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Variation in N-linked carbohydrate chains in different batches of two chimeric monoclonal IgG1 antibodies produced by different murine SP2/0 transfectoma cell subclones

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Two chimeric human/murine monoclonal antibodies were constructed by substitution of the murine constant regions with human γ1 and κ constant regions for heavy and light chains, respectively. The chimeric human/murine molecules are anti-idiotypic antibodies, meaning that they were directed against the antigen binding site in the variable region of another antibody. Antibody batches were produced under identical production conditions, using two selected SP2/0 myeloma cell subclones, which produce chimeric antibodies with different variable regions, but identical constant regions. Several samples were collected during the production of the antibodies in hollow-fibre reactors. The heavy chain, but not the light chain, of the two different chimeric IgG1 antibodies is glycosylated. Structural analysis of the enzymically released N-linked carbohydrate chains by ^{1}H-NMR spectroscopy, as well as by chromatographic profiling, demonstrated that the collection of N-glycans comprises a small amount of monoantennary, and for the greater part diantennary structures. The N-glycans are completely (α1 →6)-fucosylated at the innermost GlcNAc residue. The antennae of the neutral diantennary N-glycans are built up from GlcNAcβ1→2, Galβ1 → 4GlcNAcβ1 → 2 or Galα1 → 3Galβ1 → 4GlcNAcβ1 → 2 elements, whereas the antennae of the neutral monoantennary carbohydrate chains have only (β1 → 2)-linked GlcNAc residues. Galactosylation of the GlcNAcβ1 → 2Manα1 → 6 branch occurs four times more frequently than that of the GlcNAcβ1 → 2Manα1 → 3 branch, independently of the production batch. A small amount of the diantennary N-glycans are mono- or disialylated, carrying *N*-acetylneuraminic acid (Neu5Ac) or *N*-glycolylneuraminic acid (Neu5Gc), exclusively (α2 → 6)-linked to βGal. Analysis of the different production batches demonstrates that the structures of the N-linked carbohydrate chains are identical in the two chimeric antibodies, but that the relative amounts of the major oligosaccharide components, the degree of sialylation and the molar ratio of Neu5Ac to Neu5Gc varies with the SP2/0 cell subclone, and only slightly with cell age.

Keywords: biotechnological antibody, chimeric IgG1, N-glycosylation, cell subclones, glycoprotein

Introduction

IgG is a dimeric molecule consisting of two identical heavy chains and two identical light chains [1]. IgG consists of an antigen-binding (Fab) and an immunoregulatory (Fc) part, which can be generated as fragments by treatment with proteases. In mammalian serum IgG, diantennary *N*-acetyllactosamine-type carbohydrate chains are attached to Asn297 located in the Fc part of the heavy chain [2, 3]. In addition, N-linked oligosaccharides were also occasionally shown to occur in the Fab part (see [4] for review). O-Linked oligosaccharides have been found sporadically in the hinge region, *i.e.* between the Fab and Fc parts [5–8]. The absence of the carbohydrate chains at Asn297 in IgG destroys almost completely its immunoregulatory activity compared with the glycosylated molecule [9–13].

Animal antisera, and more recently rodent monoclonal antibodies (MABs), have been used in clinical therapy and diagnosis [14]. Administration of rodent MABs to man may elicit an anti-MAB reaction, like the human anti-mouse antibody (HAMA) response [15]. In order to minimize these reactions,

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which could interfere with clinical therapy, chimeric antibodies have been constructed, which comprise structural domains of the murine variable and human constant parts [16, 17]. Advantageously, the efficacy of the effector function of the constant part can be selected from the human isotype IgG1, 2, 3 or 4 sequences. The chimeric antibody is then expressed in a suitable cell-line, with an adequate and effective glycosylation machinery to retain the effector functions.

The carbohydrate chains of various biotechnologically produced IgGs have been investigated [5, 18–24]. Analyses showed the occurrence of predominantly diantennary *N*-acetyllactosamine type carbohydrate chains, which are ($\alpha 2 \rightarrow 3$)- and/or ($\alpha 2 \rightarrow 6$)-sialylated, or contain terminal Gal$\alpha 1 \rightarrow 3$ Galβ units. Furthermore, oligomannose type oligosaccharides [20, 25] and a tetrasialylated triantennary compound [22], not found in mammalian serum IgG, have been detected.

Glycosylation is a cell type-specific event, and the choice of a cell line therefore has implications for the expression of the final glycosylation pattern of a glycoprotein [26]. Moreover, since the biosynthesis of carbohydrate chains involves co- and post-translational events, which are not under direct genetic control [27], the cell culture conditions may have implications for the glycosylation of biotechnologically produced glycoproteins [26]. As a consequence, the pharmacokinetic behaviour of glycoproteins, like IgG, may be affected by modifications of the oligosaccharide pattern.

Here, the structures of the carbohydrate chains occurring on chimeric human/murine IgG1 antibodies produced by SP2/0 subclones are determined, and the consistency of these structures following cell culture over time is reported.

Materials and methods

Cultivation of SP2/0 cells and production of chimeric human/murine antibodies

The MABs H and M are chimeric human/murine anti-idiotypic antibodies expressed in murine SP2/0 myeloma cells, which were cultured in a Mammalian Cell Culture System AcuSyst P/3X (Endotronics, Minneapolis, USA) (Table 1), after subclone selection for growth and production rate. Cells and high-molecular-mass molecules are maintained in the extracapillary space (EC) of the reactor and are fed with medium at the same rate as product is harvested from the EC-circuit. Exchange of the low-molecular-mass nutrients occurs via the intracapillary space (IC) through a hollow-fibre membrane with a molecular weight cut-off of 10 kDa.

The serum-free EC-medium was based on the proprietary medium PFHM (Gibco) with supplementary albumin, lipids, glucose, and amino acids. The serum-free IC-medium was composed of the commercially available basic media DMEM, RPMI1640 and Ham's F12. Supplements, comprising several amino acids and a final glucose concentration of 4.5 g l^{-1}, were added to the IC-medium. Culture conditions were maintained at 37°C, pH 7.0 (pH 7.2 during the early-growth phase), and at 65–85 mmHg oxygen. Glucose and lactate levels were regulated between 1–2.5 g l^{-1} and 1–3 g l^{-1} medium, respectively. When the glucose level dropped under 1 g l^{-1} medium, a solution of 54 g l^{-1} glucose was used to supplement the IC-medium.

Table 1. Conditions used for the production of the batches of the monoclonal antibodies H and M. The SP2/0 myeloma cell subclones were cultured in serum-free media.

