

Characterization of Monoclonal Antibody Glycosylation: Comparison of Expression Systems and Identification of Terminal α -Linked Galactose

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CAMPATH-1H is a recombinant humanized murine monoclonal immunoglobulin (IgG₁) which recognizes the CDw52 antigen of human lymphocytes, and has been the subject of clinical trials for the treatment of non-Hodgkin's lymphoma and rheumatoid arthritis. Peptide mapping by liquid chromatography–mass spectrometry was used to confirm the predicted amino acid sequences and profile glycosylation for two CAMPATH isotypes expressed in a murine myeloma cell line (NS0) and a single isotype expressed in both Chinese hamster ovary (CHO) and NS0 lines. The three major glycoforms identified in CAMPATH are fucosylated biantennary structures, containing zero, one, or two galactose residues. Glycosylation of the IgG₁ form of CAMPATH expressed in CHO cells is consistent with normal human IgG. However, IgG₁ and IgG₄ expressed in NS0 cells include two potentially immunogenic glycoforms which contain either one or two nonreducing terminal α -linked galactose residues. Oligosaccharide structures were characterized by a combination of tandem mass spectrometry, methylation analysis, and exoglycosidase digestion. The strategy used here is designed to be widely applicable to recombinant glycoproteins. © 1997 Academic Press

The cell type used for expression of a recombinant therapeutic glycoprotein is rarely the native one. This is significant because differences between cell types influence the structure and distribution of protein-linked oligosaccharides (1). For example, there are variations in the glycosylation pattern of tissue plasminogen activator isolated from different cell lines (2). Changes in glycosylation may or may not adversely affect the activ-

ity, serum half-life, or immunogenicity of the recombinant protein (3, 4). The potential for significant changes in the biological activities of recombinant glycoproteins depending on their expression systems is a real concern from a pharmacological standpoint (5, 6). Aberrant glycosylation is of particular interest with respect to therapeutic monoclonal antibodies because of their obvious complexity and their intended interaction with the immune system of the patient. The observation of aberrant glycosylation of IgG in certain autoimmune disorders is one indication that antibody glycosylation is an important consideration (1, 7).

CAMPATH-1H is a recombinant humanized murine monoclonal immunoglobulin (IgG₁)² antibody which recognizes the CDw52 antigen of human lymphocytes (8). It has been the subject of clinical trials for the treatment of non-Hodgkin's lymphoma and rheumatoid arthritis (9). As is characteristic of an IgG, CAMPATH contains a single conserved consensus sequence site for N-linked glycosylation in each C_H2 domain. Conserved IgG oligosaccharides are always biantennary complex structures (10). These conserved carbohydrate chains are the only sites of glycosylation identified on CAMPATH.

The CAMPATH-1H used for most clinical trials was produced in Chinese hamster ovary (CHO) cells. However, many experimental variants have been expressed in a murine myeloma cell line (NS0). We were prompted to examine the glycosylation of a variety of

² Abbreviations used: HPLC, high-pressure liquid chromatography; LC-MS, liquid chromatography–mass spectrometry; ESMS, electrospray ionization mass spectrometry; MALDI-TOFMS, LDMS, matrix-assisted laser desorption ionization–time of flight mass spectrometry; GC-MS, gas chromatography–mass spectrometry; EI, electron impact; TIC, total ion chromatogram; MS/MS, tandem mass spectrometry; CID, collision-induced dissociation; NANA, *N*-acetyl neuraminic acid; CHO, Chinese hamster ovary; TFA, trifluoroacetic acid; DHB, dihydroxybenzoic acid; ASF, asialofetuin; IgG₁, immunoglobulin G₁.

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CAMPATH samples because of differences between isotypes and expression systems which might affect CAMPATH activity (11–13), including the potential for the presence of the immunogenic Gal α (1–3)Gal- epitope in murine-derived CAMPATH (14–17).

On-line high-pressure liquid chromatography–electrospray ionization mass spectrometry (LC-MS) was used to profile proteolytic digests of several variants of CAMPATH, in order to rapidly confirm protein primary sequence and create a site-specific profile of glycan heterogeneity (18, 19). LC-MS profiling cannot determine the sequence or branching of the oligosaccharide chains, but because IgG N-linked glycosylation follows a specific motif it was possible to surmise the probable structure of CAMPATH glycosylation from peptide molecular weights alone (20, 21). Subsequently, oligosaccharides were released from the protein by hydrazinolysis, profiled, and sequenced by MS/MS. Linkage position and anomeric configuration were determined by methylation analysis and exoglycosidase digestion.

MATERIALS AND METHODS

CAMPATH Samples

CAMPATH samples were obtained from various sources. We evaluated three separate samples of the IgG₁ isotype expressed in CHO cells. The same IgG₁ isotype of CAMPATH was also expressed in a murine myeloma cell line designated NS0, and an IgG₄ version of CAMPATH was constructed and expressed in NS0 cells. Differences between IgG₁ and IgG₄ CAMPATH are described below. Analysis of these samples permitted comparisons of (i) the glycosylation profiles of CAMPATH versus normal human IgG, (ii) IgG₁ expressed in CHO and NS0 cells, and (iii) different isotypes (IgG₁ and IgG₄) expressed in the same cell line, NS0.

Proteolysis and LC-MS Mapping

CAMPATH samples were precipitated from phosphate buffered saline by addition of cold trichloroacetic acid to 10% (v/v). After washing with acetone to remove residual acid, the protein was redissolved in 8 M urea/400 mM ammonium bicarbonate. Disulfide bonds were reduced by addition of dithiothreitol to a concentration of 4 mM and incubation at 50°C for 15 min. After addition of iodoacetamide to a concentration of 8 mM, the sample was incubated at room temperature for an additional 15 min in the dark. Following reduction and alkylation, the samples were diluted to a final urea concentration of 2 M and digested overnight (16 h) at 37°C with *Achromobacter* protease I (Lys-C) (Wako, Richmond, VA) at a 1:25 (w/w) ratio of enzyme to substrate.

