper and subjected to descending paper chromatography for 2 days using solvent I.

Radiochromatogram scanning was performed with an LB230 Berthold radiochromatogram scanner. The radioactivity remaining at the origin was subsequently eluted with water. The isolated oligosaccharides were subjected to high-voltage paper electrophoresis in pyridine/acetic acid/water (3:1:387, by vol.; pH 5.4, Whatman 3MM, 80 V/cm) in order to determine the relative proportions of neutral and acidic components. Samples of the reduced (^3H-labelled) oligosaccharides were also subjected to exhaustive neuraminidase digestion, and the products were separated by high-voltage paper electrophoresis. All radioactivity remaining at the origin following neuraminidase digestion was recovered by elution with water and desalted by using a tandem column of Chelex 100 (Na^+)/Dowex AG50X 12 (H^+)/AG3X 4A (OH^-)/QAE-Sephadex A-25. The eluate and washings were evaporated to dryness, resuspended in 175 μl of a 2 mg/ml partial dextran hydrolysate, and applied to a Bio-Gel P4 (~400 mesh) gel-permeation chromatography column (1.5 cm × 200 cm). The eluant was monitored for radioactivity by using an LB503 Berthold h.p.l.c. radioactivity monitor, and for refractive index by using an Erma ERC 7510 refractometer. Analogue signals from the monitors were digitized with Nelson Analytical ADC interfaces. The digital values were collected and analysed on Hewlett–Packard 9836 C computers. The P4 chromatograms show radioactivity (vertical axis) plotted against retention time. The numerical superscripts refer to the elution position of the glucose oligomers in glucose units, as detected simultaneously by the refractive index monitor (results not shown). V_0 is the void position. Sample elution positions (in glucose units) were calculated by cubic spline interpolation between the internal standard glucose oligomer positions. Glycosidase digestions of the recovered radiolabelled alditols were performed as described below.

Exoglycosidase digestions

Digestion of ^3H-labelled oligosaccharides (~2×10^6 c.p.m.) with exoglycosidases of defined specificities was carried out under the following conditions: jack bean β-*N*-acetylhexosaminidase, 20 μl of a 10 units/ml solution in 0.1 M-citrate/phosphate, pH 4.0; jack bean α-mannosidase, 20 μl of a 50 units/ml solution in 0.1 M-sodium acetate, pH 4.5; *Streptococcus pneumoniae* β-galactosidase, 20 μl of a 0.2 unit/ml solution in 0.1 M-citrate/phosphate, pH 6.0; *S. pneumoniae* β-*N*-acetylhexosaminidase, 20 μl of a 0.1 unit/ml solution in 0.1 M-citrate/phosphate, pH 6.0; bovine epididymis α-fucosidase, 20 μl of a 1 unit/ml solution in 0.2 M-citrate/phosphate, pH 6.0; *Arthrobacter ureafaciens* neuraminidase, 20 μl of a 10 units/ml solution in 0.1 M-sodium acetate, pH 5.0; *Achatina fulica* β-mannosidase, 20 μl of a 0.2 unit/ml solution in 0.5 M-sodium citrate, pH 4.0. Where applicable, 1 unit of exoglycosidase is defined as the amount of enzyme which, in control experiments, releases 1 μmol of 4-nitrophenol from the respective 4-nitrophenyl glycoside/min at 37 °C. All incubations were carried out for 18 h at 37 °C under toluene, and were terminated by heating to 100 °C for 2 min.

