Different Glycosylation Pattern of Human IgG1 and IgG3 Antibodies Isolated from Transiently as well as Permanently Transfected Cell Lines

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Abstract

The effector functions of IgG depend on the presence of carbohydrates attached to asparagine 297 in the Fc-portion. In this report, glycosylation profiles of recombinant wild-type and mutant IgG1 and IgG3 antibodies produced from three cell lines were analysed using LC-ESI-Orbitrap. Clear differences were detected between IgG1 and IgG3 glycoforms, where IgG1 generally contained fucosylated glycoforms, whilst IgG3 mainly were non-fucosylated. When using NS-0 and J558L cells for permanent transfection, IgG1 wt glycoforms differed between the two cell lines, whilst IgG3 wt glycoforms did not. Transiently transfected HEK 293E cells were used to produce IgG1 and IgG3 wt and mutants, affecting complement activation. Cell supernatants were harvested at early and late time points and analysed separately. IgGs harvested late showed simpler and less developed glycosylation structure compared to those harvested early. The IgG harvested early was slightly more effective in complement activation than those harvested late, whilst the antibody-dependent cell-mediated cytotoxicity was unaltered. Generally, the glycosylation pattern of the mutants tested, including a hinge truncate mutant of IgG3, did not differ significantly from the wild-type IgGs. The striking difference in glycosylation pattern of IgG1 compared to IgG3 therefore appears not to be due to the long hinge region of IgG3 (62 amino acids) relative to the IgG1 hinge region (15 amino acids). Furthermore, mutation variants at or near the C1q binding site showed similar glycosylation structure and difference in their complement activation activity observed earlier is thus most likely due to differences in protein structure only.

Introduction

IgG is the major serum immunoglobulin and is responsible for recognizing, neutralizing and eliminating toxic antigens as well as pathogens [1]. IgG is divided into four subclasses, IgG1, IgG2, IgG3 and IgG4, with specific and overlapping roles in immune protection, where IgG1 and IgG3 are generally the most important subclasses involved in interaction with Fc-receptors as well as in the capability of activation of the complement system and thereby inactivate or kill the pathogen [2–9].

Carbohydrate groups attached to asparagine 297 in the Fc-portion of IgG have a direct influence on the effector functions of the molecules [10, 11]. Recombinant monoclonal IgG antibodies are used to study structure–function relationships and also therapeutically for treatment of cancer and diseases of the immune system, and establishing their glycosylation pattern will therefore be of interest [12–19]. The field of glycosylation analysis of recombinant IgG antibodies described in the literature is very limited and motivated us to undertake the present study.

The carbohydrate molecules attached at asparagine 297 are mannose, β-N-acetylgalactosamine (GlcNAc), galactose, fucose and sialic acid, and emphasis will be put on the last three in this report. Each of these three carbohydrates affects the immune system and the immune response in different ways. A high degree of galactosylation of IgG is associated with improvement in symptoms during pregnancy for particular autoimmune patients [1, 20–25]. Fucose is present as core fucose, and a lack of fucose causes increased antibody-dependent cellular cytotoxicity (ADCC) [1, 26]. Sialic acid is believed to be responsible for the anti-inflammatory effect of intravenous immunoglobulin (IVIg), often used to treat autoimmune diseases [1, 27–29]. Without any
carbohydrates, IgG has a shorter half-life than monomeric α-glycosylated IgG. IgG without carbohydrates bind less well to FcγRs and activate complement less effectively than their carbohydrate containing counterparts [9, 20, 30].

When producing recombinant antibodies either transient gene expression (TGE) or stable gene expression (SGE) is used [31]. The antibodies studied in this article are produced using both types of expression systems to evaluate possible differences in IgG glycosylation pattern by the two procedures. The cell lines used were HEK 293E, NS-0 and J558L. HEK 293E is a human embryonic epithelial fibroblast cell line broadly used to produce recombinant proteins including antibodies [32]. J558L are mouse B myeloma cells that are unable to produce heavy chains, but produce a mouse λ1 light chain [33]. NS-0 is a mouse myeloma cell line that does not secrete light and heavy chains and is extensively used as fusion partner to produce mouse hybridoma cell lines [34–36].