Code of MAB	*Harvest time interval*	*Reactor*	*Subclone of SP2/0 cell*
H1	12/09/92–20/10/92	AcuSyst P/3X	F5-CHγ1
H2	09/11/92–20/11/92	AcuSyst P/3X	F5-CHγ1
M1	28/03/93–08/04/93	AcuSyst P/3X	MK-2 – 4
M2	08/04/93–15/04/93	AcuSyst P/3X	MK-2 – 4
M3	15/04/93–19/04/93	AcuSyst P/3X	MK-2 – 4
M4	19/04/93–25/04/93	AcuSyst P/3X	MK-2 – 4
M5	25/03/93–02/05/93	AcuSyst P/3X	MK-2 – 4
M6	03/05/93–10/05/93	AcuSyst P/3X	MK-2 – 4

Before inoculation, cells were freshly thawed from a working cell-bank and proliferated in 150 cm^2 T-flasks and 3 l cell culture bags (Stedim). During production, cells were partially drawn into the EC-medium stream upon ongoing proliferation, and removed through the continuous harvest line. The cells in the harvest mixture were regularly examined for proliferation and production characteristics. Dead and lysing cells were removed, if necessary, by a limited pressurized flushing of the reactors.

Product was collected continuously by passing the EC-medium through a combination of 0.45 μm and 0.2 μm filters. In the case of MAB M, six subsequent batches (M1–M6) each corresponding to a production period of 6 (at the end of the production process) to 15 (at the beginning of the production process) days, were collected sequentially from a 6 week production run. Likewise, two batches of MAB H (H1 and H2) were collected. The produced batches were stored at 4°C.

Isolation of chimeric human/murine IgG1

Prior to chromatography, cell culture supernatants were warmed to room temperature, and the pH was adjusted to 8.0 with NaOH or HCl. Antibodies were purified on a XK26 (Pharmacia) column, packed with 50 ml Protein A (Prosep-A, Bioprocessing, UK), fitted in a FPLC (Pharmacia) system. Before use, the column was washed with 100 ml 25 mM sodium phosphate buffer, pH 8.0, containing 0.15 M NaCl (PBS), and 100 ml 0.1 M Glycine/HCl, pH 3.5 (elution buffer), followed by re-equilibration with PBS. Then the cell culture supernatant was pumped over the column and, after washing with PBS, bound protein was eluted with elution buffer and collected as a single peak. The effluent was monitored at 280 nm. The pH of

the MAB-containing fraction was adjusted immediately to 8.0 with 2 M Trizma base (Sigma). The MAB-containing fraction was then concentrated to 10 ml by ultrafiltration using an YM30 membrane in an Amicon cell, and centrifuged at 10 000 × **g** for 20 min at 4°C. Gel filtration chromatography was performed on a XK26 (Pharmacia) column, packed with 185 ml Sephacryl S300 HR (Pharmacia), equilibrated and eluted with PBS (pH 7.5) at a flow rate of 2 ml min^{-1}. The first major eluting peak contained the antibody at a purity in excess of 90%. The final antibody preparations were stored at 4°C in the presence of 0.02% (w/v) NaN_3.

Liberation of the N-linked carbohydrate chains

The N-linked carbohydrate chains of the MABs were released by treatment with recombinant peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F (PNGase-F; Boehringer) [28]. EDTA was added to solutions of the MAB batches H1 and H2 to give a final concentration of 40 mM. Because leaving out EDTA had no detectable effect on the de-N-glycosylation process of the MABs, EDTA was not included in the solutions of the MAB batches M1–M6. Batches of 15 to 110 mg MAB were denatured in the presence of SDS, added to give a final 1:1 weight ratio of SDS to MAB, and reduced with 1% by vol 2- mercaptoethanol by incubation for 1 h at 37°C. Samples were cooled to ambient temperature before the addition of 10% (w/v) aqueous Nonidet P-40 (NP-40), giving a final 1:1 weight ratio of NP-40 to SDS, followed by the addition of 0.2 U PNGase-F per mg MAB. After incubation for 5 h at ambient temperature, a fresh aliquot of 0.2 U PNGase-F per mg MAB was introduced and incubations were continued overnight. The extent of de-N-glycosylation was checked by SDS/PAGE on a 12.5% slab gel (2.6% cross-linking) and Coomassie Brilliant Blue staining.

Isolation of the liberated N-linked carbohydrate chains

Released carbohydrate chains of each MAB were separated from the remaining protein by gel filtration on a column (2.5 × 50 cm) of Bio-Gel P-100, eluted with 0.1 M NH_4HCO_3, pH 7, containing 0.1 M NaCl, monitoring the effluent at 206 nm. The void volume, which contained the de-N-glycosylated MAB, and the retarded fraction, containing carbohydrate material, buffer salts, and detergents were collected. The retarded fraction was concentrated to 40 ml by lyophilization, and detergents were removed on a column (2.6 × 3 cm) of Extracti-Gel D (Pierce), eluted with 40 ml 0.1 M NH_4HCO_3 pH 7, containing 0.1 M NaCl. The eluate was concentrated by lyophilization and desalted on a column (3 × 55 cm) of Bio-Gel P-2, using water as eluent, and monitored at 206 nm. Carbohydrate-positive fractions (300 MHz ^{1}H-NMR analysis: *N*-acetyl region) were pooled, lyophilized and fractionated on a Q-Sepharose FF anion-exchange column (1.6 × 7 cm), using a Pharmacia FPLC system. The elution was performed with 30 ml H_2O, followed by a linear concentration gradient of 0 to 100 mM NaCl in 70 ml H_2O, and finally by a concentration gradient of 100–1000 mM NaCl in 20 ml H_2O, at a flow rate of 4 ml min^{-1}. Runs were monitored at 214 nm. Collected fractions were lyophilized and desalted on a column (1 × 44 cm) of Bio-Gel P-2 in water.

The fraction containing neutral carbohydrate chains was concentrated to 100 µl and subfractionated by HPLC on a LiChrosorb-NH_2 10-µm column (25 × 0.46 cm; Chrompack). In the case of MABs H1 and H2, the column was eluted with a linear gradient of 30% – 40% by vol water in acetonitrile for 1 h, at a flow rate of 120 ml h^{-1}, and monitoring the effluent at 205 nm. The neutral oligosaccharides from MABs M1–M6 were fractionated using a linear gradient of 32% – 40% by vol water in acetonitrile for 1 h, followed by 40% by vol water in acetonitrile for 30 min, at a flow rate of 120 ml h^{-1}. Before lyophilization, the solvent was partially removed from the collected fractions under a stream of nitrogen.

Profiling using high pH anion-exchange chromatography with pulsed amperometric detection

Chromatographic profiling studies were performed as described [29], but with some modifications. Briefly, to 200 µg of antibody M (M1–M6) dissolved in PBS pH 7.5 (see above) were added 3 µl 10% (w/v) aqueous SDS and 5 µl 1 M DTT, and the final volume was adjusted to 160 µl with 20 mM sodium phosphate buffer, pH 7. DTT was used instead of 2-mercaptoethanol, because the latter coelutes with neutral N-glycans. After heating at 95–100°C for about 5 min and the addition of 3 µl 10% (w/v) aqueous NP-40, 2 U PNGase-F were introduced. The mixture was incubated overnight at ambient temperature. Completeness of the de-N-glycosylation was checked by SDS/PAGE. The de-N-glycosylated protein was precipitated by the addition of 680 µl methanol and, after 1 h on ice, the suspension was centrifuged at 12 000 × **g** for 5 min. The supernatant was concentrated under reduced pressure, and the residue, dissolved in 100 µl water, was desalted by centrifugal size-exclusion chromatography using 1.2 ml Bio-Gel P-2 at 1 350 × **g** for 5 min [30]. For analysis 40 µl samples were injected on a Dionex CarboPac PA-1 column (0.4 × 25 cm) fitted in a Dionex BioLC system. Elutions were carried out with a linear concentration gradient from 10 mM to 110 mM NaOAc in 44 ml 0.1 M NaOH at a flow rate of 1 ml min^{-1}. Pulsed amperometric detection (PAD) was carried out using the following pulse potential and duration settings: $E_1 = 0.05$ V, 480 ms; $E_2 = 0.60$ V, 120 ms; $E_3 = -0.60$ V, 60 ms. The CarboPac PA–1 column was calibrated with carbohydrate fractions from the large scale-preparations of the MABs, identified by ^{1}H-NMR spectroscopy.