Monosaccharide composition analysis

This was performed essentially as described by Chaplin (1982). To the salt-free cryogenically dried hydrazinolysate (after re-*N*-acetylation) from 0.2 mg of salt-free cryogenically dried IgG preparation containing 10 nmol of *scyllo*-inositol was added 0.2 ml of anhydrous methanolic HCl (0.5 M). The mixture was incubated under anhydrous conditions at 85 °C for 16 h, after which pyridine (10 μl) and acetic anhydride (5 μl) were added. The reaction mixture was vortex-mixed and left at room temperature for 1 h. Reagents were removed by rotary evaporation, traces of pyridine were removed by repeated (3×) flash evaporation from methanol (100 μl), and the rotary-evaporated mixture was then again cryogenically dried prior to silylation. Silylation of the 1-*O*-methyl glycosides was performed by the addition of 25 μl of Sil-A (Sigma), brief sonication of the mixture and incubation at room temperature for 30 min. The excess Sil-A was removed by rotary evaporation, and the 1-*O*-methyl trimethylsilyl glycosides were taken up in anhydrous n-hexane. The 1-*O*-methyl TMS glycosides were separated by capillary GC (using a Hewlett–Packard 5890 Series II gas chromatograph), identified with respect to retention time and mass spectrum by reference to standard compounds, and quantified from the flame ionization detector response relative to *scyllo*-inositol.

RESULTS

Purity of IgG antibody

Each final antibody preparation was deemed to be >98% pure, as judged by SDS/PAGE analysis (Fig. 1), h.p.l.c. size-exclusion chromatography analysis for bovine IgG, and subclass testing which indicated the presence of only IgG-1 (content >95%). Endogenous murine IgG of subclasses other than IgG-1 in the ascites preparation was not revealed by the subclass testing, and is in any case likely to be irrelevant to this study since the yield of monoclonal IgG-1 from ascites fluid was >100 mg/ml of fluid, and therefore in vast excess over endogenous IgG. Radioimmunodiffusion/Ouchterlony analysis indicated an absence of bovine IgG (<0.5%), and BSA was absent as judged by SDS/PAGE analysis (results not shown). Under non-reducing conditions, a typical cluster of bands with an identical pattern was seen for all three preparations at ~160 kDa (results not shown). Under reducing conditions (Fig. 1), one band was seen at ~50 kDa (representing H chains) and two at ~25 kDa, representing a light chain contribution from the myeloma cell line. Densitometric scanning following SDS/PAGE revealed a constant ratio of the two light chains in each sample. Tryptic mapping and amino acid analysis (see the Experimental section) further indicated identity between these three IgG-1 samples. All bands visualized by SDS/PAGE are related to mouse IgG, as established by Western blot analysis

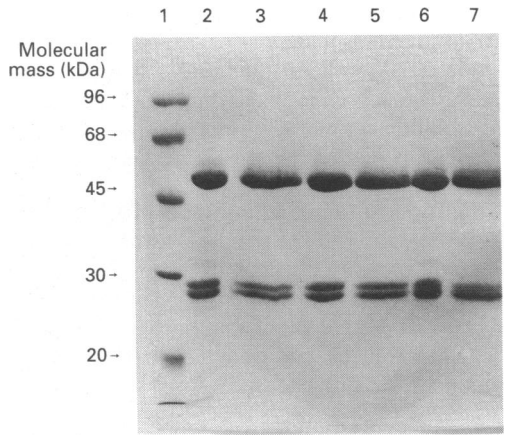

Fig. 1. SDS/PAGE (10% reducing gel) analysis of IgG preparations

Lane 1, molecular mass standards, as indicated; lane 2, IgG purified (ion-exchange) from ascites; lane 3, IgG purified from ascites and re-incubated in fresh ascites (IgG then recovered by protein A h.p.l.c.); lane 4, IgG purified (ion-exchange) from media after culture of hybridoma in serum-containing media; lane 5, IgG purified (ion-exchange) from media after culture of hybridoma in serum-containing media and then re-incubated in fresh ascites; lane 6, IgG purified (ion-exchange) from media after culture of hybridoma in serum-free media; lane 7, IgG purified (ion-exchange) from media after culture of hybridoma in serum-free media and then re-incubated in fresh ascites.

using anti-mouse reagents (results not shown). No other bands were visible. Each preparation was therefore deemed to be essentially homogeneous and monoclonal with respect to polypeptide, and suitable for a comparative carbohydrate analysis. Finally, IgG-1 samples were obtained from serum-containing and serum-free cell culture conditions under which comparable cell viability (> 95%) and integrity had been maintained, and cell-conditioned medium was harvested in both cases at the same stage of the growth cycle (early plateau phase).