The glycosylation patterns of human serum have previously been determined, and both IgG1 and IgG3 displayed heavily fucosylated glycoforms [24]. Recombinant α-NIP chimeric mouse–human antibodies from J558L cells have been analysed previously, and the glycosylation pattern did not show great differences between IgG1 and IgG3, apart from a few glycoforms. IgG2 showed a similar profile to IgG3, whilst IgG4 was closer to IgG1 [37]. Replacing certain amino acids with alanine in IgG3 produced in Chinese hamster ovary cells leads to an increase in galactose and sialic acid, providing a possibility for specific modulation of immunoglobulin production to optimize treatment [38]. Apart from this, to our knowledge, we found no reports of comparisons of recombinant IgG glycosylation regarding subclass, point mutations, transfection type or cell lines.

We captured IgG1 and IgG3 wild-type and mutant molecules by a novel microtitre plate method and analysed the glycoforms using mass spectrometry analysis of in-well trypsin-liberated glycopeptides. The mutant antibodies were produced using HEK 293E cells, and two different times of harvest were used to study possible variations in glycosylation over time during transient transfection.

**Materials and methods**

**Transient transfection**

Ten different IgG1 and IgG3 variants were tested from two different harvest times. This included 6 IgG1 variants: IgG1 wild type and 5 mutations, IgG1 L234A, IgG1 L235A, IgG1 P329A, IgG1 D270A and IgG1 L234A/L235A. For IgG3, we tested four variants: IgG3 wild type, IgG3 m15 (the hinge region had been shortened from 62 amino acids to 15), IgG3 D270A and IgG3 P329AI (see Table 1). All the mutations were done using PCR, and the DNA fragments were introduced to the vector. The vector was cotransfected into the HEK 293E cell line, and the supernatant collected to carry out the tests as previously described [39]. In this report, the naïve HEK 293E cells used were thawed, transfected and cultivated, and the supernatant was collected at two different times: an early collection (4 days after transfection) and a later collection (10 days after the transfection).

In addition, a further two mutants from HEK 293E cells were tested, but these had been harvested only once on a previous occasion before they were frozen. These were IgG1 H435A and IgG3 N297A (see Table 1).

**Permanent transfection**

A further 6 variants of IgG1 and IgG3s were analysed (see Table 1). These cells were transfected earlier, the supernatant collected and the cells and supernatant have been kept

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**Table 1** Eighteen IgG1 and IgG3 antibodies were analysed. The first 10 are transiently transfected IgG1 and IgG3 antibodies produced in HEK 293E cells harvested at two time points, one early (4 days) and one late (10 days). The next two antibodies are also produced in transiently transfected HEK 293E cells, but they were harvested only once. The last six antibodies are produced in permanently transfected NS-0 and J558L cells and were harvested only once.

| Antibody | Description of mutation | Cell line |
|----------|-------------------------|-----------|
| IgG1 wt | IgG1 wild type | HEK 293E |
| IgG1 L234A | IgG1 where Leu at position 234 is mutated to Ala | HEK 293E |
| IgG1 L235A | IgG1 where Leu at position 235 is mutated to Ala | HEK 293E |
| IgG1 L234/L235A | IgG1 where Leu at position 234 and 235 is mutated to Ala | HEK 293E |
| IgG1 D270A | IgG1 where Asp at position 270 is mutated to Ala | HEK 293E |
| IgG1 P329A | IgG1 where Pro at position 329 is mutated to Ala | HEK 293E |
| IgG3 wt | IgG3 wild type | HEK 293E |
| IgG3 D270A | IgG3 where Asp at position 270 is mutated to Ala | HEK 293E |
| IgG3 P329A | IgG3 where Pro at position 329 is mutated to Ala | HEK 293E |
| IgG3 m13 | IgG3 where the hinge is shortened to 15 amino acids | HEK 293E |
| IgG1 H435A | IgG1 where His at position 435 is mutated to Ala | HEK 293E |
| IgG3 N297A | IgG3 where Asn at position 297 is mutated to Ala | HEK 293E |
| IgG1 chimeric | IgG1 chimeric | NS-0 |
| IgG1 chimeric | IgG1 chimeric | J558L |
| IgG3 wt | IgG3 wild type | NS-0 |
| IgG3 m15 chimeric | IgG3 where the hinge is shortened to 15 amino acids | NS-0 |
| IgG3 wt α-NIP | IgG3 wild type | J558L |
| IgG3 m15 R435H | IgG3 where the hinge is shortened to 15 amino acids and Arg at position 435 is mutated to His | NS-0 |

Leu, leucine; Ala, alanine; Asp, aspartic acid; Pro, proline; His, histidine; Asn, asparagine; Arg, arginine.
frozen in liquid nitrogen for several years, they were therefore not freshly harvested for this exact experiment.

