Covalently-linked glycans on proteins have many functional roles, some of which are still not completely understood. Antibodies have a very specific glycan modification in the Fc region that is required for mediating immune effector functions. These Fc glycans are typically highly heterogeneous in structure, and this heterogeneity is influenced by many factors, such as type of cellular host and rate of Ab secretion. Glycan heterogeneity can affect the Fc-dependent activities of antibodies. It has been shown recently that increased Fc sialylation can result in decreased binding to immobilized antigens and some Fcγ receptors, as well as decreased antibody-dependent cell-mediated cytotoxicity (ADCC) activity. In contrast, increased Fc sialylation enhances the anti-inflammatory activity of antibodies. To produce antibodies with increased effector functions, we developed host cell lines that would limit the degree of sialylation of recombinantly-expressed antibodies. Towards this end, the catalytic domain of the *Arthrobacter ureafaciens* sialidase (sialidase A) was engineered for secreted expression in mammalian cell lines. Expression of this sialidase A gene in mammalian cells resulted in secreted expression of soluble enzyme that was capable of removing sialic acid from antibodies secreted into the medium. Purified antibodies secreted from these cells were found to possess very low levels of sialylation compared with the same antibodies purified from unmodified host cells. The low sialylated antibodies exhibited similar binding affinity to soluble antigens, improved ADCC activity, and they possessed pharmacokinetic properties comparable to their more sialylated counterparts. Further, it was observed that the amount of sialidase A expressed was sufficient to thoroughly remove sialic acid from Abs made in high-producing cell lines. Thus, engineering host cells to express sialidase A enzyme can be used to produce recombinant antibodies with very low levels of sialylation.

**Introduction**

Antibodies (Abs) are complex glycoproteins that play pivotal roles in the immune system. They possess the ability to bind target antigens in a highly specific manner and kill target cells that express antigen by way of either Ab-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). The latter two activities have long been known to be Fc-dependent, whereas antigen binding is thought to be Fc-independent.

The carbohydrate modification of IgG Abs occurs at a single asparagine residue in the CH2 domain of the Fc. The glycans consist mainly of complex biantennary structures and display a high degree of heterogeneity due to the absence or presence of different terminal sugars (Fig. 1). The absence of glycans in the Fc region results in a loss in ADCC and CDC activities. The Fc glycan heterogeneity can also have a significant impact on ADCC and CDC activity. For example, the absence of core fucose on the glycan moiety can greatly increase ADCC activity. In addition, terminal sialylation of Fc glycans has been shown to affect the Ab effector functions as well as immobilized antigen binding. On the other hand, an increase in terminal sialylation has been shown to increase the anti-inflammatory activity of antibodies.

A number of mammalian host cells are currently used to express recombinant antibodies (rAbs). Each of these host cell lines can differ in the extent and type of post-translational modifications. Two commonly used host cell lines for rAb expression are Chinese hamster ovary cells (CHO) and mouse myeloma cells, e.g., Sp2/O, P3x63Ag8.653 (Ag653), NS0. rAbs produced using CHO cells are greater than 95% fucosylated and do not contain bisecting GlcNAc residues, whereas rAbs produced using mouse myeloma cells are usually 70–90% fucosylated with ~10% of glycans containing bisecting GlcNAc residues. Also, CHO cells express rAbs with low levels of sialylation, whereas mouse myeloma cells express rAbs with up to 50% sialylation. These differences can have significant effects on Ab functions.

Most therapeutic rAbs that are currently marketed or in clinical trials are based on human IgG1k framework, and many of them may require Ab effector functions to be effective therapeutics. Therefore, it is desirable to develop cell lines that produce IgGs with optimized glycosylation in the Fc. Various methods have
control-transfected supernatant, but not in Ab incubated with the p3629-transfected supernatant. This strongly suggested that the sialidase enzyme was expressed in the p3629-transfected cells, secreted and actively removed sialic acid from the Ab sample (Fig. 4).