Monosaccharide compositions of the three IgG preparations

The results of monosaccharide composition analysis, expressed as nmol of monosaccharide/mg of IgG, are summarized in Table 1. Each IgG preparation was analysed in triplicate and the mean value is stated. (In all cases the difference between the highest and lowest values determined was < 10% of the mean value.) The data in Table 1 are consistent with the presence of only complex bi-antennary N-glycans on each IgG preparation. This is expected from previous reports on the analysis of murine IgG (Mizuochi *et al.*, 1987). The data further suggest that a similar number of N-glycan chains are present on each IgG preparation, as judged by the similar amounts of mannose and *N*-acetylglucosamine. The gel-filtration chromatograms (see later) showed that the glycans in all three preparations all contained the conserved trimannosyl chitobiosyl core. Assuming a content, therefore, of three mannose residues per glycan chain, and a molecular mass of 150 kDa for the IgG, the number of glycan chains can be calculated (see Table 1). These calculations indicate that, besides the presence of two chains in the Fc region, additional glycosylation occurs in the Fab region. This is supported by the detection of di-sialylated oligosaccharides in two of the IgG preparations. There are, however, clear differences with respect to the terminal processing of these chains, i.e. concerning the addition of outer-arm galactose and sialic acid residues.

Relative molar content of neutral, mono- and di-sialylated oligosaccharide chains in each IgG preparation

A sample of the total radiolabelled alditol pool from each IgG preparation was fractionated on the basis of charge by paper electrophoresis before and after treatment with neuraminidase. The resulting radioelectrophoretograms for the untreated alditols are shown in Fig. 2 (the triplicate analyses generated indistinguishable chromatograms for each preparation). By recovery of radioactivity from paper the relative contents of neutral, mono-sialylated and di-sialylated oligosaccharides in the three preparations could be determined (Table 2). These data are again as expected from previously reported analyses of murine IgG, and are consistent with the sialic acid composition data presented in Table 1. The radioelectrophoretogram after treatment with neuraminidase showed only a single peak at the origin (results not shown), indicating essentially quantitative de-sialylation. These data show that the three preparations differ with respect to their content of sialylated oligosaccharides. The ascites-derived material had no detectable sialylated oligosaccharides, the IgG derived after culture in serum-free media had a significant content of sialylated oligosaccharides, and the IgG derived after culture in serum-containing media had an intermediate content. The presence of di-sialylated oligosaccharides suggests the occurrence of Fab glycosylation (Rademacher *et al.*, 1986). Di-sialylated oligosaccharides are reported to be absent from Fc, based on both structural considerations and experimental analysis. The IgG must therefore carry an N-glycosylation site in the Fab region. Assuming the IgG preparation to be truly clonal with respect to amino acid sequence, each IgG would be expected to carry at least four N-glycan chains (two in the Fc and two in the Fab). The calculations shown in Table 1 indicate that each IgG carries only 0.3–0.4 chains in the Fab region. Attachment of N-glycans to the N-glycosylation sites in the Fab is therefore incomplete, as observed previously for another murine monoclonal antibody (Arvieux *et al.*, 1986).

Gel-filtration chromatograms of the deacidified oligosaccharides recovered from each IgG preparation

The oligosaccharide alditol pool after neuraminidase treatment was fractionated by Bio-Gel P4 (−400 mesh) gel-permeation chromatography. The resulting chromatograms are shown in Fig. 3. Individual fractions were pooled as indicated, and the

Table 1. Monosaccharide content of each IgG preparation

Figures in parentheses indicate the relative ratio of that monosaccharide to mannose in the particular IgG preparation. Monosaccharides other than those listed were not detected. n.d., not detectable.