Antibody preparations

We used either purified antibody preparations isolated as previously described [39] or unpurified cell growth supernatants.

Antibody capture before MS analysis

We used NIP-specific antibodies in this study and could therefore use microtitre plates coated with NIP16BSA to capture the antibodies. Briefly, 100 µl PBS pH 7.3 containing 1 µg/ml NIP16BSA was added to the microtitre wells and left for at least 48 h at 4 °C and then washed 4 times with 300 µl distilled water. 300 µl blocking buffer (1% dried milk powder in PBS/azide) was added to each well and left to incubate for 1 h at 37 °C. The plate was washed 4 times with distilled water, and 100 µl of each antibody preparation was added in each well, in duplicates. The 10 antibody samples from HEK 293E cells were provided as cell supernatant, and 100 µl contained around 1.5 g/ml from the first harvest and between 0.8 and 2.6 g/ml from the second. The other samples were diluted, so 100 µl contained approximately 30 µg antibody. This was incubated for 2 h in 37 °C to allow antigen–antibody binding. The plate was then emptied and washed with 300 µl 1 M NaCl 3 times and twice with 300 µl distilled water. Following the last wash, the plate was knocked vigorously against a bench covered in cell paper to remove any remaining water. The plate was sealed with tape and stored at 4 °C until trypsin digestion was started.

In-well trypsin digestion before MS analysis

One hundred and Twenty microliter trypsin digestion buffer (50 mM ammonium bicarbonate with 15% acetonitrile) was added to each well and incubated for 5 min at 80 °C and sonicated in a water bath for 30 s. 30 µl 50 mM ammonium bicarbonate containing 600 ng of trypsin in 30 [sequencing grade modified trypsin from Promega (V5111) or Roche (11418025001)] was added to each well and sonicated for 30 s in a water bath, followed by overnight incubation at 37 °C. The content of the wells was transferred to Eppendorf tubes [Rainin LiteTouch™ (Metttler-Toledo Logistik GmbH, Uznach, Switzerland) 1.7 ml Microcentrifuge Tube LTT-170_Bi(17011862)] and dried in a Speedvac centrifuge (Maxi dry Iyo F.D.1.0, Heto-Holten, Allerød, Denmark), which took approximately 2 h. 17 µl of 0.1% formic acid was added to each tube, quickly centrifuged, sonicated for 30 s, centrifuged at 16 000 g for 10 min at 4 °C and 15 µl transferred to MS tubes (VWR International microvials PP, 0.3 ml with short thread, Cat. No 548-0440 and VWR International Screw cap PP transparent, 9 mm silic.white/PTFE red, Cat. No. 548-0034), and the tubes were consequently stored at −20 °C until they were analysed.

Mass spectrometry analysis of trypsin-digested IgG samples

The samples were analysed on a LC-ESI-Orbitrap. LC separation was carried out on Agilent 1200 series capillary high-performance liquid chromatography (HPLC).

Six microliter injected into reverse phase (C18) nano online liquid chromatography coupled MS/MS analysis, length 150 mm and width 0.075 mm, GlycproSIL C18-80 Å, Glypromass, Stove, Germany. The peptides were eluted in positive ion mode and CID mode (collision-induced dissociation), where the spectra are isolated and detected in a linear ion trap, providing speed and sensitivity.

Mobile phases were A: water with 0.1% formic acid and B: acetonitrile with 0.1% formic acid. LC separation was carried out with a gradient from 10 to 95%, with a flow rate of 0.2 µl/min.

LC system connected to nanoelectrospray source of ThermoScientific LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) operated by Xcalibur 2.0.

Nanospray ionization was carried out by applying 1.2 kV between 8-µm-diameter emitter (PicoTip Emitter, New Objective, Woburn, MA, USA). The capillary temperature was 200 °C, and capillary voltage was 30 V.