HPLC was performed using a Waters (Millipore Corp., Milford, MA) Model 625 pump, Model 600E gradient controller, Model 712 WISP autoinjector, and

Model 991 photodiode array UV absorbance detector. LC-MS was carried out on a similar system equipped with a Rheodyne (Cotati, CA) Model 9125-080 injector, 50- μ l loop, and a Model 486 tunable UV absorbance detector. For all HPLC a narrowbore Vydac C18 column (2.1 $\times$ 250 mm, 300 Å) was used. Peptides were eluted using a two-part linear water/acetonitrile gradient from 1.6 to 64% organic over 100 min. Both buffers contained 0.06% trifluoroacetic acid (TFA). See the legend to Fig. 1 for details of the buffer composition and gradient program. Flow rate was 0.2 ml/min for each system. Ultraviolet chromatograms were acquired at a wavelength of 210 nm.

For LC-MS, a postdetector split was used to deliver approximately one-tenth of the column eluent to the IonSpray (electrospray) source of the API III triple quadrupole mass spectrometer (Sciex, Thornhill, Ontario, Canada). The interface heater was set to 50°C, and orifice potential was run at either 75 or 95 V. The mass spectrometer (Q1) was scanned from m/z 300 to 2400, using a 0.2-Da step size and a dwell time of 0.3 ms. Peptide spectra presented here were averaged over the width of the corresponding HPLC peak.

Hydrazinolysis and Glycan Structural Analysis

Oligosaccharides were chemically cleaved from antibodies using an automated hydrazinolysis procedure (Glycoprep 1000; Oxford Glycosystems, Rosedale, NY). After cleavage, released oligosaccharides were dissolved in 100 mM NaOH (pH 13) and passed over a 1-ml bed volume of macroporous anion exchange resin (Bio-Rad, Hercules, CA) equilibrated at pH 13. A wash of four column volumes of 100 mM NaOH removed contaminating metal ions and hydrazides introduced during hydrazinolysis, and the glycans were eluted with six to eight column volumes of ammonium formate, 100 mM, pH 3.5. The pH of each fraction was monitored, and those suspected of containing glycans were analyzed by LDMS. Glycan-containing fractions were combined and dried under vacuum. Carbohydrate samples were methylated by the method of Ciucanu and Kerek (22).

Permethylated glycans were dissolved in a 10 mM solution of sodium acetate in 70/30 methanol/water for analysis by electrospray ionization mass spectrometry (ESMS). A syringe pump (Harvard Apparatus, South Natick, MA) was used to infuse 3 μ l/min of the solution directly into the electrospray source of the API III triple quadrupole mass spectrometer. The API III was operated using the same settings described above for LC-MS. To perform collision-induced dissociation (CID) and tandem mass spectrometry (MS/MS), the ($M + 2Na$)²⁺ precursor ion was selected using Q1 and collided with argon in Q2. Q3 was adjusted to unit mass resolution to increase sensitivity. Spectra presented here were acquired in MCA mode, summing all scans.

Methylation analysis was performed by a modification of the method of Harris *et al.* (23). After reduction, the sample was dried repeatedly from 3-ml aliquots of methanol to remove residual borate. Acetylation of dry alditols employed a 1:1 mixture of acetic anhydride and pyridine, heated to 65°C for 1 h and then left at room temperature overnight. Partially methylated alditol acetates were extracted in chloroform and washed with water. The sample was evaporated to dryness, redissolved in chloroform, and injected onto the GC-MS.

Combined GC-MS was performed using a VG 70S magnetic sector mass spectrometer, equipped with an electron impact (EI) ion source. The instrument was scanned from m/z 800 to 40 at 1 second per decade. Samples were injected onto a Hewlett-Packard methylsiloxane column using an on-column injector, at 75°C, and the column temperature was ramped to 300°C over 30 minutes.

Exoglycosidase Digestion and MALDI-TOFMS

In order to confirm anomeric configuration and linkage position of nonreducing-terminal galactose residues, glycopeptides from Lys-C digestion were isolated by high-pressure liquid chromatography (HPLC) and treated with an α -galactosidase and a β -galactosidase (Oxford Glycosystems, Garden City, NY). These samples were then rechromatographed prior to analysis by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOFMS). Purified glycopeptides were incubated with 0.2 U of α -galactosidase from green coffee beans (EC 3.2.1.22) or 40 mU β -galactosidase from *Streptococcus pneumoniae* (EC 3.2.1.23) in a total volume of 50 μ l sodium phosphate/citrate buffer, pH 6, at 37°C for either 24 or 48 h, respectively. The reaction mixture was subjected to preliminary analysis by MALDI-TOFMS and was then rechromatographed and reanalyzed by MALDI-TOFMS. Rechromatography used an Aquapore C8 column (Applied Biosystems, Foster City, CA) at a flow rate of 0.2 ml/min, with linear gradients of the same buffer system as above.

MALDI-TOFMS was performed using either a Thermo Bioanalysis (Hemel Hempstead, Herts, UK) Lasermat or Micromass (Manchester, UK) Tofspec mass spectrometer operating in the linear mode. Either single targets or autosampler strips were spotted with multiple aliquots of 0.75 μ l of sample with subsequent addition of 0.60 μ l of a 10 mg/ml aqueous solution of matrix. Dihydroxybenzoic acid (DHB) and α -cyano-4-hydroxycinnamic acid were the matrices used for analysis of oligosaccharides and glycopeptides, respectively.