The goal of the study was to generate host cell lines that secrete sialidase A into the culture medium so that the enzyme can remove terminal sialic acid residues from Fc glycans of secreted antibodies co-expressed in the same cells. A CHO K1 host was transfected with the sialidase expression plasmid, p3629, and selected for stable integration of the transgene. The selected cell pools were assayed for sialidase activity in cell culture supernatant. In addition, expression of the transgene over time was monitored and compared to control-transfected cells. Sialidase activity was detected in cell culture supernatant 2–3 weeks after selection (Fig. 5). This expression was maintained for up to 8 weeks, with no decrease in enzymatic activity observed as the cells were passaged during that time. This indicated that the sialidase expression plasmid and sialidase expression were stable over time. Similar results were obtained with NS0 cells (data not shown).

To test the ability of these cells to express rAbs that are devoid of sialic acid residues in their Fc glycans, a recombinant anti-tumor necrosis factor Ab was transiently expressed in CHO cells expressing sialidase A and control CHO cells not expressing sialidase A. The recombinantly-expressed Ab was affinity-purified from conditioned cell supernatant from both hosts using a Protein A column. Analysis of the resulting purified Ab preparations from each host by MALDI-TOF-MS did not reveal any measurable differences between the Ab preparations, which suggested that the IgGs were intact (Fig. 6). In contrast, analysis of N-glycans by NP-HPLC showed that the antibodies purified from the sialidase-expressing host were devoid of sialic acid residues (Fig. 7). Clearly, the absence of sialylated glycans reduced overall glycan heterogeneity.

To confirm that this engineered expression host cell line did not affect the binding characteristics of the secreted Ab, binding studies with a surrogate antigen (S-antigen) were performed. Binding curves of 125I-labeled anti-idiotype Fab (used as S-antigen) to the three antibodies (Control, LoSA and -SA control Ab; the control Ab was treated with sialidase A to generate the -SA Ab sample) were similar, demonstrating that reduced sialic acid did not affect variable region binding to S-antigen (Fig. 8).

The amounts of Ab coated on the plate were equivalent as tested by an anti-human IgG ELISA (inset, Fig. 8). Furthermore, to confirm that antibodies with LoSA content retained similar in vivo characteristics as the untreated control Ab, a pharmacokinetic (PK) study was performed to compare circulating half-lives of three versions of the same Ab in BALB/c mice (Fig. 9). PK profiles of the three Abs (Control, LoSA and -SA Ab) were expressed

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**Results**

Expression vector p3629 was constructed for the secreted expression of the *Arthrobacter ureafaciens* sialidase A catalytic domain (Fig. 2) in mammalian cells. Since terminal sialylation also impacts Ab effector functions, we pursued a strategy to produce Abs with reduced sialylation (LoSA). We prepared CHO and NS0 cell lines that secrete both sialidase A and a rAb into the culture medium. Based on the absence of sialylated antibodies, sialidase A-coexpression appeared to efficiently remove terminal sialic acid residues from Fc glycans of rAbs.

![Figure 1](image-url)  
*Figure 1. Structure of major sialylated and asialylated glycans present in the Fc region of IgGs produced in CHO cells. NANA, N-acetylneuraminic acid; Gal, galactose; GlcNAc, N-acetylglucosamine; Man, mannose; Fuc, fucose.*

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been adopted to optimize cell lines to produce low fucosylated and/or bisecting GlcNAc containing Abs. Since terminal sialylation also impacts Ab effector functions, we pursued a strategy to produce Abs with reduced sialylation (LoSA). We prepared CHO and NS0 cell lines that secrete both sialidase A and a rAb into the cell culture medium. Based on the absence of sialylated antibodies, sialidase A-coexpression appeared to efficiently remove terminal sialic acid residues from Fc glycans of rAbs. After expression, the purified rAbs were tested for binding characteristics and in vivo pharmacokinetics.
control Abs produced in mock-transfected Ab cell lines. Cellular toxicity results (Fig. 10) showed that the two Abs expressed in sialidase A-transfected cells had greater than 10-fold improved potency compared to the control mock-transfected Abs. These results indicate that expression of antibodies in the engineered hosts only altered the sialic acid content of the protein, and the host cells can be used to produce recombinant Abs with reduced sialic acid content to improve effector functions.