Mass spectrometry data analysis

Mass spectrometric data were analysed using Thermo Scientific Xcalibur software version 2.0. The mass tolerance of the parent ion was set at 5 ppm. The MS peaks from each glycopeptides were extracted as the maximum peak height within the same retention time window based on parent ion mass, and the MS/MS spectra were manually searched using Qual Browser version 2.0.7. The transiently transfected antibodies with two harvests were all done in duplicates, and the permanently transfected and the transiently transfected with one harvest only were analysed once. Standard deviation was calculated as STDav in Microsoft Excel 2010. Before the actual samples were analysed, several test runs were carried out to make sure the results were reproducible from on analysis to the next.

Measurement of effector functions

Antibody-dependent complement-mediated cell lysis (AD-CML) and antibody-dependent cell-mediated cell lysis (ADCC) were measured as previously described [39, 40]. Briefly, Cr51-labelled sheep red blood cells (SRBC) sensitized with rabbit Fab anti-SRBC conjugated with NIP...
were used as target cells both for ADCML and ADCC. The supernatants were harvested with a harvesting device (Skatron, Norway), and the radioactivity is measured using a gamma counter, and the lytic activity is calculated.

The liberation of radioactivity coincided with effector activity. Human serum was used as complement source as previously described, and human peripheral blood lymphocytes were used as effector cells for ADCC.

**Results**

The 18 recombinant IgG molecules studied in this report were all analysed using LC-ESI-Orbitrap, searching for 20 different glycoforms based on the mass of the remaining peptide plus the carbohydrate moiety (see Fig. 1)[24,35]. The glycoforms can be in different charge states generated during the MS analysis, depending on the number of protons they have. These can be charge state 2 \((M + 2H)^{+}\) when two protons are added and charge state 3 \((M + 3H)^{+}\) when three protons are added. Even higher charge states are available, but not detected in the experiments reported here. The peaks were integrated and the values for each glycoform in charge state 2 and 3 were combined (see Table 2), and the values for all glycoforms added together, and from this, the percentage distribution of each glycoform was calculated. A spectrum for IgG1 D270A harvest 1 shows the relative distribution before integration of four of the main glycoforms (see Fig. 2).

Using transient transfection, IgG1 molecules contain strikingly more fucose than IgG3 molecules

A clear difference between IgG1 and IgG3 glycoforms was detected. IgG1 wild type and mutants all show rather similar glycoforms and IgG3 wild type and mutants all showed rather similar glycoforms, but these two profiles were opposite to each other, with IgG3 variants having non-fucosylated glycoforms (see Table 3).

IgG antibodies from transient transfection showed more complex glycoforms from the early harvest compared to the late harvest

Table 2 Twenty IgG1 and IgG3 glycoforms were searched for, and displayed below are the mass and charge state.

| IgG1 | IgG3 |
|------|------|
|      |      |

| Charge state 2 | Charge state 3 | Charge state 2 | Charge state 3 |
|----------------|----------------|----------------|----------------|
| EEQYNSTYR      | EEQFNSTFR      |                |                |
| G0F            | 1317.5270      | 878.6891       | 1301.5320      | 868.0235       |
| G1F            | 1398.5530      | 952.7044       | 1382.5581      | 922.0441       |
| G2F            | 1479.5794      | 986.7220       | 1463.5848      | 976.0587       |
| G1FS           | 1544.1007      | 1029.7362      | 1528.1058      | 1019.0729      |
| G2FS           | 1625.1271      | 1083.7538      | 1609.1322      | 1073.0905      |
| G0FN           | 1419.0663      | 946.3799       | 1403.0714      | 935.7166       |
| G1FN           | 1500.0927      | 1000.3975      | 1484.0978      | 989.7342       |
| G2FN           | 1581.1191      | 1054.4151      | 1565.1242      | 1043.7518      |
| G2FNS          | 1645.6404      | 1097.4293      | 1629.6455      | 1086.7660      |
| G2FSN          | 1726.6668      | 1151.4469      | 1710.6719      | 1140.7836      |
| G0             | 1244.4976      | 830.0008       | 1228.5027      | 819.3375       |
| G1             | 1325.5240      | 884.0184       | 1309.5291      | 873.3551       |
| G2             | 1406.5504      | 938.0360       | 1390.5555      | 927.3277       |
| G1N            | 1427.0637      | 951.7115       | 1411.0888      | 941.0483       |
| G0N            | 1346.0373      | 897.6939       | 1330.0420      | 887.0307       |
| G2N            | 1508.0901      | 1005.7291      | 1492.0952      | 1381.2423      |
| G1S            | 1471.0717      | 981.0502       | 1455.0768      | 1356.5634      |
| G2S            | 1552.0981      | 1055.6078      | 1536.1032      | 1410.3810      |
| G1NS           | 1572.6114      | 1048.7435      | 1556.6165      | 1424.2570      |
| G2NS           | 1653.6378      | 1102.7609      | 1637.6430      | 1478.2740      |