Sialic acid analysis

The analysis of sialic acids was carried out essentially as previously described [31]. MAB (0.5 mg) in PBS, pH 7.5 (see above), was lyophilized, and the residue, dissolved in 200 µl 2 M acetic acid, was heated for 3 h at 80°C. Neu5Ac, Neu5Gc, human serum transferrin (Sigma) and equine fibrinogen (Sigma) were taken through the procedure as standards. HPLC analysis was carried out on a Chromspher C_{18} reversed-phase column (0.46 × 25 cm; Chrompack) as described [32].

Monosaccharide analysis

In order to remove buffer salts, solutions containing 1 mg MAB were passed through a column (1 × 28 cm) of Bio-Gel P-6 (200–400 mesh, Bio-Rad), eluted with 25 mM, NH_4HCO_3. The void volume was collected and lyophilized twice to remove residual NH_4HCO_3. The trimethylsilylated (methyl ester) methyl glycosides were prepared by methanolysis (1.0 M methanolic HCl, 16 h, 85°C), *N*-acetylation, and trimethylsilylation. Monosaccharide analysis was carried out by GLC on a capillary SE-30 WCOT fused silica column (25 m × 0.32 mm, Chrompack) using a Varian Aerograph 3700 gas chromatograph [33].

^{1}H-NMR spectroscopy

Prior to ^{1}H-NMR spectroscopy, oligosaccharide samples were repeatedly treated with 99.8% ^2H_2O (MSD Isotopes) at p^{2}H with repetitive lyophilization, finally using 99.96% ^2H_2O. One-dimensional (1D) ^{1}H-NMR spectra were recorded 300 MHz, 500 MHz and 600 MHz at on Bruker AC-300 (Department of Organic Chemistry, Utrecht University), AMX-500 and AMX-600 (Bijvoet Center, Department of NMR-spectroscopy, Utrecht University) spectrometers, respectively, at a probe temperature of 300 K [34, 35]. Chemical shifts are expressed in ppm relative to internal acetone (δ 2.225 in ^2H_2O at 300 K).

Quantification of N-linked carbohydrate chains

The molar ratio of constituent oligosaccharide components was determined on the basis of FPLC (Q-Sepharose) and HPLC (LiChrosorb-NH_2) peak intensities at 214 and 205 nm, respectively, corrected for the number of C=O groups. ^{1}H-NMR spectra were used to determine the relative amounts of N-glycans in charged FPLC fractions **N1** and in overlapping HPLC peaks.

Results

Monosaccharide analyses of the various batches of monoclonal human/mouse chimeric antibodies revealed in each case a carbohydrate content of about 2% (by mass) and the presence of Fuc, Gal, Man, GlcNAc and sialic acid. The absence of GalNAc implies that neither mucin-type carbohydrate chains nor N-linked chains containing the GalNAcβ1→4GlcNAcβ element occur. Sialic acid analyses of the MAB preparations show that the sialic acids comprise Neu5Gc and Neu5Ac. Neu5Gc is predominantly present in the MAB

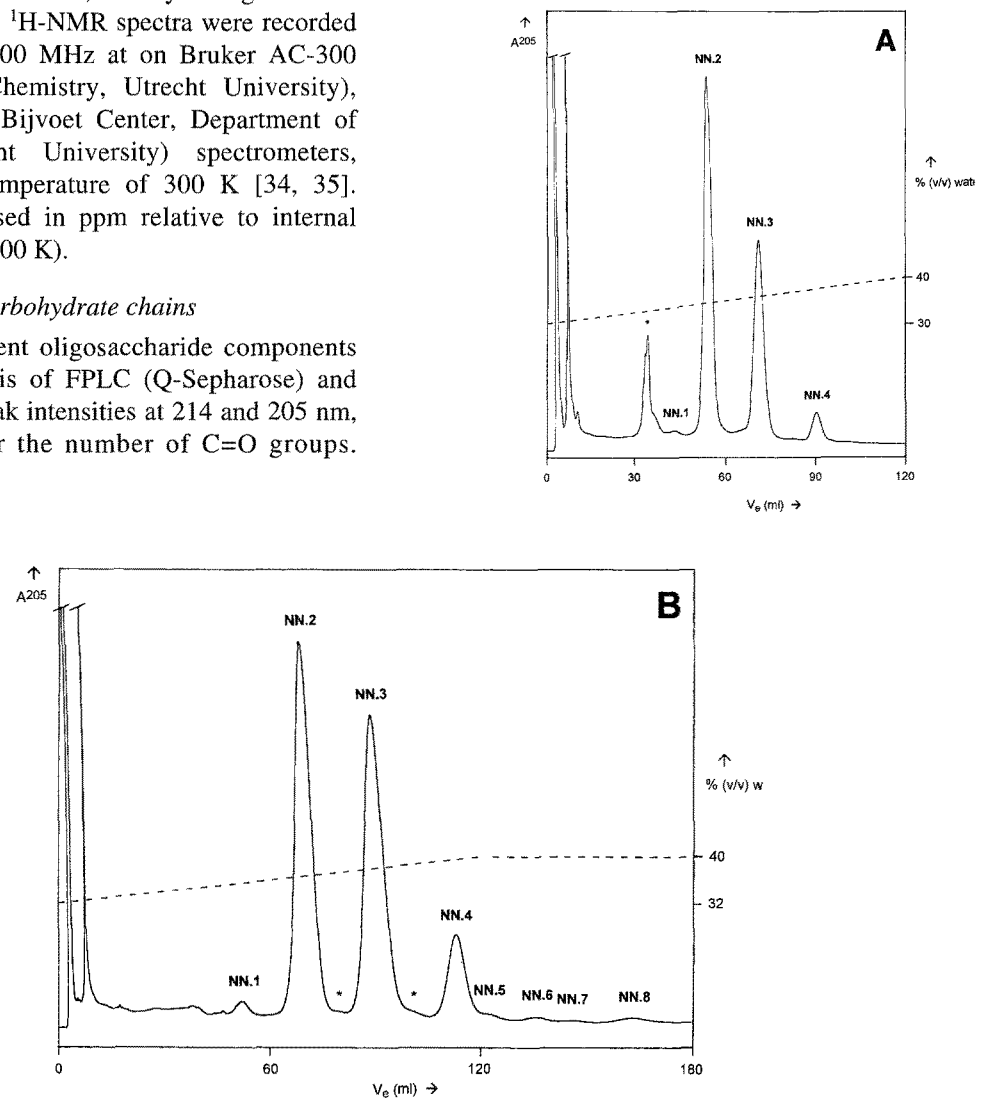


Figure 1. HPLC fractionation patterns of neutral FPLC fractions NN from MAB batches (A) H1 and (B) M4 at 205 nm on a LiChrosorb-NH_2 10 μm column (0.46 × 25 cm). The elution was carried out at a flow rate of 120 ml/h^{-1} using concentration gradients of water in acetonitrile as indicated. The HPLC fractions marked by an asterisk did not contain carbohydrate material.