RESULTS

LC-MS Confirmation of the Primary Structure of CAMPATH Isotypes

Figure 1 shows the total ion chromatogram (TIC) for a Lys-C digest of CHO-derived IgG₁ CAMPATH.

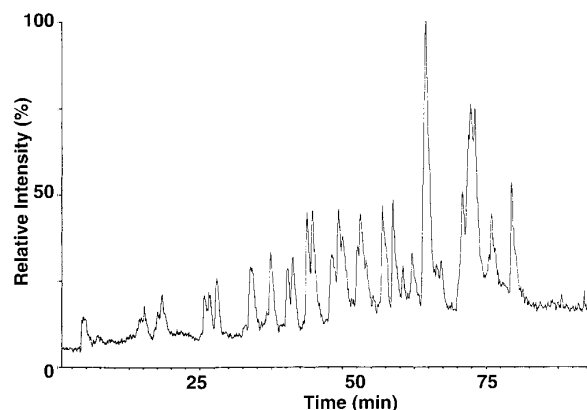


FIG. 1. TIC from LC-MS of CAMPATH Lys-C digest, IgG₁ from CHO cells. HPLC conditions: Vydac C18 column, 2.1 $\times$ 250 mm; mobile phase, flow rate = 0.2 ml/min: (solvent A) 0.064% TFA in water and (solvent B) 0.060% TFA in 80:20 (v/v) acetonitrile/water. Gradient: 0 min/98%A, 60 min/63%A, 90 min/25%A.

Comparison of the TIC and UV chromatograms demonstrates excellent correlation between the two traces. The IgG₁ and IgG₄ isotypes have identical light chains and their heavy chain sequences are essentially identical in the variable and C_H1 regions. Significant differences occur in the hinge region, and there are several other single amino acid substitutions. In the LC-MS analysis, homologous Lys-C peptides eluted in the same positions for each isotype. For each sample, all expected Lys-C peptides greater than four amino acids in length were detected. For example, of the 665 residues in the heavy and light chains of the IgG₁ isotype, 95% were accounted for by LC-MS of the Lys-C digest. Likewise, the masses for all major HPLC peaks were consistent with Lys-C cleavage peptides of CAMPATH. The minor differences observed among the HPLC profiles were due to the sequence differences in the heavy chains of the two CAMPATH isotypes.

Glycosylation Profiles of CAMPATH Isotypes

Figure 2 shows a portion of the electrospray spectrum of the glycosylated peptide of CHO-derived IgG₁. Those peaks in the spectrum not specifically labeled as corresponding to the glycosylated peptide are due to other coeluting peptides. Multiply charged ions which correspond to different glycoforms of the glycopeptide were observed at m/z 1228, 1269, and 1309 (+4 charge state) and at m/z 1637, 1691, and 1745 (+3 charge state). A coeluting peptide ion overlapped a glycoform in the +4 charge state. Consequently, the +3 charge envelope is shown because it is a more accurate indicator of the abundances of the various glycoforms.

Because IgG glycosylation follows specific patterns, it is reasonable to propose carbohydrate structures for the glycopeptide based only on the expected bianten-

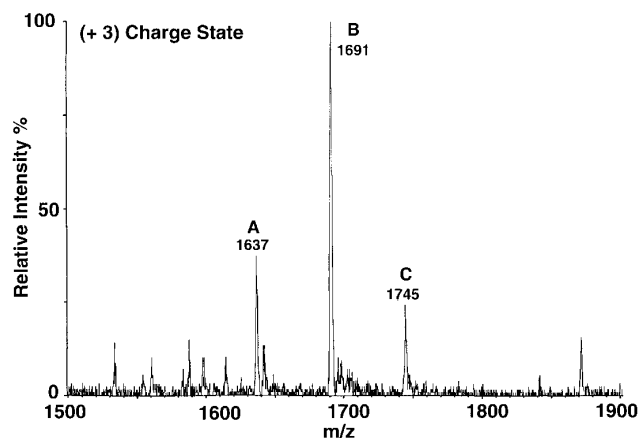


FIG. 2. ESMS spectrum of the glycosylated peptide of CHO-derived IgG₁. The mass spectrum is shown from m/z 1500 to 1900. The three main peaks correspond to the (+3) charge state of the glycopeptide. Ions corresponding to glycoforms are indicated.

nary motif and molecular weight data. Possible structures for the peaks marked A, B, and C in Fig. 2 are shown in Fig. 3. Letter codes correspond to the peak labels in Fig. 2. These biantennary core-fucosylated structures contain zero to two galactose residues, and virtually no *N*-acetyl neuraminic acid (NANA), all of which is typical of IgG glycosylation (7, 10, 24). In the case of structure B, the location of the galactose residue could not be determined from the LC-MS data. A second series of peaks, detected at a low level, correspond to the masses of the afucosyl forms of glycoforms A, B, and C.

In order to determine whether expression in CHO versus mouse myeloma cells would alter glycosylation of this antibody, the glycosylation profiles of IgG₁ expressed in NS0 cells and CHO-derived IgG₁ were compared. Figure 4 (top) shows the TIC for a Lys-C digest of NS0-derived IgG₁ CAMPATH. Because the amino acid sequence was the same as for the CHO-expressed antibody, the map is essentially the same. However there were differences in the mass spectrum of the glycopeptide. A portion of the ESMS spectrum for the NS0-derived IgG₁ glycopeptide is shown in Fig. 4 (bottom). The same three core-fucosylated biantennary glycoforms found in the CHO-derived material were present and appear to be well conserved between CHO- and NS0-expressed CAMPATH. However, NS0-derived IgG₁ contained two additional glycoforms at higher m/z values relative to the other ions in the mass spectrum. These peaks are labeled D and E and differ in mass from the other glycosylated peaks by the addition of one and two hexose residues respectively ($\Delta m = 162$ Da). Peaks D and E are not as abundant as the other glycoforms, but may be significant if they represent an immunogenic epitope such as the Gal α (1–3)Gal- motif mentioned above.