Source of IgG	Monosaccharide content (nmol/mg)					Glycan chains/IgG
	Sialic acid	Gal	Man	Fuc	GlcNAc	
Ascites	n.d.	8.2 (0.17)	47 (1)	12 (0.36)	72 (1.5)	2.3
Serum-containing media	3.4 (0.07)	5.1 (0.11)	46 (1)	14 (0.3)	75 (1.6)	2.3
Serum-free media	6.9 (0.14)	5.5 (0.10)	49 (1)	11 (0.22)	79 (1.6)	2.4

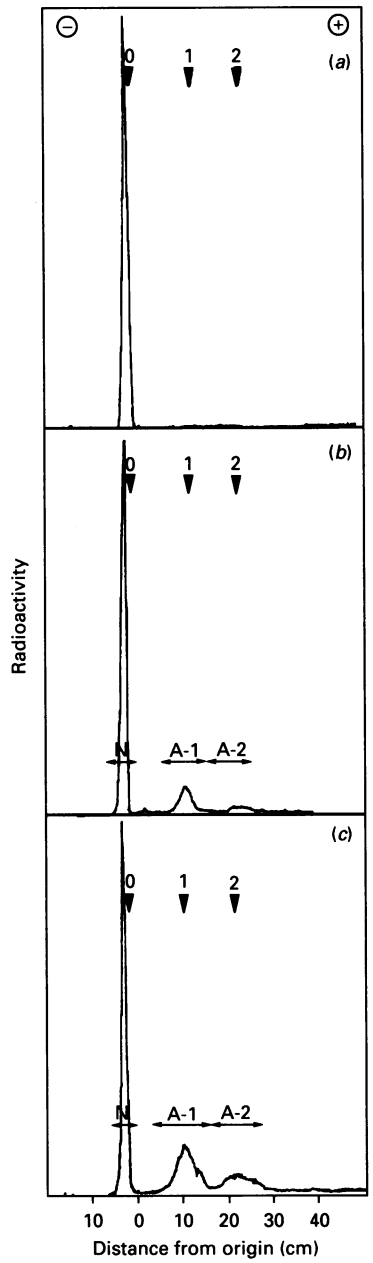

Fig. 2. High-voltage radioelectrophoretograms of the radiolabelled alditols derived from each IgG preparation

The labels 0, 1, and 2 mark the migration positions of authentic neutral, mono-sialylated, and di-sialylated oligosaccharide alditols (derived from human serum IgG) respectively. (*a*) Ascites, (*b*) serum-containing medium, (*c*) serum-free medium. The indicated regions N, A-1 and A-2 were eluted (with water) and the total radioactivity in each eluate was determined by liquid scintillation counting.

relative radioactivity in each was determined (Table 3). These chromatograms indicate that the three IgG preparations differ not only with respect to sialylation but also with respect to other structural determinants. To define these differences further, fractions A, B, C and D from each IgG preparation were analysed by sequential exoglycosidase digestion (Kobata, 1984).

Exoglycosidase analysis of fractions A, B, C and D recovered from the gel-filtration chromatograms

Each fraction was incubated sequentially with individual exoglycosidases. After each incubation, the products were separated by P4 gel filtration, collected and incubated with the next exoglycosidase. Assignment of monosaccharide sequence, anomeric configuration of individual glycosidic linkages and, in the case of outer-arm galactose and *N*-acetylglucosamine residues,

Table 2. Relative molar content of neutral, mono-sialylated (A-1) and di-sialylated (A-2) oligosaccharides in the glycan population obtained from each IgG preparation

Triplicate analyses for each IgG preparation yielded the same value (to ±1%) for the percentage of neutral, A-1, and A-2 structures. n.d., not detectable.