batches M1–M6 (≥ 99 mol%), whereas Neu5Ac is the major residue in the batches H1 (71 mol%) and H2 (73 mol%). Incubation of the various MAB batches with PNGase-F afforded the complete release of N-glycans from the heavy chain, as checked by SDS/PAGE. The apparent molecular mass difference (2 kDa) of the heavy chain before and after PNGase-F treatment is in agreement with the occurrence of one N-glycosylation site in the heavy chain. SDS/PAGE did not show a molecular mass difference in the light chain before and after PNGase-F treatment of the MABs, which is in accordance with the absence of N-linked carbohydrate chains in the Fab part. Anion-exchange chromatography on Q-Sepharose FF of the desalted Bio-Gel P-100 oligosaccharide fractions from the MAB batches H1 and H2 gave rise to a neutral and a charged carbohydrate-containing fraction, denoted **NN** and **N1**, respectively. The charged fraction eluted at the same position as the reference monosialylated diantennary carbohydrate chain *N1.2* [28]. In the case of the MAB batches M1–M6, an additional carbohydrate-positive fraction **N2**, eluting at the position of the reference disialylated diantennary carbohydrate chain *N2.2* [28], was collected. On the basis of the absorbance at 214 nm of the FPLC fractions, the degree of sialylation in the MAB batches H1 and H2 is determined to be less than 4%, and about 14% in batches M1–M6. The charged carbohydrate chains in M1–M6 comprise equimolar amounts of mono- (**N1**) and disialylated (**N2**) compounds.

Each of the neutral fractions **NN** was subfractionated by HPLC on LiChrosorb-NH_2. Subfractionation of the neutral N-glycans from MAB batch M4 yielded eight carbohydrate-positive subfractions, **NN.1**–**NN.8** (Fig. 1B), which were investigated by ^{1}H-NMR spectroscopy. The HPLC fractions **NN.5** and **NN.7** did not contain enough carbohydrate material for a reliable structural analysis. On the basis of the chromatographic profile, subfractions obtained by HPLC of fractions **NN** from the MAB batches H1 (Fig. 1A), H2, M1–M3, M5 and M6, eluting at a position corresponding to one of the subfractions **NN.1**–**NN.8** from MAB batch M4, were uniformly designated. In order to verify this designation, subfractions **NN.3** and several additional systematically selected subfractions were investigated by ^{1}H-NMR spectroscopy. The ^{1}H-NMR data are compiled in Tables 2 and 3, and a list of the identified N-linked carbohydrate chains and their corresponding codes are given in Table 4. The structure determination will be given briefly, in an order that is convenient for discussing the ^{1}H-NMR data. The series of N-linked carbohydrate chains have the (α1→6)-fucosylated *N,N′*-diacetylchitobiose element in common, as is evident from the H-1 signals of αGlcNAc-**1**, βGlcNAc-**1**, GlcNAc-**2** and Fuc, together with the NAc signals of GlcNAc-**1** and GlcNAc-**2**, and the Fuc CH_3 signals [35].

^{1}H-NMR analysis of the subfractions **NN.1** of the MAB batches M4 and M6 shows in each the presence of a mixture of two carbohydrate chains **CN1A** and **CN1B**, in a molar ratio of 4 : 1 and 1 : 1, respectively. The NMR data of component **CN1A** are in accordance with those from reference compound *{0,2+F}* [36], revealing that component **CN1A** is a monoantennary N-glycan, wherein the Manα1 → 3 branch of the pentasaccharide core is extended with a (β1 → 2)-linked GlcNAc residue. Component **CN1B** represents a structural isomer of **CN1A**, with the additional (β1 → 2)-linked GlcNAc residue at the Manα1→6 branch. The structure of **CN1B** could be deduced from a comparison of the NMR data of **CN1B** with those of the reference non-fucosylated analogue *{2, 0}* [36].

The ^{1}H-NMR spectra of the HPLC subfractions corresponding to **NN.2** revealed the presence of a fucosylated diantennary carbohydrate chain having a terminal (β1→2)-linked GlcNAc residue in each antenna, referred to as **CN2**. The NMR data of the antennae are in agreement with those of reference pyridylaminated compound *V* [37].

Comparison of the structural-reporter-group ^{1}H-NMR data of carbohydrate chain **CN4** in fractions **NN.4** with those of reference glycopeptide 8 [34] indicated the occurrence of an (α1 → 6)-fucosylated diantennary oligosaccharide with terminal Galβ1 → 4GlcNAcβ1 → 2 elements in each antenna.

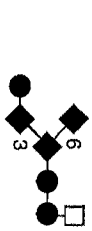
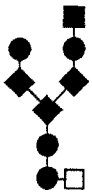
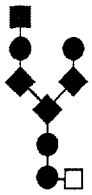
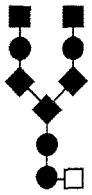
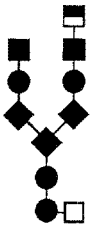
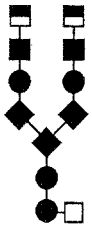
^{1}H-NMR analysis of the subfractions **NN.3** showed the presence of a mixture of two isomers **CN3A** and **CN3B**, representing monogalactosylated extensions of oligosaccharide **CN2**. In the major component **CN3A** of subfractions **NN.3**, the GlcNAc-**5′** residue is (β1 → 4)-galactosylated, whereas in component **CN3B** GlcNAc-**5** is (β1 → 4)-galactosylated. The ^{1}H-NMR data of the galactosylated branches in **CN3A** and **CN3B** coincide with those of the corresponding branches in **CN4**, whereas the ^{1}H-NMR data of the non-galactosylated branches fit those of the corresponding branches in **CN2**. Using the ratio of the intensities of the H-1 signals of Gal-**6** (**CN3B**) and Gal-**6′** (**CN3A**), together with that of Man-**4′** (**CN3A**) and Man-**4′** (**CN3B**), the relative molar ratio of **CN3A** and **CN3B** in the subfractions **NN.3** was calculated to be 4 : 1 for each production batch.

Inspection of the ^{1}H-NMR spectra of the subfractions **NN.6** revealed that their major component **CN6** is an (α1→6)-fucosylated diantennary carbohydrate chain containing terminal Gal-**6** and (α1→3)-galactosylated Gal-**6′**. The presence of the Galα1→3Galβ1→4GlcNAcβ1→2Manα1→6 branch was deduced from the matching of the ^{1}H-NMR parameters of this branch, namely αGal H-1, Gal-**6′** H-1, GlcNAc-**5′** H-1, GlcNAc-**5′** NAc, Man-**4′** H-1 and Man-**4′** H-2, with those of the corresponding branch in reference monosialylated compound *1-7* [38]. Likewise, the structural-reporter-group data of the Galβ1→4GlcNAcβ1→2Manα1→3 branch fit those of the corresponding branch in compound **CN4**.