To demonstrate that the LoSA Ab have improved effector functions, ADCC assays were performed with two different Abs (Ab1 and Ab2) produced in cell lines transfected with sialidase A and compared their ADCC activity with the corresponding.

**Figure 2.** Nucleic acid and deduced amino acid sequence of the *Arthrobacter ureafaciens* catalytic domain. Restriction enzyme sites used for cloning into the parent vector, p2815, are indicated.

**Figure 3.** Schematic representation of expression plasmid p3629 used to express the catalytic domain of the *Arthrobacter ureafaciens* sialidase. (A) Restriction enzyme sites used for cloning into the parent vector, p2815, along with the hGH (human growth hormone) signal sequence used to facilitate secretion, are indicated.
modification is especially important for Ab therapeutics, where it has been shown that N-linked glycosylation in the Fc domain of antibodies has a profound effect on Ab effector function.\textsuperscript{1,2} Various culture parameters such as nutrient levels, pH, temperature and oxygen and ammonia content have been shown to alter glycosylation patterns on rAbs, with levels of sialylation and galactosylation often being affected.\textsuperscript{2,3,13,16-18} Microheterogeneity has also been observed in Fc glycans of antibodies even when expressed under similar culture conditions.\textsuperscript{3}

The types of glycosylation that occur in mammalian cells might have an effect on product activity. Human antibodies contain glycan structures that vary by gender and change with age and disease status.\textsuperscript{19} The glycan structures commonly seen in human antibodies are depicted in Figure 1. The sialylated glycans found in human IgGs contain $\alpha_2,6$-linked sialic acid residues,\textsuperscript{4,7,8} but CHO cells do not often express antibodies with terminal $\alpha_2,6$-sialylation because they lack the $\alpha_2,6$-sialyltransferase enzyme ($\alpha_2,6$-ST).\textsuperscript{17} CHO cells, however, have been shown to express antibodies with terminal sialic acid with an $\alpha_2,3$-linkage.\textsuperscript{17}

The role of sialic acid on Ab function has been recently challenged.\textsuperscript{6-8} Terminal sialic acid was thought to mask glycoproteins by capping galactose residues and prevent recognition by galactose-specific lectins such as asialoglycoprotein receptor on hepatocytes.\textsuperscript{20} This process was shown to increase serum half-life of such sialylated serum glycoproteins.\textsuperscript{20} Hence, various attempts to increase the amount of sialic acid on glycoproteins have been made, including overexpressing sialyltransferases, process optimization to manipulate culture conditions and host cell engineering with RNA interference.\textsuperscript{10} Recently, sialylated Fc glycans of antibodies have been shown to negatively influence proteolytic resistance and Fc specific effector functions.\textsuperscript{2} Antibodies with terminal sialic acid can have reduced ADCC as a result of either reduced FcγRIIIa binding on natural killer cells or lower affinity binding to cell-surface antigen.\textsuperscript{6} In addition, sialylated antibodies are more susceptible to protease digestion, which might influence ADCC.\textsuperscript{12}

Many of the currently marketed Abs are produced using CHO cells.\textsuperscript{2,3} Although CHO cell-derived Abs are poorly sialylated, they contain $\alpha_2,3$-linked sialic acid residues instead of $\alpha_2,6$-linked sialic acid residues present in human IgGs. Hence, it may be advisable to express sialidase A in CHO cells to remove $\alpha_2,3$-linked sialic acid residues. However, manufacturing processes have been manipulated to improve the production yield of Abs produced in CHO cells from 0.2 to over 1.0 g/liter. To examine whether catalytic amounts of sialidase A enzyme were enough to remove sialic acid residues from Abs at high production levels, cell culture fluids from CHO cell lines expressing active sialidase A were spiked with an anti-IL-12 Ab (containing ~40% sialylation) at various concentrations ranging from 0.1 to 10.0 mg/mL and incubated at 37°C for 24 h. Analysis of N-glycans by NP-HPLC revealed that the enzyme was able to remove sialic acid residues at all concentrations (data not shown) suggesting that the catalytic amounts of sialidase A is enough to produce LoSA Abs even at high production levels.

