

# Human uptake and incorporation of an immunogenic nonhuman dietary sialic acid

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Humans are genetically unable to produce the sialic acid *N*-glycolylneuraminic acid (Neu5Gc), because of a mutation that occurred after our last common ancestor with great apes. Although Neu5Gc is presumed absent from normal humans, small amounts have been claimed to exist in human tumors and fetal meconium. We have generated an antibody with high specificity and avidity for Neu5Gc. Fetal tissues, normal adult tissues, and breast carcinomas from humans showed reactivity to this antibody, primarily within secretory epithelia and blood vessels. The presence of small amounts of Neu5Gc was confirmed by MS. Absent any known alternate pathway for its synthesis, we reasoned that these small amounts of Neu5Gc might originate from exogenous sources. Indeed, human cells fed with Neu5Gc incorporated it into endogenous glycoproteins. When normal human volunteers ingested Neu5Gc, a portion was absorbed and eliminated in urine, and small quantities were incorporated into newly synthesized glycoproteins. Neu5Gc has never been reported in plants or microbes to our knowledge. We found that Neu5Gc is rare in poultry and fish, common in milk products, and enriched in red meats. Furthermore, normal humans have variable amounts of circulating IgA, IgM, and IgG antibodies against Neu5Gc, with the highest levels comparable to those of the previously known anti- $\alpha$ -galactose xenoreactive antibodies. This finding represents an instance wherein humans absorb and metabolically incorporate a nonhuman dietary component enriched in foods of mammalian origin, even while generating xenoreactive, and potentially autoreactive, antibodies against the same molecule. Potential implications for human diseases are briefly discussed.

Sialic acids (Sias) are nine-carbon sugars typically found as outermost units on the mammalian cellular glycocalyx and on secreted glycoproteins (1–4). The most common Sias are *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc). Cellular Neu5Gc is generated by hydroxylation of the sugar nucleotide donor CMP-Neu5Ac to CMP-Neu5Gc, catalyzed by CMP-Neu5Ac hydroxylase (CMAH) (5–7). Although Neu5Gc is a major Sia in most mammals (including our closest evolutionary relatives, the great apes) (8), it is thought to be absent in healthy humans (1–3). Indeed, humans generate immune responses against i.v.-administered molecules carrying Neu5Gc, e.g., the “serum sickness” reaction to equine antithymocyte globulin therapy (9, 10). These findings are explained by a human-specific inactivating mutation in the *CMAH* gene (11–13) that occurred  $\approx$ 2.5 million to 3 million years ago (14).

Despite no known alternate pathway for Neu5Gc synthesis in humans, antibodies have been used to claim its presence in some human cancers (15–20) and human fetal meconium (15). However, the specificity of the polyclonal antibodies used was not well defined. One study using mAbs failed to detect Neu5Gc in human tumors and tissues (21). However, these mAbs were specific for Neu5Gc only in the context of underlying structural motifs. Another mAb thought to be specific for Neu5Gc cross-reacts with some sulfated glycolipids (22). Meanwhile, some reports claim chemical proof for Neu5Gc in human tumors (18, 19, 23).

Human biosynthetic pathways could theoretically allow exogenous Neu5Gc to be metabolically incorporated (13, 24). Indeed, human cells cultured in FCS express cell surface Neu5Gc in small amounts (8, 21). However, it is not known whether this expression represents passive adsorption of serum glycoconjugates or metabolic incorporation. Although earlier studies claimed the absence of Neu5Gc from normal human tissues, we noted a small HPLC peak at the elution time of Neu5Gc in extracts from human organs (8). Here we explore whether Neu5Gc is actually present in normal and/or pathological human tissues, if normal humans can take up Neu5Gc from dietary sources, and if we spontaneously express antibodies against it.

## Materials and Methods

**Western Blot Analysis.** Serum proteins or total proteins extracted from Neu5Gc-fed or nonfed Caco-2 cells were either treated or sham-treated with 10 milliunits of *Arthrobacter ureafaciens* sialidase (EY Laboratories) in 100 mM sodium acetate, pH 5.5, at 37°C for 3 h. After SDS/PAGE, the separated proteins were transferred to nitrocellulose membrane, and binding of the anti-Neu5Gc antibody [1:10,000 in Tris-buffered saline with 0.1% Tween 20 (TBST)] was detected by using a secondary horseradish peroxidase-conjugated donkey anti-chicken IgY antibody (1:30,000 in TBST) and Super-signal West Pico (Pierce).