^{1}H-NMR analysis of fraction **NN.8** in the MAB batch M4 demonstrated the presence of an (α1→6)-fucosylated diantennary oligosaccharide **CN8**, having a terminal Galα1→3Galβ unit in both antennae. The ^{1}H-NMR data of the antennae in **CN8** are in good agreement with those of the reference (α1 → 6)-fucosylated oligosaccharide alditol *FN* [39].

The ^{1}H-NMR spectra of the FPLC fractions **N2** from the MAB batches M1, M4 and M6 indicated the presence of an

Table 2. ^{1}H-Chemical shifts in structural-reporter-group protons of constituent monosaccharides of the neutral N-linked oligosaccharides derived from different MAB preparations. Chemical shifts are given in ppm relative to internal acetone (δ 2.225) in ^2H_2O at 300 K and at p^{2}H 7 [34]. Compounds are represented by shorthand symbolic notation: □, L-Fuc; ■ D-βGal; ◧, D-αGal; ●, D-GlcNAc; ◆, D-Man. For numbering of the monosaccharide residues, see structure **CN4** in Table 4. ND, not determined. α and β stand for the anomeric configuration of GlcNAc-**1**.

Reporter	*Residue*	*Chemical shift in group*							
		CN1A	**CN1B**	**CN2**	**CN3A**	**CN3B**	**CN4**	**CN6**	**CN8**
									
		ppm							
H-1	GlcNAc-**1**α	5.180	5.180	5.180	5.180	5.180	5.180	5.181	5.181
	GlcNAc-**1**β	ND	ND	4.694	4.694	4.694	4.694	ND	ND
	GlcNAc-**2**α	ND	ND	4.664	4.665	4.665	4.664	4.664	4.666
	GlcNAc-**2**β			4.669	4.667	4.667	4.668	4.668	
	Man-**3**	ND	ND	4.771	4.767	4.767	ND	ND	ND
	Man-**4**	5.119	5.099	5.116	5.117	5.117	5.119	5.119	5.119
	Man-**4′**	4.916	4.915	4.916	4.925	4.915	4.925	4.927	4.924
	GlcNAc-**5**	4.552	–	4.555	4.556	4.582	4.582	4.583	4.583
	GlcNAc-**5′**	–	4.552	4.555	4.582	4.556	4.582	4.583	4.583
	Gal-**6**	–	–	–	–	4.468	4.467	4.469	4.540
	Gal-**6′**	–	–	–	4.473	–	4.473	4.541	4.544
	αGal	–	–	–	–	–		5.147	5.146[a]
	$Fuc_α$	4.892	ND	4.895	4.897	4.897	4.897	4.898	ND
	$Fuc_β$	4.886	ND	4.888	4.890	4.890	4.889	4.891	ND
H-2	Man-**3**	4.251	4.254	4.250	4.250	4.250	4.250	4.248	4.250
	Man-**4**	4.184	4.067	4.188	4.189	4.189	4.189	4.188	4.194
	Man-**4′**	3.971	4.104	4.106	4.108	4.108	4.108	4.107	4.11
H-4	Gal-**6′**	ND	ND	ND	ND	ND	ND	4.185	4.183[a]
H-5	$Fuc_α$	4.10	4.098	4.096	4.096	4.096	4.096	4.100	ND
	$Fuc_β$	4.13	4.134	4.132	4.133	4.133	4.133	4.134	ND
	αGal	–	–	–	–	–	–	4.192	4.19
NAc	GlcNAc-**1**	2.039	2.039	2.039	2.039	2.039	2.039	2.040	2.039
	GlcNAc-$\mathbf{2}_α$			2.095			2.096		
	GlcNAc-$\mathbf{2}_β$	2.094	2.094	2.092	2.096	2.096	2.094	2.096	2.096
	GlcNAc-**5**	2.052	–	2.053	2.053	2.053	2.051	2.050	2.052
	GlcNAc-**5′**	–	2.051	2.053	2.047	2.053	2.047	2.050	2.048
CH_3	$Fuc_α$	1.209	1.209	1.208	1.210	1.210	1.210	1.210	1.209
	$Fuc_β$	1.220	1.220	1.219	1.221	1.221	1.222	1.222	1.220

[a] Signal has the intensity of two protons.

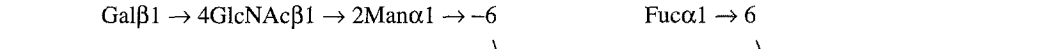
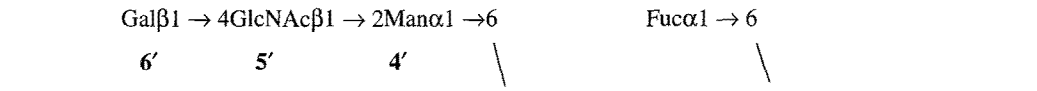
(α1 → 6)-fucosylated diantennary oligosaccharide **C2** with an (α2 → 6)-linked Neu5Gc residue in each antenna. The structural-reporter-group ^{1}H-NMR data of the antennae of **C2** match those of the reference disialylated (Neu5Gc), non-fucosylated diantennary oligosaccharide analogue *N2.4* [40]. On the basis of the identical sialic acid composition in the MAB batches M1–M6, the identical elution position of fractions **N2**, together with the detection of **C2** in the investigated MAB batches M1, M4 and M6, compound **C2** is proposed to be present in the MAB batches M2, M3 and M5 as well.

Table 3. ^{1}H-Chemical shifts in structural-reporter-group protons of constituent monosaccharides of the sialylated N-linked oligosaccharides derived from different MAB preparations. Chemical shifts are given in ppm relative to internal acetone (δ2.225) in ^2H_2O at 300 K and at p^{2}H 7 [34]. Compounds are represented by shorthand symbolic notation: □, L-Fuc; ■, D-βGal; ◧, D-αGal; ●, D-GlcNAc; ◆, D-Man; ⊘, D-Neu5Gcα(2-6). For numbering of the monosaccharide residues, see structure **CN4** in Table 4. ND, not determined. α and β stand for the anomeric configuration of GlcNAc-1.