The glycosylated Lys-C peptide of the IgG₄ variant of CAMPATH has the same primary structure as the IgG₁ form, except for a tyrosine to phenylalanine substitution at residue 300, adjacent to the glycosylated asparagine. The retention times of the IgG₁ and IgG₄ glycopeptides differ by approximately thirty seconds. Figure 5 shows the Lys-C proteolytic map and expanded mass spectrum of the glycopeptide from the NS0-expressed IgG₄ isotype. Comparison of the mass spectra in Fig. 4 (NS0-derived IgG₁) and Fig. 5 (NS0-expressed IgG₄) shows that in this expression system, glycosylation was essentially the same for the IgG₁ and IgG₄ isotypes of CAMPATH, although the IgG₄ isotype appeared to be galactosylated more extensively. Peaks in Fig. 5 (bottom) which correspond to the IgG₄ glycoforms occur at m/z values corresponding to a 16-Da mass difference relative to the IgG₁ glycopeptide, in agreement with the expected difference in sequence of the glycopeptides (i.e., Y300F, $\Delta m = 16$ Da). This observed mass difference provides confirmation of the as-

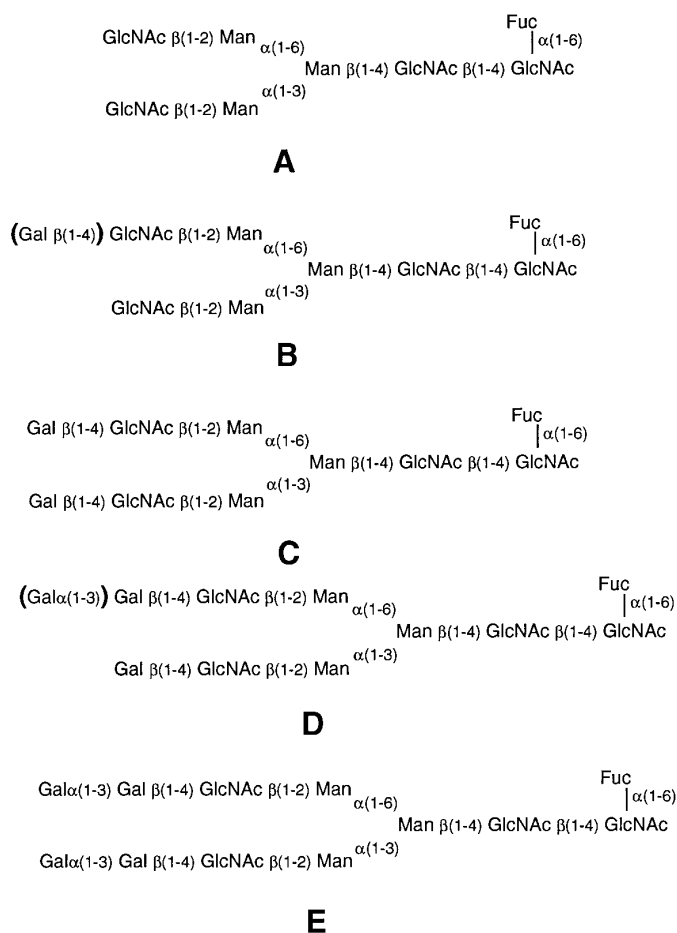


FIG. 3. Structures of Asn-linked oligosaccharides from CHO-derived IgG₁ and NS0-derived IgG₁ and IgG₄. Letter codes correspond to glycoforms labeled in Figs. 2, 4, and 5.

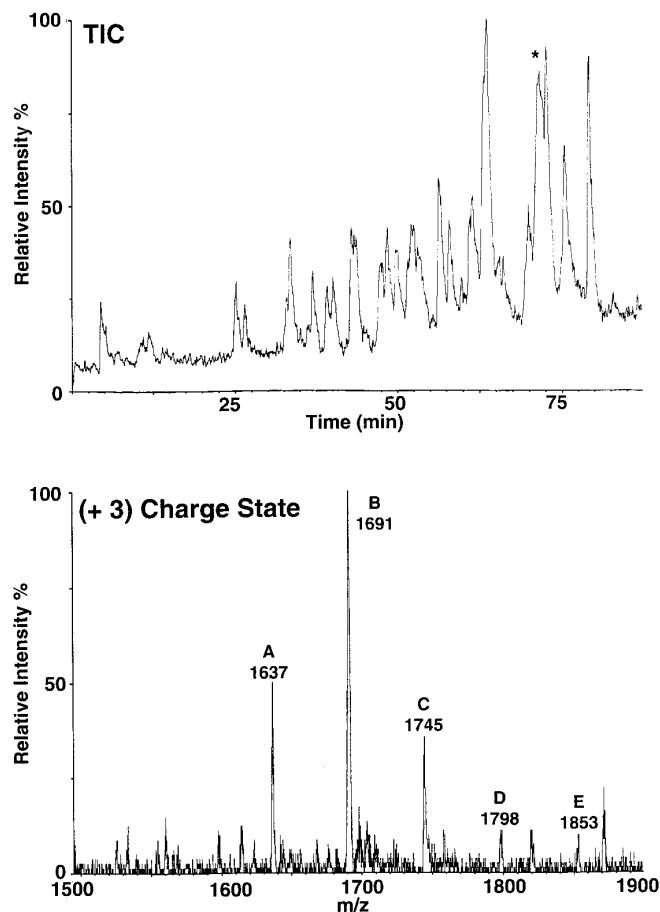


FIG. 4. LC-MS of CAMPATH Lys-C digest, IgG₁ from NS0 cells: (top) TIC and (bottom) mass spectrum of the glycopeptide over the range of the (+3) charge state. HPLC conditions were as described in the legend to Fig. 1.

signments made for each ion corresponding to the glycopeptide in these spectra.