**Immunohistochemistry Using the Chicken Anti-Neu5Gc Antibody.** Human tissues were collected by an approved Institutional Review Board protocol, from autopsies or unused pathological material, frozen in OCT compound (Sakura Finetek, Torrance, CA), and archived at  $-70^{\circ}\text{C}$ . Harold McClure (Yerkes Primate Center, Atlanta) generously provided frozen chimpanzee tissues. Frozen tissue sections were air-dried for 30 min and fixed in 10% buffered formalin for 30 min. Endogenous peroxidase activity was quenched, and nonspecific binding sites were blocked with 5% (Neu5Gc-free) human serum in PBS for 30 min. Sections were then incubated with the anti-Neu5Gc antibody in 5% human serum/PBS at a 1:200 dilution at room temperature for 2 h (see *Supporting Text*, which is published as supporting information on the PNAS web site, www.pnas.org, for antibody production). After washing, horseradish peroxidase-conjugated donkey anti-chicken IgY antibody in 5% human serum/PBS at a 1:100 dilution was applied for 1 h. Control sections were incubated with normal IgY. Specific binding was detected by using the Nova Red substrate kit (Vector Laboratories), followed by hematoxylin counterstaining.

**Detection of Neu5Gc by HPLC Analysis and MS.** Human autopsy tissues (0.5 g) were subjected to sequential organic extractions and glycopeptides preparation (8). Sias in glycopeptides and lipid

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Abbreviations: Sia, sialic acid; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; CMAH, CMP-Neu5Ac hydroxylase; DMB, 1,2-diamino-4,5-methylene dioxybenzene;  $\alpha$ -Gal,  $\alpha$ -galactose; PAA, polyacrylamide.

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extracts were released by acid and purified by ion exchange chromatography (25), derivatized with 1,2-diamino-4,5-methylene dioxybenzene (DMB), and analyzed by HPLC (DMB-HPLC) and MS (14, 26).

**Purification of Neu5Gc from Porcine Submaxillary Mucin.** See *Supporting Text*.

**Feeding of Human Epithelial Cells with Neu5Gc.** Caco-2 cells (American Type Culture Collection, human epithelial cells isolated from a primary colon carcinoma) were propagated in  $\alpha$ -MEM (GIBCO/Invitrogen) supplemented with 20% heat-inactivated FCS, at 37°C, 5% CO<sub>2</sub>. Before feeding, the cells were split and cultured for 4 days in  $\alpha$ -MEM supplemented with 20% heat-inactivated premium human serum type AB (Irvine Scientific). Cells were then cultured in various concentrations of free Neu5Gc for 3 days at 37°C. Cells were then washed with cold PBS, scraped into 20 mM sodium phosphate, pH 7.5, and sonicated. The protein extract was quantified and analyzed by Western blot by using the anti-Neu5Gc antibody.

**Human Neu5Gc Ingestion Study.** Human subjects signed informed consent documents approved by the University of California at San Diego Institutional Review Board. They avoided animal foods for 2 days and all medications for 1 day, used a lanolin-free (Neu5Gc-free) shampoo for 2 days, and ingested only fruit juice on the morning of study. Subjects then drank 150 mg of porcine submaxillary mucin Sias (95% Neu5Gc) dissolved in 100 ml of water. For the next 6 h they drank a 1:1 mixture of fruit juice and soymilk (Sia-free) at  $\approx$ 2 ml/kg per h. Serum, urine, and saliva samples were obtained at multiple time points. Human saliva was collected by chewing parafilm, after washing the mouth out well with water. Urine volume and pH were recorded before centrifugation at 1,000  $\times$  g for 10 min and collection of the supernatant. All samples were stored frozen at  $-20^\circ\text{C}$ . Head hairs ( $\approx$ 20) and/or facial hair trimmings (from men) were collected before and several days after Neu5Gc ingestion.

**Purification and Analysis of Sias and N-Acylmannosamines from Human Samples.** Human salivary mucin was precipitated by adjusting to pH 3 with 100 mM acetic acid, stirring at 4°C overnight, collected at 14,000  $\times$  g for 10 min, and washed twice with 1 ml of ice-cold PBS. Human hair clippings were washed in 100 mM ice-cold acetic acid for 2 h and crushed in a clean ceramic mortar. Bound Sias from saliva, hair, or serum were released with mild acid as above, filtered through Microcon 10 filters (Millipore), dried down, reconstituted in water, and analyzed for Sia content by DMB-HPLC.

Five-milliliter urine aliquots were diluted 5-fold and loaded onto 10-ml columns of AG50W-X2 resin (H<sup>+</sup> form, Bio-Rad) equilibrated in water. The run-through and washes (2  $\times$  10 ml of water) were pooled and loaded onto 10-ml columns of AG1  $\times$  8 resin (Formate form, Bio-Rad) equilibrated in water. The combined run-through and washes (2  $\times$  10 ml of water) were lyophilized. After washing with 5 vol of 10 mM formic acid, free Sias were eluted with 5 vol of 1 M formic acid, diluted 2-fold with water, and lyophilized.