**Discussion**

Mammalian cells such as CHO and NS0 cells have become the host cells of choice for production of rAbs as human therapeutics. As a result, many technological and process advancements have been made to optimize the expression levels in these hosts to maximize yield.\textsuperscript{16,17} Many of these advancements have been shown to have effects on product homogeneity, especially with regard to post-translational modifications.\textsuperscript{15,16} This type of data demonstrate that Abs produced in sialidase A-expressing cell lines can have improved ADCC activity.

Figure 4. NP-HPLC profile of PNGase F released glycans from a recombinant antibody. Purified anti-IL-12 Ab samples were incubated with cell culture medium from cells transiently transfected with: (A) control vector (mock transfected medium) or (B) expression plasmid p3629 (sialidase A-transfected medium). The Ab samples were then re-purified and the glycans were characterized as described in Materials and Methods. Structure of G0, G1, G2, G2S1 and G2S2 glycans are shown in Figure 1.
Sialidases have been described from viruses, protozoa, bacteria, fungi, mycoplasma, birds and higher animals. They have many diverse roles, including viral replication and release, bacterial infection, molecular transport, proliferation and differentiation. The two types of sialidases are endo-α-sialidases that cleave internal sialic acid linkages and exo-α-sialidases that cleave terminal sialic acid linkages. The bacterium *Arthrobacter ureafaciens* is a non-pathogenic organism that expresses a sialidase that is suspected to have a nutritional function. The enzyme is frequently used in various biochemical applications due to its broad substrate specificity, cleaving α2,3-, α2,6- and α2,8-bonds of terminal sialic acids in glycoproteins and glycolipids. This enzyme was recently cloned and a catalytically active soluble fragment was identified.

Using the soluble enzyme fragment, we engineered host cell lines that overexpress soluble sialidase A from *Arthrobacter ureafaciens*, capable of cleaving terminal sialic acid residues in the medium. This enzyme was engineered for secreted expression in mammalian cells and is present and active in culture supernatant from either HEK293 cells or CHO cells. We demonstrated that lower sialic acid-containing Abs potentially can have improved ADCC activity compared to their corresponding untreated Abs. In the example described here, we showed that Abs from sialidase-expressing cell lines had similar soluble antigen binding, as well as similar pharmacokinetic properties compared to the untreated Ab produced in normal host cell lines that contain higher level of terminal sialylation. These host cell lines can be used to express rAbs that are virtually devoid of sialic acid residues, thus limiting any negative impact of sialylated glycan on Ab effector functions, as well as increase product homogeneity during the large-scale production of Ab therapeutics.

**Materials and Methods**

**Sialidase A expression plasmid assembly.** The nucleic acid sequence encoding the catalytic domain of the *Arthrobacter ureafaciens* sialidase, residues 40 to 535, was synthesized by Blue Heron Biotechnology, Inc. (Bothell, WA, USA), based on GenBank accession number AY934539 (Fig. 2). The synthesized gene was cloned in plasmid p2815 using unique BamHI and Not I restriction sites. This plasmid possesses a CMV promoter, coding sequence for human growth hormone signal sequence for secretion and a neomycin resistance gene for stable selection. The completed expression plasmid, p3629, was analyzed by restriction enzyme digestion and sequencing (Fig. 2).

**Transient transfection.** HEK293 cells were transfected with 15 μg of purified plasmid p3629 or control plasmid (same vector without sialidase coding sequence) using Lipofectamine 2000. Plasmid DNA and 90 μL of Lipofectamine 2000 was diluted in OptiMem, combined, and then incubated for 20 min at room temperature. The transfection cocktail was then added to 70% confluent HEK293 cells in growth medium overnight. After 24 h, growth medium was replaced with 293SFM medium and cells incubated for 5 days for medium harvest and analysis. Similarly, CHO cells were also transfected with antibody-expressing plasmid and or sialidase A-expressing plasmid for transient expression. All transfection reagents, cell culture materials and medium were obtained from Invitrogen, Inc., (Carlsbad, CA, USA).