Reporter group	*Residue*	*Chemical shift in*				
		C1A	C1B	C1C	C1D	C2
		ppm				
H-1	GlcNAc-**1**α	5.181	5.181	5.181	5.181	5.180
	GlcNAc-**1**β	4.694	4.694	4.694	4.694	ND
	GlcNAc-**2**$_\alpha$	4.666	4.666	4.666	4.666	4.666
	GlcNAc-**2**$_\beta$	4.668	4.668	4.668	4.668	4.669
	Man-**3**	4.771	4.771	4.771	4.771	ND
	Man-**4**	5.135	5.135	5.119	5.135	5.134
	Man-**4′**	4.927	4.918	4.937	4.927	4.939
	GlcNAc-**5**	4.609	4.609	4.555	4.609	4.606
	GlcNAc-**5′**	4.582	4.555	4.609	4.582	4.606
	Gal-**6**	4.451	4.451	–	4.451	4.449
	Gal-**6′**	4.472	–	4.451	4.545	4.449
	αGal	–	–	–	5.147	–
	Fuc$_\alpha$	4.890	4.890	4.890	4.890	4.888
	Fuc$_\beta$	4.898	4.898	4.898	4.898	4.896
H-2	Man-**3**	4.253	4.253	4.253	4.253	4.256
	Man-**4**	4.197	4.197	4.187	4.197	4.197
	Man-**4′**	4.11	4.11	4.11	4.11	4.11
H-3a	Neu5Gc	1.735	1.735	–	1.735	1.734
	Neu5Gc′	–	–	1.735	–	1.734
H-3e	Neu5Gc	2.687	2.687	–	2.687	2.685
	Neu5Gc′	–	–	2.691	–	2.689
H-5	Fuc$_\alpha$	4.11	4.11	4.11	4.11	4.100
	Fuc$_\beta$	4.135	4.135	4.135	4.135	4.131
NAc	GlcNAc-**1**	2.039	2.039	2.039	2.039	2.038
	GlcNAc-**2**	2.096	2.096	2.096	2.096	2.097
	GlcNAc-**5**	2.072	2.072	2.053	2.072	2.070
	GlcNAc-**5′**	2.048	2.053	2.072	2.048	2.070
NGc	Neu5Gc	4.119	4.119	4.119	4.119	4.118
CH_3	Fuc$_\alpha$	1.210	1.210	1.210	1.210	1.209
	Fuc$_\beta$	1.222	1.222	1.222	1.222	1.221

The ^{1}H-NMR spectra of FPLC fractions **N1** from the MAB batches M1, M4, M5 and M6 showed the presence of a mixture of four monosialylated compounds **C1A, C1B, C1C** and **C1D**, in molar ratios of 4 : 1 : 3 : 1, as evidenced by the occurrence of four different sets of structural-reporter-group signals (Table 3). The diantennary character of the compounds was deduced from the resonance positions of the Man-**4/4′** H-1 and H-2 signals [28, 34]. The presence and

Table 4. Identified carbohydrate chains occurring in recombinant chimeric monoclonal antibody production batches H1, H2 and M1–M6 and their corresponding oligosaccharide codes. The monosaccharide numbering system is indicated in carbohydrate chain **CN4.**

Carbohydrate chain	*code*
	**CN1A**
	**CN1B**
	**CN2**
	**CN3A**
	**CN3B**
	**CN4**
	**CN6**

continued

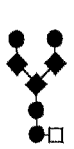
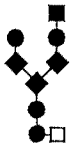
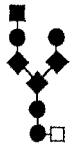
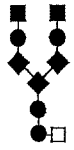
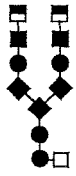
Table 4. Identified carbohydrate chains occurring in recombinant chimeric monoclonal antibody production batches H1, H2 and M1–M6 and their corresponding oligosaccharide codes. The monosaccharide numbering system is indicated in carbohydrate chain **CN4. – (*cont'd*)**

Carbohydrate chain	*code*
Galα1 → 3Galβ1 → 4GlcNAcβ1 → 2Manα1 → 6 (Fucα1 → 6) Manβ1 → 4GlcNAcβ1 → 4GlcNAc	**CN8**[a]
Galα1 → 3Galβ1 → 4GlcNAcβ1 → 2Manα1 → 3	
Galβ1 → 4GlcNAcβ1 → 2Manα1 → 6 (Fucα1 → 6) Manβ1 → 4GlcNAcβ1 → 4GlcNAc	**C1A**[b]
Neu5Gcα2 → 6Galβ1 → 4GlcNAcβ1 → 2Manα1 → 3	
GlcNAcβ1 → 2Manα1 → 6 (Fucα1 → 6) Manβ1 → 4GlcNAcβ1 → 4GlcNAc	**C1B**[b]
Neu5Gcα2 → 6Galβ1 → 4GlcNAcβ1 → 2Manα1 → 3	
Neu5Gcα2 → 6Galβ1 → 4GlcNAcβ1 → 2Manα1 → 6 (Fucα1 → 6) Manβ1 → 4GlcNAcβ1 → 4GlcNAc	**C1C**[b]
GlcNAcβ1 → 2Manα1 → 3	
Galα1 → 3Galβ1 → 4GlcNAcβ1 → 2Manα1 → 6 (Fucα1 → 6) Manβ1 → 4GlcNAcβ1 → 4GlcNAc	**C1D**[b]
Neu5Gcα2 → 6Galβ1 → 4GlcNAcβ1 → 2Manα1 → 3	
Neu5Gcα2 → 6Galβ1 → 4GlcNAcβ1 → 2Manα1 → 6 (Fucα1 → 6) Manβ1 → 4GlcNAcβ1 → 4GlcNAc	**C2**[b]
Neu5Gcα2 → 6Galβ1 → 4GlcNAcβ1 → 2Manα1 → 3	

[a]Detected in the MAB batch M4 only
[b]Not detected in the MAB batches H1 and H2

intensity of two sets of signals for H-3a, H-3e and NGc demonstrate that the mixture contains components with (α2 → 6)-linked Neu5Gc located in the Manα1 → 3 branch (major) as well as in the Manα1 → 6 branch (minor) (*cf.* compound **C2**). The (α1→6)-fucosylated, diantennary oligosaccharide **C1A**, containing Neu5Gcα2 → 6Galβ1 → 4GlcNAcβ1→ 2Manα1 → 3 and Galβ1 → 4GlcNAcβ1 → 2Manα1 → 6 branches, is indicated by the coincidence of its structural-

Table 5. Relative molar amounts of neutral N-linked carbohydrate chains occurring in recombinant chimeric human/murine monoclonal antibodies batches H1 and H2, and M1 – M6. The amounts are given as percentages of the total molar amount of neutral N-glycans. The relative amounts were determined on basis of the peak intensities of the LiChrosorb-NH^2 subfractions **NN.1** to **NN.8** at 205 nm, and of the components **CN3A** and **CN3B** from the ^{1}H-NMR spectra of fractions **NN.3**. Compounds are represented by short-hand symbolic notation: □, L-Fuc; ■, D-βGal; ◧, D-αGal; ●, D-GlcNAc; ◆, D-Man. ND, not detected.