Figure 1 The 20 different glycoforms studied in this report. Black square = peptide, blue square = N-acetylglucosamine, green circle = mannose, red triangle = fucose, yellow circle = galactose and pink diamond = sialic acid.
Table 5 The average percentage distribution for the different glycoforms found for IgG1 and IgG3 produced in transiently transfected HEK 293E cells from two harvests, harvest 1 (early) and harvest 2 (late). The last two IgG antibodies were also produced in HEK 293E cells, but these had only one harvest. Of these, IgG3 N297A had no glycoforms, as would be expected, as the oligosaccharides are situated at asparagine 297. Glycoforms that were not detected in any of the antibodies were not included in the table, and glycoforms detected in some, but not all, were marked nd, not detectable. Glycoforms present in <0.4% were excluded.

| Antibodies from HEK 293E cells – 2 harvests | G0F | G1F | G2F | G1N | G1 | G2 | G0FN | G1FN | G0 | G2FS |
|---------------------------------------------|-----|-----|-----|-----|----|----|------|------|----|-----|
| IgG1 wt harvest 1                           | 52  | 45  | 3   | nd  | nd | nd | nd   | nd   | nd | nd  |
| IgG1 wt harvest 2                           | 87  | 13  | nd  | nd  | nd | nd | nd   | nd   | nd | nd  |
| IgG1 P329A harvest 1                         | 50  | 45  | 5   | nd  | nd | nd | nd   | nd   | nd | nd  |
| IgG1 P329A harvest 2                         | 81  | 17  | 2   | nd  | nd | nd | nd   | <0.4 | <0.4| <0.4|
| IgG1 D270A harvest 1                         | 33  | 46  | 15  | nd  | nd | nd | nd   | 2    | 4   | nd  |
| IgG1 D270A harvest 2                         | 66  | 27  | 2   | 1   | nd | nd | nd   | 4    | nd  | nd  |
| IgG1 L234A harvest 1                         | 49  | 42  | 9   | nd  | nd | nd | nd   | nd   | nd | nd  |
| IgG1 L234A harvest 2                         | 76  | 21  | 2   | nd  | nd | nd | nd   | <0.4 | <0.4| 1   | nd  |
| IgG1 L235A harvest 1                         | 50  | 45  | 5   | nd  | nd | nd | nd   | nd   | nd | nd  |
| IgG1 L235A harvest 2                         | 78  | 20  | 1   | nd  | nd | nd | nd   | <0.4 | <0.4| 1   | nd  |
| IgG1 L234A/L235A harvest 1                   | 42  | 51  | 7   | nd  | nd | nd | nd   | nd   | nd | nd  |
| IgG1 L234A/L235A harvest 2                   | 75  | 22  | 2   | nd  | nd | nd | nd   | <0.4 | <0.4| 1   | nd  |
| IgG3 wt harvest 1                            | nd  | nd  | nd  | nd  | 60 | 40 | nd   | nd   | nd | nd  |
| IgG3 wt harvest 2                            | nd  | nd  | nd  | 1   | 86 | 13 | nd   | nd   | nd | nd  |
| IgG3 P329A harvest 1                         | nd  | nd  | nd  | nd  | 44 | 56 | nd   | nd   | nd | nd  |
| IgG3 P329A harvest 2                         | nd  | nd  | nd  | 1   | 80 | 19 | nd   | nd   | nd | nd  |
| IgG3 D270A harvest 1                         | nd  | <0.4| <0.4| <0.4| 55 | 45 | nd   | nd   | nd | nd  |
| IgG3 D270A harvest 2                         | nd  | nd  | nd  | 2   | 85 | 13 | nd   | nd   | nd | nd  |
| IgG3 m15 harvest 1                           | nd  | nd  | nd  | nd  | 50 | 50 | nd   | nd   | nd | nd  |
| IgG3 m15 harvest 2                           | nd  | nd  | nd  | <0.4| 83 | 17 | nd   | nd   | nd | nd  |
| Antibodies from HEK 293E cells -1 harvest    |     |     |     |     |    |    |      |      |    |     |
| IgG1 H435A HEK 293E                         | 56  | 32  | 9   | nd  | nd | nd | nd   | 2    | nd | 1   |
| IgG3 N297A HEK 293E                         | nd  | nd  | nd  | nd  | nd | nd | nd   | nd   | nd | nd  |