**Stable transfection.** CHOK1 cells were transfected with 15 μg of p3629 using FUGENE. Transfected cells were maintained in growth medium with 700 μg/mL Geneticin antibiotic to select for stable transfectants. Antibiotic resistant clones were expanded and tested for sialidase activity. Source Abs, Ab1 and Ab2, are recombinant human monoclonal IgG1k that were produced either in stable CHOK1 or mouse myeloma cells with or without p3629.

**Sialidase A activity assay.** Sialidase A activity was detected using 2’-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid as substrate as previously described. Briefly, a fluorometric assay on cell supernatant from viable cell cultures was performed by mixing cell supernatant with 200 μL of 150 μM 2’-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid in 100 mM citrate-phosphate buffer. Sialidase activity was measured by determining the released substrate activity using the fluorometric substrate and conditions detailed in the text. Top part represents activity for cells three weeks post selection, while bottom part represents activity from cells 8 weeks post selection.

**Figure 5.** Sialidase activity of C1836A CHO cells after stable transfection of p3629 encoding the *Arthrobacter ureafaciens* sialidase A gene. Sialidase activity was determined using the fluorometric substrate and conditions detailed in the text. Top part represents activity from cells three weeks post selection, while bottom part represents activity from cells 8 weeks post selection.
buffer, pH 6.5 at 37°C; followed by the addition of 2 mL of 0.5 M Na₂CO₃ to stop the reaction. Excitation at 366 nm and emission at 446 nm were measured using a spectrofluorometer. Fluorometric units were normalized against viable cell counts. Alternatively, sialidase activity in culture medium was determined by overnight incubation of sialylated infliximab (Centocor Ortho Biotech, Inc.) with medium from transfected cells and testing for sialic acid by NP-HPLC as described below.

**Sialic acid determination.** Sialidase A activity in the harvested cell culture medium was determined by measuring sialic acid content of purified antibodies before and after spiking the Ab samples to the medium. Briefly, purified Ab samples (~1.0 mg) were spiked to the cell culture medium from either sialidase A expressing cells or from control cells and incubated at 37°C. After 24 h, the Ab samples were purified on a Protein A column. The N-linked oligosaccharides were released by treating Ab samples (0.05–0.5 mg in 0.1 mL) with PNGase F in 20 mM Tris-HCl buffer, pH 7.0 at 37°C for 4–6 h. An aliquot of this solution (~0.01 mL) was passed through a column containing cation exchange resin and analyzed by MALDI-TOF-MS as described previously. The remaining portion of the sample was subjected...
Analysis of oligosaccharides using MALDI-TOF-MS. IgG samples (~50 μg) were digested with PNGase F in 10 mM Tris-HCl buffer (pH 7.0) for 4 hr at 37°C. The digestion was stopped by acidifying the reaction mixture with 50% acetic acid (~5 μL) and then passed through a cation-exchange resin column as described previously.12 These samples containing a mixture of acidic and neutral oligosaccharides were analyzed by MALDI-TOF-MS in the positive and negative ion modes, as described elsewhere.15 The MALDI-TOF-MS was carried out using a Voyager DE instrument from Applied BioSystems (Foster City, CA, USA).

Analysis of intact IgG samples. IgG samples were buffer exchanged into 10 mM Tris-HCl buffer, pH 7.0 and adjusted to reductive amination with anthranilic acid and subsequent analysis by NP-HPLC as described by Anumula.14

Antibody expression and purification. 100% confluent cells in a 10-tier CellSTACK™ were split into a maximum of five new 10-tier CellSTACK™ units, each with 1 L of transfection medium and 350–450 x 10⁶ cells. Cells were incubated at 37°C overnight. The next day, 40 mL of Optimem (stored at room temperature) was aliquoted into two conical tubes. Approximately 1.8 mL Lipofectamine 2000 was added to one tube and 300 μg of Ab expression plasmids (150 μg per chain) was added to the other tube. The two conicals were incubated at room temperature for 5 min, then mixed together and allowed to sit for 20 min at room temperature. Optimem transfection mixture was added to the CellSTACK™, which was then placed back into the incubator. On day three, the medium was removed and replaced with production medium containing 293SFM II, 4 mM L-glutamate and 5 mM sodium butyrate. On day seven, the conditioned medium was clarified by centrifugation and filtered through a 0.8 μm filter unit. Recombinant-expressed antibodies in conditioned medium were purified using standard affinity chromatography methods using conjugated Protein A resin. Purified antibodies were analyzed by SDS-PAGE and SEC-HPLC for purity and integrity.