MAB	*Carbohydrate chain*							
								
	CN1A	**CN1B**	**CN2**	**CN3A**	**CN3B**	**CN4**	**CN6**	**CN8**
H1		+	59	30	6	5	+	ND
H2		+	57	30	7	6	+	ND
M1		+	39	39	10	12	+	ND
M2		+	50	32	8	10	+	ND
M3		+	49	30	8	13	+	ND
M4		1	45	34	8	11	<1	<1
M5		+	42	35	9	14	+	ND
M6		+	46	35	9	10	+	ND

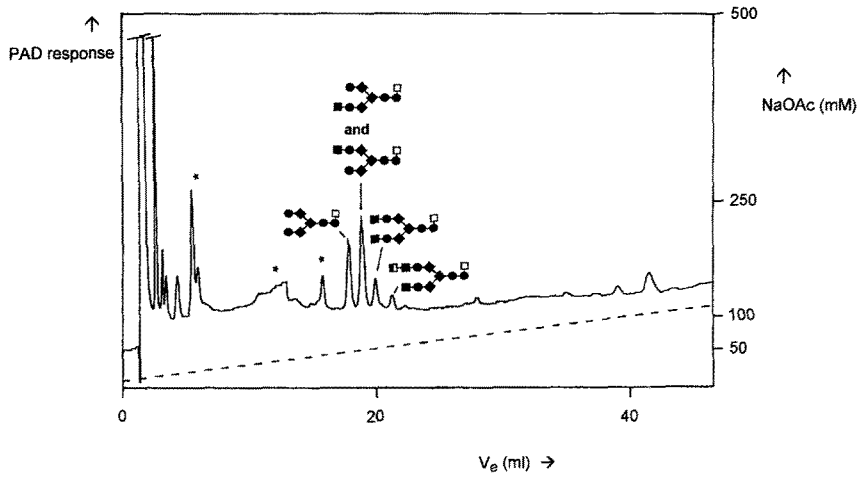


Figure 2. A typical HPAEC profile of the N-linked carbohydrate chains of MAB M on a CarboPac PA-1 anion-exchange column (0.4 × 25 cm). In this case the HPAEC profile of MAB batch M1 is shown. The N-glycans of MAB M1 were enzymically released using PNGase-F. Protein was precipitated with 80% methanol. Salts were then removed from the carbohydrate-containing supernatant by centrifugal gel-permeation chromatography prior to injection. The elution was carried out at a flow rate of 60 ml h^{-1} using a gradient of NaOAc in 0.1 M NaOH as indicated. Structures are represented by shorthand symbolic notation: □, L-Fuc; ■, D-βGal; ◧ , D-αGal; ●, D-GlcNAc; ◆, D-Man.

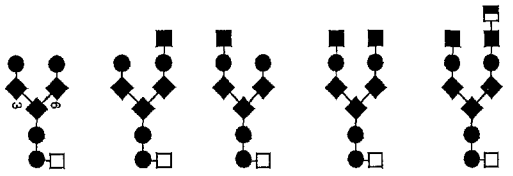
reporter-group signals with those of reference compound *1–4* [38]. In fractions **N1** the structural isomer of **C1A**, having Galβ1 → 4GlcNAcβ1 → 2Manα1 → 3 and Neu5Gcα2 → 6Galβ1 → 4GlcNAcβ1 → 2Manα1 → 6 branches, does not occur, because a Gal-**6** H-1 signal at δ 4.467 is absent in the ^{1}H-NMR spectrum (cf. compound **CN6**). Component **C1B** is a monosialylated (Neu5Gc), diantennary carbohydrate chain, containing a terminal GlcNAc residue in the Manα1→6 branch. The parameters of the branches in component **C1B** fit those of the GlcNAcβ1 → 2Manα1 → 6 branch in **CN2**, and

the Neu5Gcα2 → 6Galβ1 → 4GlcNAcβ1 → 2Manα1 → 3 branch in **C1A**, respectively. Component **C1C** is an isomer of **C1B**, carrying a terminal (α2 → 6)-linked Neu5Gc residue at the Manα1 → 6 branch, instead of at the Manα1 → 3 branch. The ^{1}H-NMR data of the Neu5Gcα2→6Galβ1→4GlcNAcβ1 →2Manα1→6 branch in **C1C** are in accordance with those of **C2**, whereas the data of the GlcNAcβ1 → 2Manα1→3 branch match those of compound **CN2**. Component **C1D** is a structural variant of component **C1A** extended with a terminal (α1 → 3)-linked Gal residue in the Manα1 → 6 branch. The structure of component **C1D** was deduced from the correspondence of its ^{1}H-NMR data with those of reference compound *1-7* [38]. Because no ^{1}H-NMR signal was found for Gal-**6** H-1 at δ 4.540, like the one found in the ^{1}H-NMR spectrum of **CN8** (Table 2), it was concluded that the structural isomer of **C1D**, containing Galα1 → 3Galβ1 → 4GlcNAcβ1 → 2Manα1 → 3 and Neu5Gcα2 → 6Galβ1 → 4GlcNAcβ1 → 2Manα1 → 6 branches, did not occur in the fractions **N1**. On the basis of the identical sialic acid composition in the MAB batches M1–M6, the identical elution position of fractions **N1**, together with the identical relative quantities of **C1A, C1B, C1C** and **C1D** found in the investigated MAB batches M1 and M4–M6, these compounds are proposed to be also present in the MAB batches M2 and M3. Since the MAB batches H1 and H2 contain predominantly Neu5Ac instead of Neu5Gc, as found in MAB batches M1–M6, and fractions **N1** from MAB batches H1 and H2 did not contain enough material for ^{1}H-NMR analysis, no definitive structures can be given for these FPLC fractions.

The relative amounts of the neutral oligosaccharides, as summarized in Table 5, were determined using ^{1}H-NMR spectra (in the case of overlapping HPLC peaks), and the absorbance of HPLC peaks at 205 nm. Comparison of the relative amounts of the neutral oligosaccharides in MABs H and M shows that **CN2, CN3A, CN3B** and **CN4** represent the major N-glycans in the collection of carbohydrate chains. The distribution of **CN2, CN3A, CN3B** and **CN4** shows only very moderate changes following cell culture over time (MAB batches H1 and H2, and MAB batches M1–M6).

In addition to the isolation of N-glycans and their structural identification by ^{1}H-NMR spectroscopy, a profiling method was developed allowing relatively fast analysis on small amounts of MABs. This microscale method includes an enzymic cleavage with PNGase-F, a limited purification of released N-glycans, and a fractionation on CarboPac PA-1 with PAD detection. Chromatographic profiles of the major N-glycans were generated for the MAB batches M1–M6, and a typical HPAEC profile is depicted in Fig. 2. The HPAEC profiles of M1 to M6 are very similar and conform to the profiles obtained by HPLC fractionation and UV detection, showing structures **CN2, CN3A/CN3B** and **CN4** as the major components. Because each component gives rise to a structure-specific PAD response, the relative HPAEC peak intensities of **CN2, CN3A, CN3B** and **CN4** cannot be compared directly with their relative molar quantities, determined on the basis of HPLC peak intensities at 205 nm and ^{1}H-NMR spectroscopy. Nevertheless, the consistency of the distribution of the carbohydrate chains is shown by the similar relative HPAEC peak intensities (Table 6), from MAB batches M1–M6, in accordance with those obtained by HPLC fractionation of fractions **NN** obtained from large scale preparations.

Table 6. The relative HPAEC peak intensities of the neutral N-linked carbohydrate chains **CN2, CN3A/CN3B, CN4**, and **CN6** occurring in recombinant chimeric human/murine monoclonal antibodies batches M1 to M6. Compounds **CN3A** and **CN3B** eluted in one subfraction. Structures are represented by short-hand symbolic notation: □, L-Fuc; ■, D-βGal; ◧, D-αGal; ●, D-GlcNAc; ◆, D-Man.