	Molar content (%)		
Source of IgG	Neutral	A-1	A-2
Ascites	> 98	n.d.	n.d.
Serum-containing media	81	15	4
Serum-free media	71	18	11

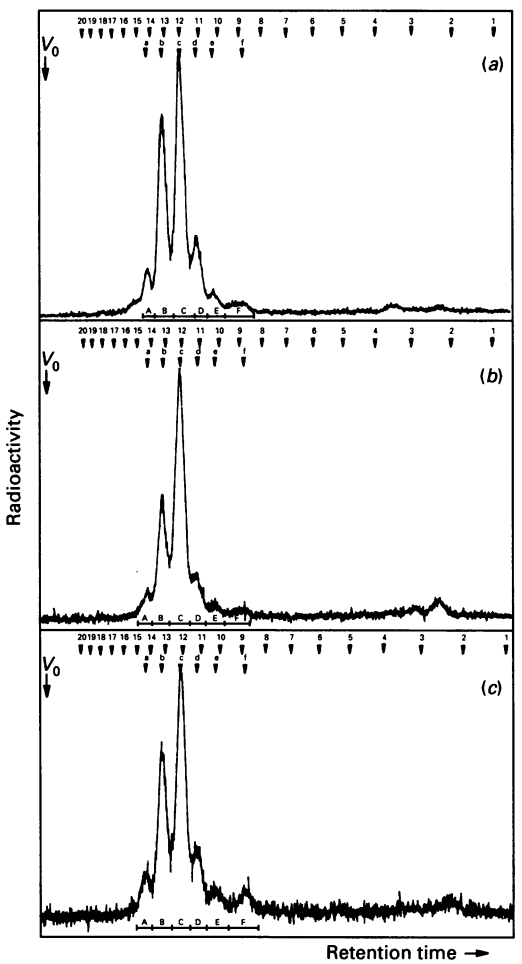

Fig. 3. P4 (-400 mesh) gel filtration chromatograms of the alditols derived from each IgG preparation

(*a*) Ascites, (*b*) serum-containing medium, (*c*) serum-free medium. Numbers represent the elution positions of co-applied non-radioactive linear glucose oligomers (see the Experimental section) detected simultaneously by an in-line differential refractometer. V_0 is the void elution position. Peaks a, b, c, d, e and f were clearly identified and corresponding fractions A, B, C, D, E and F were pooled as indicated, and the total radioactivity in each fraction was determined by liquid scintillation counting.

Table 3. Relative content of individual fractions in the gel filtration chromatograms of the de-acidified oligosaccharide alditols obtained from each IgG preparation

Triplicate analyses for each IgG preparation yielded the same percentage incidence to ±0.8% for each of fractions A, B, C and D, and the same percentage incidence to ±1% for fractions E and F.

Source of IgG	Relative content of fraction (%)					
	A	B	C	D	E	F
Ascites	7.5	32.1	42.8	12.3	3.7	1.6
Serum-containing media	6.2	25.3	57.5	6.8	2.7	1.5
Serum-free media	7.6	29.7	43.6	11.6	4.1	3.4

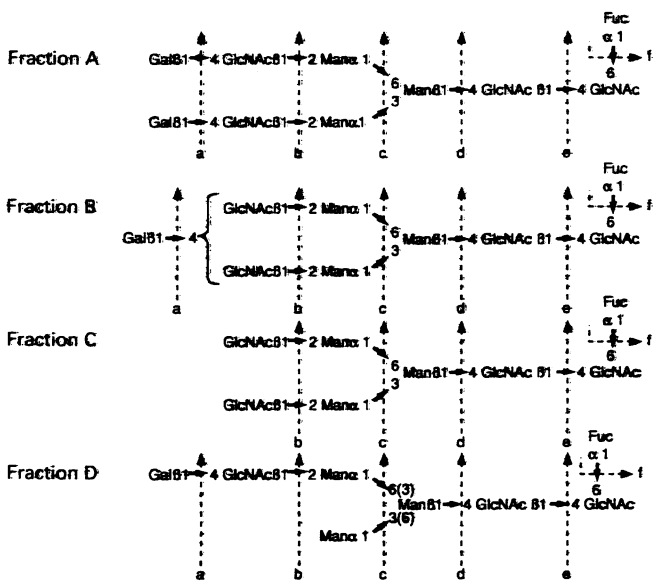

Fig. 4. Structures of the oligosaccharides in fractions A, B, C and D obtained by gel filtration of the total oligosaccharide alditol pool (after desialylation) from each IgG preparation