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subcell in the early harvest. A few stood out, as IgG1 D270A displayed more G2F than any other IgG1, whilst the presence of G1 was slightly higher in IgG3 wt and IgG3 D270A compared to the other two IgG3 antibodies from the early harvest (see Table 3).

The same trends were seen for the late harvest; the mutant IgG antibodies showed similar glycosylation patterns to their wild-type IgG subclass, although a decrease in the more complex glycoforms was seen here (see Table 3).

Choice of cell line for permanent transfection influences glycosylation pattern for IgG1, but not for IgG3

A further 6 wild types and mutant IgG1 and IgG3s were analysed, 2 IgG1 and 4 IgG3 (see Table 4). The two IgG1 variants differ in their glycoforms, in that chimeric IgG1 from J558L cells hardly has any of the G0F glycoform but more G2F and the exact opposite are seen for chimeric IgG1 from NS-0. The amount of G0 and the presence of G2 also separate the two (see Table 4).

The four IgG3 antibodies from permanently transfected cell lines show similar glycocalyx profiles, with only two glycoforms being present, G1 and G2 (see Table 4). Three of the antibodies show a very similar percentage distribution of these two glycoforms, whilst the last antibody, IgG3 wild-type z-NIP from J558L, deviates from this.

Transient and permanent transfection affects glycosylation pattern of both IgG1 and IgG3

Chimeric IgG1 from NS-0 portrays a glycosylation profile with a similar trend compared to the other transiently transfected immunoglobulins from harvest 1 (see Tables 3 and 4). Chimeric IgG1 from J558L on the other hand has a completely different profile, with G2F as the main glycoform.

IgG3 wild types and IgG3 m15 from transiently transfected HEK 293E cells had equal amounts of G1 and G2 in the first harvest, but mainly G1 in the late harvest, whilst IgG3 wild-type NS-0 and IgG3 m15 NS-0 have G2 as their main glycoform (see Table 3). Similarities are seen between mutants of different transfection form, such as IgG3 wild-type z-NIP J558L and IgG3 wild type from NS-0 and IgG3 m15 R435H from NS-0 and IgG3 m15 from NS-0 (see Tables 3 and 4). IgG3 glycoforms isolated from NS-0 and J558L are therefore rather similar, but differ from the ones produced by HEK 293E cells.

Some of the transiently transfected IgG mutants from the early harvest displayed higher complement-mediated lysis compared to IgGs from the late harvest

All 10 transiently transfected antibodies from two harvests as well as the 6 permanently transfected antibodies were tested using ADCML, and some were also tested using ADCC.

ADCML analysis for the 10 transiently transfected antibodies showed that IgG1 P329A, IgG3 P329A and IgG1 L234A/L235A had very low or no cytotoxic activity. IgG1 wild type, IgG3 wild type, IgG3 D270A and IgG3 m15 all showed good activity, but with very little variance between the two harvests. IgG1 D270A, IgG1 L234A and IgG1 L235A all showed higher cytotoxic index for the early harvest compared to the late harvest; hence, a more active effector function was seen (see Fig. 3A, B).

When the permanently transfected antibodies were tested for ADCML activity, they all gave a high response that did not seem influenced by the choice of cell line. Both chimeric IgG1 z-NIP and IgG3 wild type produced in NS-0 cells showed similar results to their J558L-produced counterparts. The two IgG3 m15 variants from NS-0 cells: IgG3 m15 and IgG3 m15 R435H also gave similar results (see Fig. 3C). IgG1 D270A, IgG1 L234A and IgG1 L235A were also tested for ADCC activity and did not show any significant difference between early and late harvest (data not shown).