Because of the unglycosylated peptides which coelute with the glycopeptide in Lys-C digests of CAMPATH, the spectra were somewhat complex, and despite the presence of two charge envelopes (+3 and +4), isobaric ions made characterization more difficult. In order to provide an additional confirmation of the glycoforms observed here, the NS0-derived IgG₄ isotype was also subjected to Asp-N digestion and LC-MS mapping (data not shown). The glycosylation pattern observed in this case is identical to that observed for the Lys-C digests.

Both isotypes of the antibody expressed in NS0 cells appear to be glycosylated differently from the clinical material grown in CHO cells. Although the addition of either one or two hexose residues might theoretically result in a number of possible structures, the overall glycosylation pattern should still be consistent with that observed for human IgG. One possibility for the

structures of the D and E glycoforms is that they are hypergalactosylated forms observed previously in non-human mammalian systems. In some species of nonprimate mammals, as well as new world monkeys, Asn-linked glycans sometimes contain a terminal α -linked galactose residue instead of the more typical *N*-acetylneuraminic acid end cap (Gal α (1-3)Gal β (1-4)GlcNAc-R rather than NANA α (2-6)Gal β (1-4)GlcNAc-R) (17).

The masses of the D and E glycoforms identified on NS0-expressed CAMPATH correspond to structures D and E shown in Fig. 3. Occurrence of these glycoforms in the samples characterized here correlates with the observation that murine systems express an active α -galactosyl transferase. While relatively common in murine systems, these structures are not generally seen in CHO cells. CHO cells have been well characterized, and while they do have an intact gene for the α -galactosyl transferase, this protein is not expressed. Only one case has been documented of α -galactosylation in CHO cells (25). It apparently resulted from selection of a clone in which the transferase gene was accidentally turned on.

Oligosaccharide Structural Analysis

Hydrazinolysis was used to release all Asn-linked glycans from CHO-derived CAMPATH IgG₁, NS0-de-

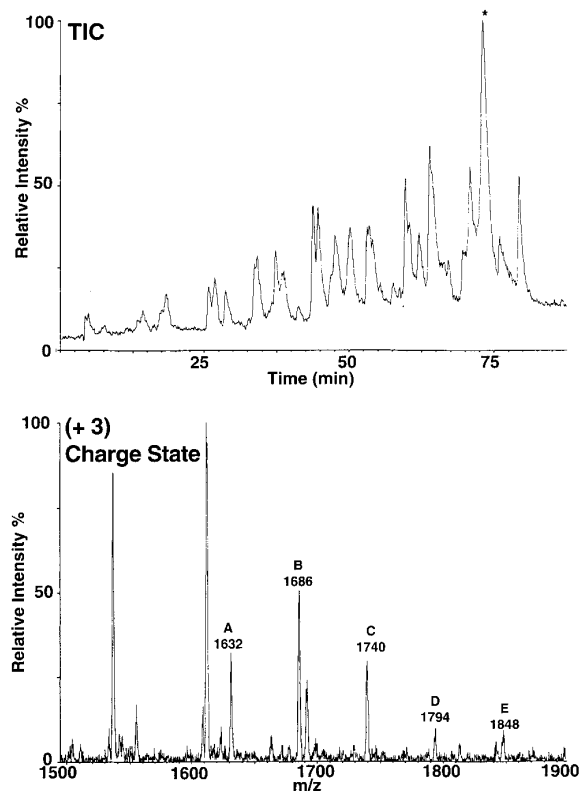


FIG. 5. LC-MS of CAMPATH Lys-C Digest, IgG₄ from NS0 cells: (top) TIC and (bottom) mass spectrum of the glycopeptide over the range of the (+3) charge state. HPLC conditions were as described in the legend to Fig. 1.

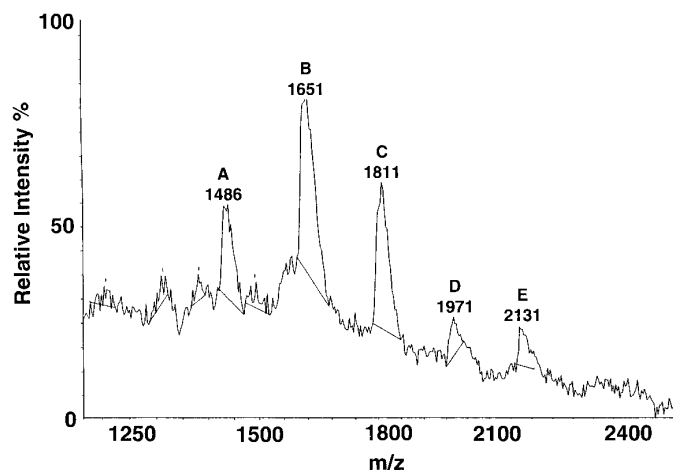


FIG. 6. LDMS spectrum of oligosaccharides isolated from NS0-derived CAMPATH IgG₄. Approximately 500 fmol were spotted. DHB was the matrix. 50 shots were summed.

rived CAMPATH IgG₄, and a standard glycoprotein, asialofetuin (ASF) (Sigma, St. Louis, MO). Biantennary glycans from asialofetuin were used here as standards for low-energy CID tandem mass spectrometry (MS/MS) and linkage analysis because they are similar in structure to the glycans found on IgG.