MAB	*Carbohydrate chain*				
					
	CN2	**CN3A**	**CN3B**	**CN4**	**CN6**
M1	30	48		15	7
M2	39	42		13	6
M3	41	41		10	8
M4	39	42		13	6
M5	37	43		13	7
M6	40	42		12	6

Discussion

Analysis of the pools of N-linked carbohydrate chains, enzymically released from chimeric human/murine IgG1 antibodies H and M, led to the identification of 13 oligosaccharides. The glycosylation patterns of the MABs H and M show similar arrays of microheterogeneity. However, between the two antibodies, produced by distinct SP2/0 subclones under identical production conditions, significant differences in glycan distribution are observed (Table 5). The neutral diantennary oligosaccharides **CN2, CN3A, CN3B** and **CN4** account for the majority of the carbohydrate chains of each MAB H and M. The relative amount of the galactosylated N-glycans **CN3A, CN3B** and **CN4** in the MAB batches M1–M6 is generally higher than in the MAB batches H1 and H2 (Table 5, Fig. 1A, B). Moreover, the degree of sialylation of oligosaccharides in the MAB batches M1–M6 is higher than that in the MAB batches H1 and H2. These differences indicate that the N-linked carbohydrate chains of the batches M1-M6, expressed in the SP2/0 MK-2-4 cell subclone, are

more processed than those of the batches H1 and H2, expressed in the SP2/0 F5-CHγ1 cell subclone. Furthermore, the molar percentage of Neu5Gc in the MABs fluctuates dramatically between more than 99% in the batches M1 – M6 and approximately 28% in the batches H1 and H2. Since no sialic-acid-containing additives were used in the serum-free media, the variation in the molar ratio of Neu5Ac to Neu5Gc of the two MABs can be ascribed to different expression levels of CMP-*N*-acetylneuraminate mono-oxygenase activity [41], responsible for the conversion of CMP-Neu5Ac into CMP-Neu5Gc, in the transfected subclones of the SP2/0 cell.

The occurrence of disialylated carbohydrate chains, as in the batches M1–M6, has been proposed to be dependent on the idiotype of IgG, invariably present in the Fab part of serum IgG [3]. In the present study, a single disialylated carbohydrate chain (**C2**) has been found to be attached only to the heavy chain. The heavy chain is unmodified and of human origin, containing the conserved glycosylation site at Asn297 in the Fc part, indicating that the proposed dependence on idiotype is not valid for biotechnologically produced recombinant IgG.

Despite the observed branch specificity of β-galactosyltransferase, transferring preferentially Gal onto the Manα1 → 3 branch in diantennary structures [42, 43], in each production batch of the MABs H and M galactosylation occurs predominantly on the Manα1→6 branch (80%; **CN3A**), instead of on the Manα1→3 branch (20%; **CN3B**). This phenomenon is in accordance with the galactosylation in human IgG1 from healthy persons [37]. However, this distribution of βGal over the branches changes dramatically in favour of the Manα1→ 3 branch, going from human serum IgG1 to IgG2 [37]. The same holds when going from chimeric human/murine IgG1 (predominantly Manα1→6 branch galactosylation) to chimeric human/murine IgG2 (predominantly Manα1→3 branch galactosylation), which are both expressed in murine J558L cells [19]. Although β-galactosyltransferase acts preferentially on the Manα1→6 branch in native IgG1, in denatured IgG1 a higher preference is observed for the Manα1→3 branch [37]. Apparently, the conformation of the isotype-dependent polypeptide of the Fc part (IgG1 or IgG2), and not the properties of the involved β-galactosyltransferase, gives rise to the observed distribution of βGal over the Manα1→6 and Manα1→3 branches [19, 37].

The chimeric MABs H and M, produced by murine transfectoma cells, are glycosylated in a murine-specific manner. First of all this is reflected by the presence of Neu5Gc and Galα1 → 3Galβ elements (≤ 1%), which are normally not found in N-glycans on human glycoproteins, including serum IgG [44, 45]. Furthermore, a bisecting GlcNAc, present in the N-glycans on human [2] but not on murine serum IgG [46], is absent in the MAB H and M preparations, despite the expression of the human heavy chain constant region.

Although so far no data are available about the immunogenicity of Neu5Gc-containing glycoproteins, antibodies directed against Neu5Gcα2 → 3Galβ–containing glycolipids have been detected in normal human adults after exposure to animal sera [47, 48]. The Galα1 → 3Galβ element, being an antigenic determinant for humans, has been identified in the MABs H and M, namely in the carbohydrate chains **CN6** and **CN8** (MAB batch M4 only). The percentage of N-glycans containing this determinant is less than or about 1% of the total amount of carbohydrate chains (MAB batch M4 only), whereas this relative amount is 22% of the N-glycans in chimeric murine/human IgG3 produced by murine J558L B cells [18]. About 1% of the circulating antibodies in healthy human individuals are directed against the Galα1 → 3Gal element [45], and may recognize this determinant on therapeutic recombinant glycoproteins, such as MABs. Nevertheless, Galα1→3Galβ-containing recombinant human t-PA and factor VIII did not show decreased *in vivo* half-life or bioactivity compared to the corresponding wild-type human glycoproteins, lacking this element [45]. Since in IgG the N-glycans are enclosed by the peptide chain of the Fc part [49], Neu5Gcα2 → 6Galβ and Galα1 → 3 Galβ epitopes in the MABs H and M may be masked in the immune system. Therefore, the relevance of the occurrence of these carbohydrate determinants for the immunogenicity and clearance of parenterally administered therapeutic MABs, remains to be evaluated.

Slight or no effects had previously been observed on the oligosaccharide profiles of MABs secreted by several murine hybridomas following the production over time [21]. Cell age and changes of the cell's environment, like increasing cell density and number of dead and lysing cells, have apparently only a minor effect on the glycosylation pattern of the MABs. However, it was also shown for MABs produced by a murine hybridoma cell line, that the degree of sialylation of the Asn297-linked carbohydrate chains increased with cell culture age [50]. Such cell culture variations indicate a requirement for fast and reliable oligosaccharide analytical procedures in order to assess glycosylation changes during the production of a glycoprotein, like the HPAEC method used here. Very similar HPAEC chromatographic profiles were obtained following the production of MABs H and M over time. These results match those obtained by HPLC of the FPLC-purified neutral oligosaccharide fraction **NN**.

Our results indicate a relatively constant glycosylation pattern over at least the 6 weeks production cycle of the producing cells. It should be noted that the Fc part of the two chimeric MABs, and therefore the protein matrix surrounding glycosylation site Asn297, is equivalent and of human origin. Moreover, the culture media, reactor type and isolation procedure following the production of the two antibodies remained unchanged. Therefore, the observed changes in the degree of processing and in the molar ratio of Neu5Ac to Neu5Gc between MAB H and M are most likely to be explained by the utilization of different transfectoma cell subclones. This work shows that the choice of cell subclones, usually based on superior growth or production behaviour, may also be relevant in the production of MABs with respect to the optimization of

carbohydrate structures, which may affect their pharmacokinetic parameters.

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