In vitro desialylation of antibodies. Ab samples (0.1 mg/mL to 10.0 mg/mL) were incubated with 5 μL of sialidase A (SelectinBio, Pleasant Hill, CA, USA) either in 50 mM Tris-HCl buffer or in harvested cell culture fluid from p3629 cells. After incubation at 37°C for 24–72 h, Ab samples were purified on a Protein A column and the purified samples were subjected to glycan analysis by MALDI-TOF-MS and/or NP-HPLC.
carried out as described previously. Briefly, a solution of 4% gosaccharides with anthranilic acid (2-aminobenzoic acid) was
PNGase F at 37°C for 4–8 h. Derivatization of the released oli-
saccharides with anthranilic acid (AA) reagent solution in 1.6 mL polypropylene
tester tubes (Sigma) was mixed with 2 µL of the
substrate (Sigma) and the reactions stopped by addition of
0.5 M HCl. The resulting OD490 values were determined
by ELISA assays using human IgG-specific
binding reagents. Briefly, 96-well plates were coated with
culture supernatant from sialidase A-transfected
cell lines and their corresponding mock-transfected control Abs were incubated with PBMC
(effector) and target cells at an effector: target ratio of 50:1 for 2 h, 37°C. Cellular
toxicity expressed as % specific lysis are shown with Ab concentrations ranging
from 0.2–10 µg/mL. (A) Ab1 comparisons. Samples were tested in triplicates
(error bars represent SD). (B) Ab2 comparisons. Samples were tested in
duplicates (error bars represent ranges).

Figure 10. ADCC potency of two Abs derived from sialidase A-transfected
cell lines. Ab1 or Ab2 produced in sialidase-transfected cell lines and their
corresponding mock-transfected control Abs were incubated with PBMC
(effector) and target cells at an effector: target ratio of 50:1 for 2 h, 37°C. Cellular
toxicity expressed as % specific lysis with Ab concentrations ranging
from 0.2–10 µg/mL. (A) Ab1 comparisons. Samples were tested in triplicates
(error bars represent SD). (B) Ab2 comparisons. Samples were tested in
duplicates (error bars represent ranges).

concentration to ~1 mg/mL buffer. About 2 µL of IgG solution
was mixed with 2 µL of matrix solution (the matrix solution was
prepared by dissolving 10 mg sinnapinic acid in 1.0 mL of 50% ace-
tonitrile in water containing 0.1% trifluoroacetic acid) and 2 µL of this solution was loaded onto the target and allowed to air dry.
MALDI-TOF-MS was acquired using a Voyager DE instrument
from Applied BioSystems (Foster City, CA, USA).

HPLC analysis of oligosaccharides. IgG samples (~50 µg)
in 10 mM Tris-HCl buffer (~50 µL) pH 7.0 were digested with
PNGase F at 37°C for 4–8 h. Derivatization of the released oli-
saccharides with anthranilic acid (2-aminobenzoic acid) was
carried out as described previously. Briefly, a solution of 4% sodium acetate 3H2O (w/v) and 2% boric acid (w/v) in methanol
was prepared first. The derivatization reagent was prepared fresh
dissolving -30 mg of anthranilic acid (Aldrich) and -20 mg of sodium cyanoborohydride (Aldrich) in 1.0 mL of methanol-
sodium acetate-borate solution. IgG derived oligosaccharides
(~3 nmol in 20–50 µL of water) were mixed with 0.1 mL of the
anthranilic acid (AA) reagent solution in 1.6 mL polypropylene
cap \(n\) cap freeze vials with “O” rings (Sigma, St. Louis, MO, USA)
and capped tightly. The vials were heated at 80°C in an oven or
heating block (Reacti-Therm, Pierce) for 1–2 h. After cooling the
vials to room temperature, the samples were diluted with water to bring the volume to ~0.5 mL. Derivatized oli-
saccharides were purified by using NAP-5 columns as
described previously.