Anion-exchange chromatography was used for purification of oligosaccharides released by hydrazinolysis. This step was necessary because hydrazinolysis on the Glycoprep 1000 introduces metal salts and hydrazine derivatives which interfere with subsequent derivatization chemistry. MALDI-TOFMS was used to monitor column fractions from anion exchange and profile the purified pool of oligosaccharides released from each CAMPATH sample. Figure 6 is a representative MALDI-TOFMS spectrum of glycans released from NS0-derived IgG₄. The five glycoforms observed previously in LC-MS of the glycopeptide were present at levels which were in agreement with the LC-MS results.

After MALDI-TOFMS analysis, samples were permethylated for ESMS and linkage analysis (26, 27). Samples were dissolved in a 70/30 mixture of methanol/water, in the presence of sodium acetate (1–10 mM) in order to generate exclusively $(M + Na)^+$ and $(M + 2Na)^{2+}$ molecular ions, rather than the mixture of natriated and protonated species which occur in acidic solution.

The ESMS spectrum of the permethylated CHO-derived CAMPATH glycans showed the $(M + Na)^+$ and $(M + 2Na)^{2+}$ molecular ions for each of the three major glycoforms (data not shown). No fragmentation was observed. This concentration of ion current in the molecular ions favors the use of MS/MS for sequence analysis. As expected (28), low-energy CID of the $(M + Na)^+$ precursor ion did not generate structurally relevant

fragment ions. This may be due to the labile nature of the Na^+ adduction, resulting in loss of the sodium cation and no other fragmentation. However, when the $(M + 2Na)^{2+}$ precursor was selected, abundant sequence related fragment ions were produced. Fragmentation observed here for permethylated biantennary glycans was consistent with results obtained by other workers (27), with fragmentation limited to glycosidic bonds on the reducing-end side of GlcNAc residues. Comparable spectra were obtained for the ASF standard and CAMPATH-derived glycans. Oligosaccharides from CHO-derived IgG₁ and NS0-derived IgG₄ were each analyzed, and results for glycoforms A, B, and C were identical between these two samples.

MS/MS results were similar for the agalactosyl, monogalactosyl, and bigalactosyl biantennary structures (structures A, B, and C, Fig. 3). The MS/MS spectrum of the $(M + 2Na)^{2+}$ parent of glycan B is shown in Fig. 7, and the fragmentation of this ion is diagrammed above the spectrum. Ions at m/z 1837, 2041, and 2245 which occurred in the MS/MS spectra of structures A, B, and C, respectively, were due to the loss of one Na^+

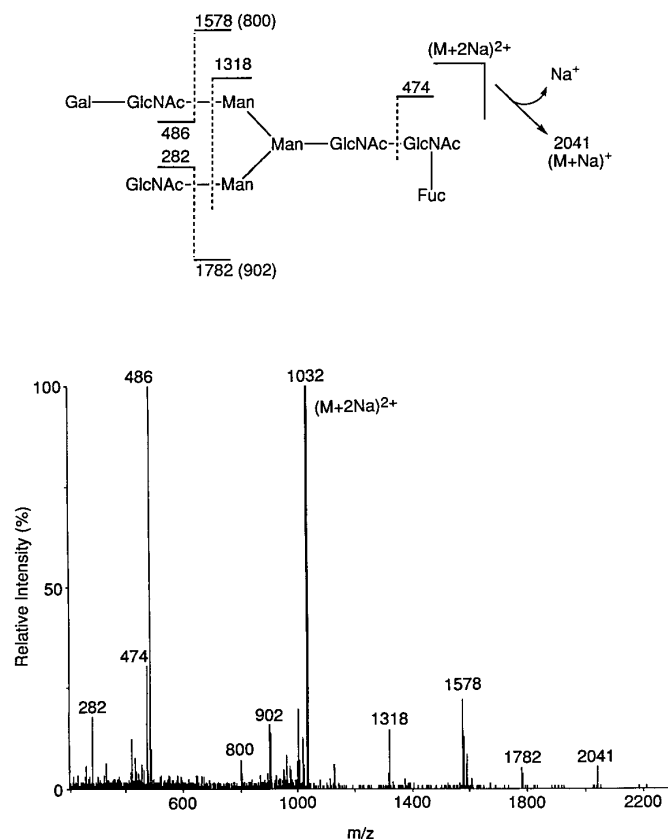


FIG. 7. Product ion spectrum obtained for the $(M + 2Na)^{2+}$ parent ion of structure B, containing one galactose residue. The sample was infused directly from a 10 mM solution of sodium acetate in 70/30 MeOH/H₂O. Q3 was scanned from 2400 to 100 over 5 s, at unit mass resolution. 50 scans were summed.

ion and corresponded to the singly charged molecular ions. A significant fragment ion in each of these spectra occurred at m/z 474, representing the loss of the reducing-terminal core GlcNAc residue and an attached fucose. This ion showed that in the CAMPATH glycans, a fucose residue is attached to the reducing-terminal core GlcNAc rather than on an outer arm.

As seen in Fig. 7, a series of fragment ions occurred at m/z 282, 486, 1578, and 1782. These were two sets of complimentary ions representing cleavage of the nonreducing-terminal GlcNAc (m/z 282 and 1782) and Gal-GlcNAc (m/z 486 and 1578) residues on either arm of the monogalactosylated biantennary structure. The doubly charged species of the m/z 1578 and 1782 ions were observed at m/z 800 and 902. A related ion which occurred at low abundance was the double-cleavage product ion at m/z 1318, representing loss of both Gal-GlcNAc- moieties, with charge retention on the reducing end of the molecule. In the product ion spectrum for the agalactosyl glycoform (structure A) analogous ions at m/z 282, 1578, and 800 were observed, resulting from loss of either nonreducing-terminal GlcNAc residue. The bigalactosyl structure (C) showed only the m/z 486, 1578, and 902 ions of this series, indicating that one galactose residue was attached to each nonreducing-terminal arm.