IgG1 H435A and IgG3 N297A from transiently transfected HEK 293E cells – one harvest only

Two more mutants were transiently transfected from HEK 293E, but these had only been harvested once and then frozen, IgG3 N297A and IgG1 H435A. IgG3 N297A did not contain any glycoforms, as would be expected as 297 is the position where the sugar moiety is attached.

IgG1 H435A portrays a quite similar pattern to chimeric IgG1 from NS-0 with regard to the major glycoforms (see Table 4).

Discussion

It has lately been accepted that the structure of carbohydrates attached to asparagine 297 in the Fc-portion of the IgG molecule has a profound influence on effector functions.
and thus immune protective capacity. The study of the variation of the glycosylation pattern of recombinant IgG antibodies is therefore important for a more comprehensive understanding of the relationship between structure and function as DNA-based construction of antibodies is becoming the fastest growing therapeutic class in the pharmaceutical industry. The aim of our study was to reveal possible variation in glycosylation related to differences in subclasses, point mutations, hinge truncation, host cell line, permanent versus transient transfection and the time of harvest for transient transfection. We chose to study IgG1 and IgG3 as they are generally the most immune protective human subclasses. The recombinant antibodies used in this study were chimeric, mouse V-region and human C-region constructed molecules containing a full mouse \( \lambda \)-1 light chain. Although not formally documented in the literature, it is highly unlikely that neither the V-region nor the light chain will influence glycosylation structure at position N297 in the IgG Fc-region. Our choice of recombinant antibodies should therefore reflect glycosylation of fully human IgG under the same conditions.

Several striking observations came out of the present study. First of all, there was a clear difference in the glycosylation pattern of recombinant human IgG1 and IgG3, revealed using three different cell lines as host for the production and using permanent and transient transfection of IgG coding DNA vectors. The most striking difference was a predominant presence of fucosylated glycoforms in IgG1, whilst IgG3 was mainly non-fucosylated (see Tables 3 and 4). Observations of human serum IgG did not portray the same trend, as IgG3 did not show this lack of fucose [24]. Similar glycosylation structure analysis of the same IgG1 and IgG3 NIP antibodies produced from J558L cells has been performed in some old studies, but with conflicting results compared to ours. In that analysis, the glycosylation pattern did not show great differences between IgG1 and IgG3, apart from a few glycoforms [37]. They did not detect this heavily fucosylation of IgG1 and non-fucosylation of IgG3. The reason for this is not obvious, but might be due to a different analytical procedure used then and our state-of-the-art mass spectrometry equipment, giving rise to more accurate and sensitive analysis.

The differences in glycosylation pattern of IgG1 and IgG3 we observed could possibly be due to a very large hinge region of IgG3 (62 amino acids) compared to IgG1 with a hinge of 15 amino acids [41, 42]. This was, however, apparently not the case as the hinge truncated mutant IgG3 m15 containing only one of the hinge exons coding for 15 amino acids exhibits essentially the same glycosylation pattern as wild-type IgG3 (see Table 3) [43]. Furthermore, the IgG3 m15 mutant has an enhanced ADCML activity compared to IgG3 wt [43]. A possible explanation could be an altered glycosylation profile of IgG3 m15. We addressed that question in this report and have clearly shown no major difference in glycosylation structure, which can explain the extraordinary high ADCML activity of IgG3 m15.

Secondly, the time of harvest seemed to impact glycosylation. The antibodies produced in transiently transfected cell lines that were harvested 4 days after transfection showed more complex glycoforms compared to the antibodies harvested 10 days after transfection. Similar observations have been seen previously, as Chinese hamster