Purified AA labeled oligosaccharides were separated on an amine (NH4) bonded polymeric column (Polymer-NH4, 5 µm, 0.46 x 25 cm, Astec,
Whippany, NJ, USA). Solvent system A consisted of 2% acetic acid and 1% tetrahydrofuran (inhibited) in aceto-
nitrile and solvent system B consisted of 5% acetic acid, 3% triethylamine and 1% tetrahydrofuran (inhibited)
in water. The following gradient for mapping was used for optimum resolution of the oligosaccharides present in
IgG samples: 30% B isocratic for 2 min followed by a linear increase to 95% B over 80 min and was held at 95% B
for additional 15 min. The column was equilibrated with initial conditions for 15 min prior to the next injection.
HPLC systems consisted of Agilent 1100 system. The anthranilic acid derivatives were detected using 230 nm
excitation and 425 nm emission wavelengths.

Determination of serum levels of human IgG. Concentrations of human IgG in mouse serum were determined by ELISA assays using human IgG-specific
binding reagents. Briefly, 96-well plates were coated with
culture supernatant from sialidase A-transfected
cell lines and their corresponding mock-transfected control Abs were incubated with PBMC
(effector) and target cells at an effector: target ratio of 50:1 for 2 h, 37°C. Cellular
toxicity expressed as % specific lysis are shown with Ab concentrations ranging
from 0.2–10 µg/mL. (A) Ab1 comparisons. Samples were tested in triplicates
(error bars represent SD). (B) Ab2 comparisons. Samples were tested in
duplicates (error bars represent ranges).
determined using the linear region of an IgG1 standard curve. Secondly, $^{125}$I-labeled anti-Id Fab at varying concentrations in 1% BSA-PBS was added to test wells coated with Ab and the plate incubated for 2 hr. After washing the plate, individual wells were removed and counted in the gamma counter (Wallac Wizard 1470-010, Perkin Elmer Life Science Inc., Boston, MA, USA). Non-specific binding was determined using a 100-fold excess of unlabeled S-antigen and that value subtracted to establish specific counts bound.

**Antibody-dependent cell-mediated cytotoxicity (ADCC) assay.** Target cells for assays involving Ab1 was a human melanoma cell line obtained from ATCC. These cells were cultured in DMEM medium containing 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate and 0.1 mM non-essential amino acids. Target cells for assays involving Ab2 were prepared at Centocor by transfecting Sp2/O mouse myeloma cells with cell surface, transmembrane-anchored form of human antigen Ag2. Ag2-expressing cells were cultured in Iscove's Modified Dulbecco's medium containing 5% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids.

ADCC assays were performed using a DELFIA EuTDA-based cytotoxicity assay kit (Perkin-Elmer, Boston, MA) according to the manufacturer's instructions. Target cells were loaded with BATDA (bis-(acetoxymethyl)-2,2':6',2"-terpyridine-6,6" -dicarboxylate) for 30 min at 37°C, washed with PBS and resuspended in the appropriate culture medium at 2 x 10^5 cells/mL. Peripheral blood mononuclear cells (PBMC) were isolated from blood of healthy donors by density gradient centrifugation on Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden), washed twice with PBS and resuspended at 1 x 10^5 cells/mL in the same culture medium used for the target cells.

Ab dilutions in 100 μL culture medium were added in a round-bottom 96-well plate with 50 μL PBMC and 50 μL target cells at a ratio of 50:1. The plate was centrifuged for 5 min at 500x g and then incubated for 2 hr at 37°C. DELFIA europium solution was added to cells and then fluorescence was measured on an EnVision instrument (Perkin-Elmer). The percentage of specific cytotoxicity was calculated as (experimental release - spontaneous release)/(maximum release - spontaneous release) x 100. Spontaneous release was determined by incubating the targets with medium instead of effector cells and maximum release was determined by incubating target cells with with 10 μL of DELFIA lysis solution. Data were fitted to a sigmoidal dose-response curve using GraphPad Prism v5 (GraphPad Software, San Diego, CA).