These results for structures A, B, and C were consistent with previous characterization of IgG glycosylation (7, 10, 24). These mass spectra were consistent with the structures reported in the literature, and provided a basis for comparison with results obtained for glycoforms D and E present on the NS0-derived CAMPATH. Figure 8 shows the MS/MS spectrum for one of the lower abundance hypergalactosylated species (structure D). Because the parent ion abundance was significantly lower for this glycan than for structure B, note that the ion abundances in this spectrum were lower. The MS/MS spectrum of this species was consistent with results from structures A, B, and C, except for the masses of the sequence-specific ions related to cleavage at outer arm GlcNAc residues.

As expected, structure D contained one additional nonreducing-terminal galactose residue. It exhibited two pairs of complimentary ions, as structure B did, but at correspondingly higher masses, m/z 486 and 1986, and at 690 and 1782. These fragments were assigned to cleavage of Gal-GlcNAc or Gal-Gal-GlcNAc. In the product ion spectrum of structure E, the 486 and 1986 ions were absent, indicating that the second additional galactose residue was attached to the second arm of the glycan, giving two Gal-Gal-GlcNAc branches rather than one Gal-Gal-Gal-GlcNAc. These results were consistent with the literature for α -linked terminal galactose and supported the presence of the Gal α (1-3)Gal- epitope on structures D and E.

Linkage analysis was performed on the CHO-derived

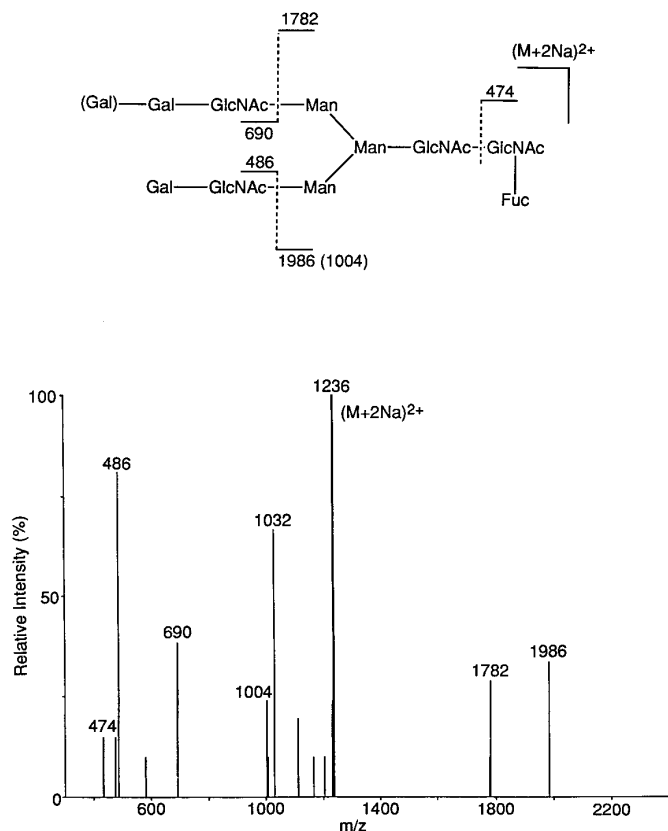


FIG. 8. Product ion spectrum obtained from MS/MS of the $(M + 2Na)^{2+}$ parent ion of structure D. Conditions were as described in the legend to Fig. 7.

IgG₁ isotype, in order to confirm the monomer identity and linkage positions of hexoses present in this material. This confirmed that the oligosaccharides were complex fucosylated biantennary structures and provided support for the sequence information obtained by MS/MS. Galactose and mannose alditols were identified by their EI spectra and comparison of GC retention times with similar glycans from asialofetuin, which contain substantially the same linkages. The core-linked fucose residue was also identified as a deoxyhexose by its EI mass spectrum, although no standard was used for GC retention time.

Exoglycosidase Digestion and LDMS

Two exoglycosidases with specificity for α - and β -linked terminal galactose residues were used to determine that the terminal galactose residues present in the hypergalactosylated glycoforms D and E were α -linked. To do this, the Lys-C glycopeptide of CAMPATH from NS0 cells was isolated. Although the purified fraction still contained some additional peptides, the glycopeptide was found to be the major component by both LDMS and ESMS analysis.