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Figure 3 ADCML results for transiently and permanently transfected antibodies. (A) Transiently transfected IgG1 L234A and IgG1 L235A from both early and late harvest where the early harvest is represented by a continuous line and the late harvest by a dotted line. (B) Transiently transfected IgG1 wild type and IgG1 D270A from two harvests, where the early harvest is represented by a continuous line and the late harvest by a dotted line. (C) Permanently transfected chimeric IgG1 ?-NIP from NS-O (continuous line) and J558L (dotted line) and IgG3 wild-type ?-NIP from NS-O cells (continuous line) and J558L cells (dotted line).
ovary (GS-CHO) cells producing chimeric IgG4 monoclonal antibody cB72.3 showed increasingly shorter chained and simpler glycoforms with increasing growth period [44]. A possible explanation is that the different enzymes involved in the glycosylation are being put under more strenuous pressure and not functioning as well as in the beginning of the production, leading to uncompleted sugar composition [44]. When we tested the antibodies for ADCML activity, we observed a general trend of higher activity in the early harvest compared to the late harvest (see Fig. 3A, B). Thus, the more short-chained carbohydrates observed in the late harvest seem to depress ADCML activity, whilst this was not the case for ADCC, which was unaltered between the two harvests. It therefore seems that more complex and longer chained carbohydrates are required for effective C1q binding or C1 activation. ADCC, on the other hand, seems more independent of this carbohydrate structure variation.

Thirdly, it was clear that choice of permanent cell line affected the glycosylation, as NS-0 and J558L caused different glycosylation profiles for IgG1, whilst no major differences were seen for IgG3 (see Table 4). Monoclonal chimeric mouse/human IgG produced in J558L cells caused human heavy chain glycosylation to show typical mouse antibody glycosylation pattern; hence, a species-specific cell line will in itself affect the glycosylation [45]. Differences in both O- and N-glycosylation in 12 proteins produced by transient transfection in CHO and HEK cells have previously been observed, but immunoglobulins were not included. A major difference between the proteins from these two cell lines was that the majority of proteins expressed in CHO cells had more sialic acid–rich glycosylation compared to their HEK counterparts. The differences were found to be caused by the choice of cell line and not the culture conditions as they varied only slightly between the two cell lines [46]. Sialic acid–rich proteins tend to have an extended half-life as non-sialylated proteins are likely to be eliminated quicker as they bind to a liver receptor [47]. In pilot studies, HEK cells are often used, and when larger quantities are required, a change to CHO cells often occurs, but this transfer may not be as seamless as shown in the study. Whether the glycosylation is determined exclusively by cell type or whether post-translational changes are equally important is not clear [46]. As the list of these 12 proteins did not include immunoglobulins [46], the results cannot be directly related to our findings, but we observe with interest the reduced sialic acid content in the HEK-produced proteins. As the transiently transfected cell lines produced simpler glycoforms with time, possibly because of overutilization of the enzymes required to make the glycoforms, this could indicate that the same occur for IgG1 in NS-0 cells, but not J558L cells, as that cell line produced more complex glycoforms. A similar observation concerning NS-0 cells was seen with the humanized IgG antibody Campath-1H when expressed in three different cell lines as the glycosylation pattern from NS-0 showed less complex and less overall glycosylation compared to CHO and Y0 myeloma cells [48].

Biological activity in the form of complement activation has previously been studied by us using the same wild-type and mutant molecules as in this report. The main findings in the previous report were that IgG3 was more resilient to mutations near the C1q binding site than IgG1, as point mutations in IgG3 did not affect ADCML, whilst for IgG1, particularly the mutant D270A was affected [39]. The difference in ADCML between the mutants could possibly be due to glycosylation differences as the mutations introduced are close to the glycosylation site. Here, we show no striking differences in glycoforms when comparing the wild type and mutants, and thus, the differences in ADCML activity seem only to be due to differences in protein structure.

IgG3 has been shown to possess a stronger ADCC activity compared to IgG1 [49]. As ADCC is mainly activated through FcγRIIIα and removal of fucose caused a 50-fold increase in receptor binding, the dominance of non-fucosylated IgG3 we detected in the present report is consistent with IgG3s stronger ADCC activity [26, 49].

In conclusion, we used a new isolation technique with a high resolution, sensitive LC-ESI-Orbitrap method for glycosylation profile analysis of human IgG and revealed a striking difference between IgG1 and IgG3. IgG1 was mainly fucosylated, whilst IgG3 was mainly non-fucosylated, probably explaining the stronger ADCC activity of IgG3 compared to IgG1. Furthermore, this difference in fucosylation between IgG1 and IgG3 was apparently not due to the large hinge region of IgG3 and mutations near the C1q binding site did not significantly influence glycosylation. When employing transiently transfected cell lines to produce IgG, the glycosylation structure was more complex earlier in production compared to later.

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