**In vivo pharmacokinetics of antibodies with low sialic acid content.** The pharmacokinetics of antibodies with low sialic acid (LoSA) and minimal sialic acid (-SA) were compared with the normal untreated Ab (control Ab) after intraperitoneal injection of a weight-adjusted dose of 1.5 mg/kg in BALB/c mice (N = 12 animals, 4 animals per time points/group, 8–10 wks old, Charles River Laboratories). Blood samples (60 μL) were collected from CO$_2$-anesthetized mice by serial retro-orbital bleeds following dosing at 2 hr, 5 hr, 24 hr, 48 hr, 3 d, 7 d, 9 d and by cardiac puncture at 14 d after dosing. Serum was prepared from each blood sample and stored at -70°C until the assay for anti-human IgG by ELISA. Results were expressed as % maximal dose (at 2 h) over time and plotted using a 4-parameter fit with GraphPad Prism 5.0. To determine Ab half-life ($t_{1/2}$), the terminal phase slope for each animal was calculated using unweighted linear regression of time points from days 2 to 14, where $t_{1/2}$ = ln2/slope. One-way analysis of variance was used to determine whether slopes were significantly different between the groups. Statistical significance was defined as p < 0.05.

**References**

1. Wright A, Morrison SL. Effect of glycosylation on antibody function: implications for genetic engineering. Trends Biotechnol 1997; 15:26-32.
2. Raju TS. Terminal sugars of Gc glycans influence antibody effector functions of IgGs. Curr Opin Immuun 2008; 20:471-8.
3. Raju TS. Glycosylation variations with expression systems and their impact on biological activity of therapeutic immunoglobulins. BioProcess Int 2003; 1:44-53.
4. Jefferis R. Antibody therapeutics: isotype and glycoform selection. Expert Opin Biol Ther 2007; 7: 1401-13.
5. Satoh M, Lida S, Shiota K. Non-fucosylated therapeutic antibodies as next-generation therapeutic antibodies. Expert Opin Biol Ther 2006; 6:1161-73.
6. Scallon BJ, Tam SH, McCarthy SG, Cai AN, Raju TS. Higher levels of sialylated Fc glycans in immunoglobulin G molecules can adversely impact functionality. Mol Immunun 2007; 44:1525-34.
7. Kaneko Y, Nimmejanzahn F, Ravetch JV. Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. Science 2006; 313:670-3.
8. Anthony RM, Nimmejanzahn F, Ashline DJ, Reinhold VN, Paulson JC, Ravetch JV. Recapitulation of IVIG anti-inflammatory activity with a recombinant IgG Fc. Science 2008; 320:373-6.
9. Hooiser P, Khartak SF, Li ZJ. Optimal and consistent protein glycosylation in mammalian cell culture. Glycobiology 2009; 19:936-49.
10. Ngourtang FA, Miller PG, Brunnert FR, Tang GL, Wang DL. RNA interference of sialidase improves glycoprotein sialic acid content consistency. Biotech Bioeng 2006; 95:106-19.
11. Winter AJ, Comis SD, Osborne MF, Tarlow MJ, Stephen J, Andrew PW, et al. A role for pneumolysin but not neuraminidase in the hearing loss and cochlear damage induced by experimental pneumococcal meningitis in guinea pigs. Infect Immun 1997; 65:4411-8.
12. Raju TS, Scallon B. Fc glycans terminated with N-acetylgalactosamine residues increase antibody resistance to papain. Biotechnol Progr 2007; 23:964-71.
13. Warner TG. Enhancing therapeutic glycoprotein production in Chinese hamster ovary cells by metabolic engineering endogenous gene control with antisense DNA and gene targeting. Glycobiology 1999; 9: 841-50.
14. Anumula KR. High-sensitivity and high-resolution methods for glycoprotein analysis. Anal Biochem 2000; 283:17-26.
15. Raju TS, Scallon BJ. Glycosylation in the Fc domain of IgG increases resistance to proteolytic cleavage by papain. Biochem Biophys Res Commun 2006; 341:797-803.
16. Kozlowski S, Swann P. Current and future issues in the manufacturing and development of monoclonal antibodies. Adv Drug Deliv Rev 2006; 58:767-22.