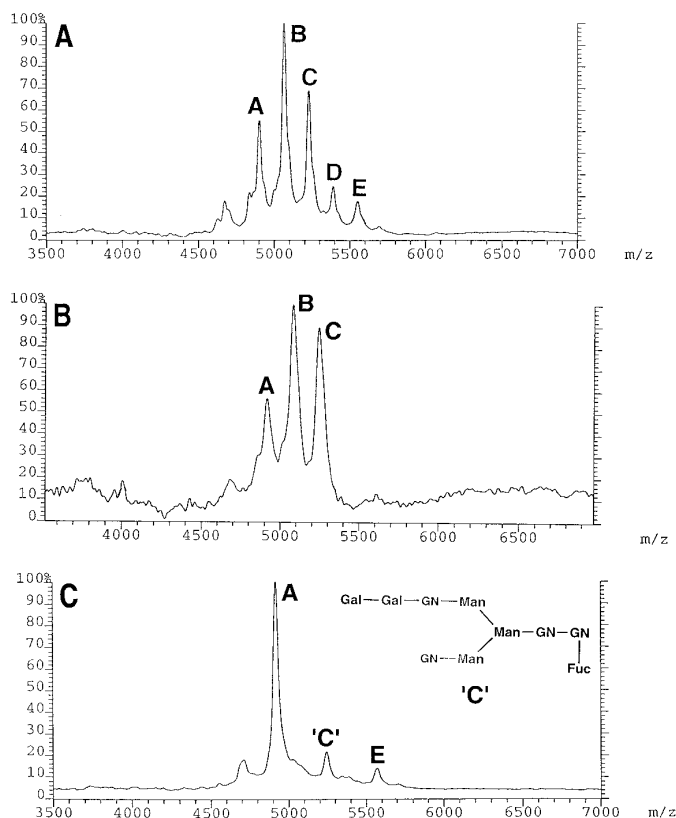


FIG. 9. (A) LDMS spectrum of the NS0-derived CAMPATH IgG₄ glycopeptide. (B) LDMS spectrum of the NS0-derived CAMPATH IgG₄ glycopeptide, following α -galactosidase digestion. (C) LDMS spectrum of the NS0-derived CAMPATH IgG₄ glycopeptide, following β -galactosidase digestion. For each spectrum, approximately 500 fmol were spotted. DHB was the matrix. 50 shots were summed.

Figure 9A shows the LDMS spectrum of the NS0-derived glycopeptide after rechromatography. Five major glycoforms were present, as discussed above. Following treatment with an α -galactosidase, which is specific for cleavage of α -linked nonreducing-terminal galactose residues, the two highest molecular weight glycoforms were no longer detected, consistent with the structures assigned earlier for these glycans (Fig. 9B). Also, in Fig. 9B, glycoform C, the bigalactosyl form, is more abundant relative to glycoform B than in the original distribution shown in Fig. 9A. This is consistent with the loss of terminal α -linked galactose, to give form C.

A second aliquot of the glycopeptide was then treated with a β -galactosidase, which is specific for β (1–4)-linked nonreducing-terminal galactose. In this case, glycoforms A and E should remain intact, because A and E contain no terminal β -linked galactose. In addition, a peak isobaric with glycoform C should be formed by removal of one terminal β -linked galactose from form D, while the hypergalactosylated arm remained intact.

In Fig. 9C most of the ion current was represented by the agalactosyl form (A), due to the loss of β -linked galactose from glycoforms B and C. Two other species are present. One peak is due to the presence of glycoform E, which contains no terminal β (1–4)-linked galactose residues. The other peak represents a structure isobaric with glycoform C, designated C'. As described above, this structure results from the loss of a β -linked galactose from structure D. This structure is shown in Fig. 9C. The results from exoglycosidase digestion strongly support the presence of the Gal α (1–3)Gal epitope in this glycopeptide.

DISCUSSION

We have found that the glycosylation of each expressed form of CAMPATH is consistent, and the major glycoforms are identical, although there are certain clear differences. This study provides evidence that CAMPATH expressed in NS0 cells is hypergalactosylated. It is well documented that murine cell-expressed proteins are sometimes α -galactosylated. However, while one previous paper has shown indirect evidence for the presence of α -linked galactose in monoclonal antibodies from murine expression systems (14), this is the first time that such hypergalactosylation has been documented in a monoclonal antibody. It also appears from this study that the normal galactosylation pattern of the IgG₁ and IgG₄ forms of CAMPATH are slightly different. The bigalactosyl form (C) is somewhat more abundant in the IgG₄ isotype. This is consistent with observations that the protein primary structure of an IgG may affect its glycosylation, specifically the level of terminal β -linked galactose present (11). This may contribute to the more abundant hypergalactosylated glycoforms observed in the IgG₄, by providing a greater abundance of substrate for α -galactosylation.

Although CAMPATH glycosylation is substantively similar to the glycosylation of other immunoglobulins, the presence of an α -linked terminal galactose in NS0-expressed IgG is significant because of current widespread interest in therapeutic monoclonal antibodies. Murine myeloma cell lines are extensively used in immunological research, and have been a logical choice for the expression of such antibodies. Recently, however, there has been discussion, centered in the xenotransplantation community, of aberrant glycosylation found in nonprimate species. With regard to the α -linked galactose moiety, it has been reported that up to 1% of all circulating IgG may be specific for binding to the α -Gal epitope (16). It has been proposed that antibodies carrying this epitope may be highly immunogenic, resulting in extremely low circulatory half-life (14). This has raised questions concerning the structure of carbohydrate on murine-derived antibodies and the possible deleterious effects of this glycosylation. There have

been no reports in the literature of a demonstrated immune response in humans to a murine-derived monoclonal antibody which was specifically related to the presence of α -linked terminal galactose residues. There is evidence, however, for the presence of a surprisingly high level of IgG specific for this epitope in human serum (16). It is important to address the question of whether the presence of the α -Gal epitope on therapeutic or diagnostic antibodies presents a problem and, if so, at what level of expression.

We have employed a broad-based analytical strategy for the characterization of the glycosylation of a monoclonal antibody produced in two cell lines. For monoclonal antibodies such as CAMPATH, which are glycosylated with a known structural motif and where the amino acid sequence has been determined, significant structural information can be determined from molecular weight alone. In addition to allowing the prediction of glycan structures, peptide mapping by LC-MS confirmed the predicted amino acid sequences for CAMPATH with high efficiency, identified discrepancies, and facilitated their characterization. Low-energy CID product ion spectra (MS/MS) of glycans released by hydrazinolysis as well as digestion with specific exoglycosidases confirmed the sequence, branching, and linkages of the carbohydrate portion of the molecule, and established the presence in glycosylation from one cell line of a potentially immunogenic structure.

These results demonstrate the capabilities of mass spectrometry for carbohydrate structural characterization. In addition, examination of samples from different cell lines highlights a potential difficulty associated with the widespread use of mouse hybridoma cell lines for the production of therapeutic monoclonal antibodies.

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