Exoglycosidases used were: a, *S. pneumoniae* β-galactosidase; b, *S. pneumoniae* β-*N*-acetylhexosaminidase; c, jack bean α-mannosidase; d, *A. fucila* β-mannosidase; e, jack bean β-*N*-acetylhexosaminidase; f, bovine epididymis α-fucosidase. Changes in the hydrodynamic volume of oligosaccharide structures were induced by exoglycosidases when used in the following order: fraction A, a, b, c, d, e, f; fraction B, a, b, c, d, e, f; fraction C, b, c, d, e, f; fraction D, c, a, b, c, d, e, f. The deduced points of hydrolysis of each structure by individual exoglycosidases are indicated.

the position of glycosyl linkages, is based on a consideration of the specificities of the glycosidases used and the change in hydrodynamic volume induced by each one (Kobata *et al.*, 1987). In the case of the core region, the position of glycosyl linkages is presumed to be consistent with the known biosynthetic pathway for N-glycans (Kornfeld & Kornfeld, 1985). The sequences of oligosaccharides in each of fractions A, B, C and D are shown in Fig. 4. The sequence of the oligosaccharide in fraction A was the same for all three IgG preparations, and likewise for fractions B, C and D. This analysis indicates the following: first, the major desialylated oligosaccharides associated with each IgG preparation are the same; second, the IgG derived from the culture in serum-containing media has a decreased molar content of outer-arm galactosylated oligosaccharide structures.

DISCUSSION

The results presented here suggest that the glycosylation pattern of the monoclonal IgG is dependent on the culture method employed. Specifically, ascites-derived material and serum-free-culture-derived material differ with respect to the content of sialic acid (Tables 1 and 2 and Fig. 2), but not with respect to the sequence and relative incidence of the neutral portion of their attached oligosaccharides (Table 3 and Fig. 3). Material derived from culture in serum-containing media had an intermediate sialic acid content (Tables 1 and 2 and Fig. 2) compared with those derived from ascites and culture in serum-free media, and a lower incidence of galactosylated oligosaccharides (Table 3 and Figs. 3 and 4). Similarities in glycosylation between the three IgG preparations include the number of attached N-glycan chains and the occurrence of the same set of oligosaccharide structures (although in different relative amounts), all of the complex biantennary form. The low content of sialic acid in the ascites-derived material cannot be explained by the action of a neuraminidase in ascites fluid, since incubation of *in vitro*-culture-derived IgG with IgG-depleted ascites fluid led to no change in the sialic acid content of the *in vitro*-culture-derived IgG (results not shown). These differences are considered to be experimentally significant, for the reason that triplicate analyses on each IgG preparation yielded very similar results (see Tables 1, 2 and 3), much closer than the differences between the preparations. These differences are not ascribed to differences in the three IgG preparations with respect to contamination by endogenous murine IgG (in the case of ascites IgG) or bovine IgG (in the case of cell-culture-derived IgG), since neither murine IgG of subclasses other than IgG-1 nor bovine IgG were detected in any of the IgG preparations. Contamination by non-immunoglobulin glycoproteins was not revealed by SDS/PAGE analysis (Fig. 1), and all three IgG preparations were indistinguishable by tryptic mapping and amino acid composition analysis.

Numerous factors have now been reported to influence glycosylation (Yet *et al.*, 1988). Traditionally, most emphasis has been on the effect of polypeptide structure (Sheares & Robbins, 1986) and cell type (Parekh *et al.*, 1987) on the glycosylation of the secreted polypeptide. It is, however, becoming increasingly clear that environmental factors can also profoundly influence glycosylation (for a review, see Goochee & Monica, 1990). These include the effects of hormones (Goochee & Monica, 1990), culture method (Anderson *et al.*, 1985), age of cell culture (Curling *et al.*, 1990), pH (Matlin *et al.*, 1988), buffers (Oda *et al.*, 1988), and the content of added serum during culture in serum-containing media (Megaw & Johnson, 1979). For example, Anderson *et al.* (1985) observed major differences in the glycosylation of a monoclonal IgM derived after culture in ascites and after cell culture *in vitro*. Megaw & Johnson (1979) found major differences in the glycosylation of the major secreted glycoprotein after culture of a murine parietal yolk sac carcinoma in media containing different levels of foetal calf serum, but there were no detectable changes in the amino acid composition of this glycoprotein. While in no case are the details of the molecular mechanisms by which such extracellular factors influence the intracellular glycosylation process understood, the results presented here lend support to the view that production method has an influence on the glycosylation of a secreted monoclonal antibody. To establish the generality or otherwise of this observation, however, will require similar analysis of other monoclonal antibodies. In this regard, one similar study has been

recently reported (Moellering *et al.*, 1990) which suggested that glycosylation of a murine IgG-1 monoclonal antibody was not influenced by production method. In this latter study, peptide *N*-glycosidase F (PNGase F) was used to liberate oligosaccharides from native, undenatured IgG. Given the current knowledge on the activity of this enzyme (Nuck *et al.*, 1990) and the relative inaccessibility of Fc N-glycans in native IgG (Sutton & Phillips, 1982), it is likely that PNGase F released only a small, possibly Fab-associated, population of the N-glycans from each IgG preparation.

Finally, the results presented here have implications for the production of monoclonal antibodies for therapeutic administration. In many cases, clincial trials on a monoclonal antibody are initiated using material derived from hybridoma culture in ascites. This preparation is then set as the reference material against which subsequent preparations, usually obtained from large-scale *in vitro* cell culture, are measured both functionally and structurally. If structural changes such as alterations in glycosylation can occur during a change in the method of preparation, any such changes need to be defined prior to entry of altered material into the clinic. Such changes in glycosylation could not only alter the physicochemical properties of the monoclonal antibody but also affect its pharmacokinetic behaviour *in vivo*. For example, in Fc the two oligosaccharide chains are attached to Asn-297 in the CH_2 domains. The oligosaccharides interact both with each other and with the polypeptide of the CH_2, and serve to bridge the two domains (Diesenhofer, 1981; Sutton & Phillips, 1982). The glycosylation of Fc therefore reflects the pairing of oligosaccharides on each heavy chain. To a first approximation, therefore, the incidence of Fc molecules devoid of galactosylated oligosaccharides in ascites- and serum-free-culture-derived IgG is ~ 18% (the square of the molar incidence of fraction C), and in IgG derived from culture in serum-containing media it is ~ 33%. IgG devoid of galactosylated oligosaccharides in the Fc portion is elevated in several human diseases (Parekh *et al.*, 1988), and has been suggested to contribute to the pathology of rheumatoid arthritis, perhaps through a predisposition to self-associate into immune complexes (Rademacher *et al.*, 1989). Several other studies have also indicated an effect of Fab and Fc glycosylation on pharmacologically important parameters (Hymes & Mullinax, 1979; Malaise *et al.*, 1987; Middagh & Litman, 1987).

We thank Emily Tate, Patrick Balsmann and Dr. Randy Rupp for providing valuable cell culture support.

REFERENCES

Adams, R. L. P. (1990) in Laboratory Techniques in Biochemistry and Molecular Biology (Burdon, R. H. & Von Knippenberg, P. H., eds.), vol. 8, 2nd edn., Chapter 7, Elsevier, London

Anderson, D. R., Atkinson, P. H. & Grimes, W. J. (1985) Arch. Biochem. Biophys. **243**, 605–618

Arvieux, J., Willis, A. C. & Williams, A. F. (1986) Mol. Immunol. **23**, 983–990

Brown, J. P., Wright, P. W., Hart, E. C., Woodbury, R. G., Hellstrom, K. G. & Hellstrom, I. (1980) J. Biol. Chem. **255**, 4980–4983

Chaplin, M. F. (1982) Anal. Biochem. **123**, 336–341

Curling, E. M. A., Hayter, P. M., Baines, A. J., Bull, A. T., Gull, K., Strange, P. G. & Jenkins, N. (1990) Biochem. J. **272**, 333–337

Diesenhofer, J. (1981) Biochemistry **20**, 2361–2370

Goochee, C. F. & Monica, T. (1990) Bio/Technology **8**, 421–427

Hymes, A. J. & Mullinax, F. (1979) J. Biol. Chem. **254**, 3148–3151

Kobata, A. (1984) Anal. Biochem. **100**, 1–14

Kobata, A., Yamashita, K. & Takasaki, S. (1987) Methods Enzymol. **138**, 84–94

Kornfeld, R. & Kornfeld, S. (1985) Annu. Rev. Biochem. **54**, 631–634

Laemmli, U. K. (1970) Nature (London) **227**, 680–685

Leatherbarrow, R. J., Rademacher, T. W., Dwek, R. A., Woof, J. M., Clark, A., Burton, D. R., Richardson, N. & Feinstein, A. (1985) Mol. Immunol. **22**, 407–415

Malaise, M. G., Franchimont, P., Gomez, F., Bouillene, C. & Mahieu, P. R. (1987) Clin. Immunol. Immunopathol. **45**, 1–11

Matlin, K. S., Skibbens, J. & McNeil, P. L. (1988) J. Biol. Chem. **263**, 11478–11485

Megaw, J. M. & Johnson, L. D. (1979) Proc. Soc. Exp. Biol. Med. **161**, 60–65

Middagh, C. R. & Litman, G. N. (1987) J. Biol. Chem. **262**, 3671–3674

Mizuochi, T., Hamako, J. & Titani, K. (1987) Arch. Biochem. Biophys. **257**, 387–394

Moellering, B. J., Tedesco, J. L., Townsend, R. R., Hardy, M. R., Scott, R. W. & Prior, C. P. (1990) Biopharmacology **3**, 30–38

Nose, M. & Wigzell, H. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 6632–6636

Nuck, R., Zimmerman, M., Sauvageot, D., Josic, D. & Reutter, W. (1990) Glycoconjugate J. **7**, 279–286

Oda, K., Ogata, S., Korigama, Y., Yamada, E., Mifune, K. & Ikehara, Y. (1988) J. Biol. Chem. **263**, 12576–12583

Parekh, R. B., Dwek, R. A., Sutton, B. J., Fernandes, D. L., Leung, A., Stanworth, D., Rademacher, T. W., Mizuochi, T., Taniguchi, T., Matsuta, K., Takeuchi, F., Nagano, Y., Miyamoto, T. & Kobata, A. (1985) Nature (London) **316**, 452–457

Parekh, R. B., Tse, A. G. D., Dwek, R. A., Williams, A. F. & Rademacher, T. W. (1987) EMBO J. **6**, 1233–1244

Parekh, R. B., Isenberg, D., Rook, G., Roitt, I., Dwek, R. A. & Rademacher, T. W. (1989) J. Autoimmun. **2**, 101–114

Porstmann, B., Porstmann, T., Nugel, E. & Evers, U. (1985) J. Immunol. Methods **79**, 27–37

Rademacher, T. W., Homans, S. W., Parekh, R. B. & Dwek, R. A. (1986) Biochem. Soc. Symp. **51**, 131–148

Rademacher, T. W., Parekh, R. B., Dwek, R. A., Isenberg, D., Rook, G., Axford, J. S. & Roitt, I. (1988) Springer Semin. Immunopathol. **10**, 231–249

Sheares, B. T. & Robbins, P. W. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 1993–1997

Sox, H. C., Jr. & Hood, L. (1970) Proc. Natl. Acad. Sci. U.S.A. **66**, 975–982

Spiegelberg, H. L., Abel, C. A., Fishkin, B. G. & Grey, H. M. (1970) Biochemistry **9**, 4217–4222

Sutton, B. J. & Phillips, D. C. (1982) Biochem. Soc. Trans. **11**, 130–132

Winkelhake, J. L. (1978) Immunochemistry **15**, 695–714

Yet, M. G., Shoo, M. C. & Wold, F. (1988) FASEB J. **2**, 22–31

Received 10 September 1991/10 February 1992; accepted 20 February 1992