The neutral fraction from urine was dissolved in water, and reduced peracetylated volatile derivatives of free sugars were prepared (27). Samples were dissolved in 10  $\mu$ l of acetone, and gas-liquid chromatography for N-acylmannosamines was performed on 1- $\mu$ l aliquots (27). Eluting ions were detected and fragmented by using a HP5971 Mass Selective Detector (Hewlett-Packard).

**Detection of Anti-Neu5Gc and Anti- $\alpha$ -Galactose ( $\alpha$ -Gal)-Specific Antibodies in Human Sera.** Microtiter plate (Costar) wells were coated with Neu5Ac-polyacrylamide (PAA), Neu5Gc-PAA, or

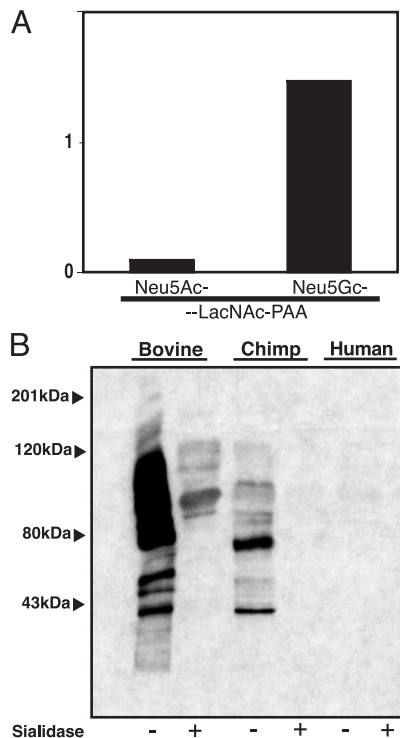
Gal $\alpha$ 1-3Galbeta1-4GlcNAc-PAA (Glycotech, Rockville, MD) at a concentration of 500 ng per well, in 50 mM sodium carbonate-bicarbonate buffer, pH 9.5, at 4°C overnight. After washing with TBS, pH 7.5, and blocking with TBST for 2 h at room temperature, triplicate wells were incubated with 1:50 dilutions of serum in TBST at room temperature for 4 h. Wells were washed five times with TBS and incubated with horseradish peroxidase-conjugated mouse anti-human IgA (Calbiochem), anti-human IgG (Jackson Immuno-Research Laboratories), or anti-human IgM (Kirkegaard & Perry Laboratories) each diluted in TBST at 1:20,000, at room temperature for 1.5 h. The IgG anti-Neu5Gc and anti- $\alpha$ -Gal antibodies were quantitated by using a standard curve of normal human IgG coated to the wells under the same general conditions.

**Purification and Analysis of Sias from Food Samples.** One-gram portions of samples were homogenized in 2 M acetic acid, and total Sias were released at 80°C for 3 h. After clarification at 50,000 g, the supernatant was filtered through a Microcon 10 unit, dried down, reconstituted in water, de-O-acetylated with 0.1 M NaOH for 30 min at 37°C, and neutralized with HCl. Aliquots were derivatized with DMB, and Neu5Gc content was analyzed by HPLC and MS as described above.

## Results

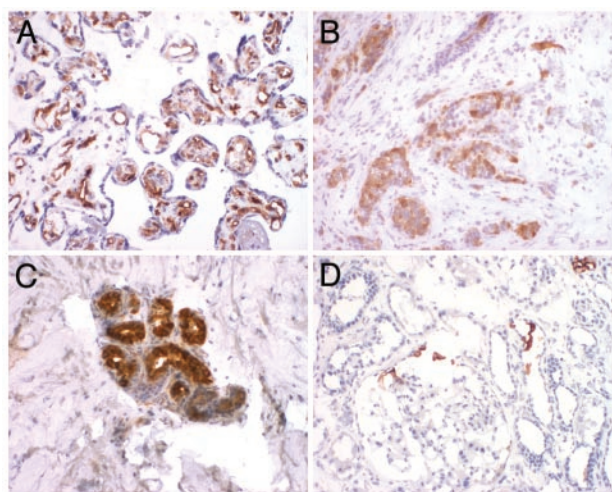
**Affinity Purification and Characterization of a Monospecific Antibody Against Neu5Gc.** Chickens are the only species other than humans known to generate an immune response to injected Neu5Gc (20, 28–30). A previous attempt at affinity purification of such antibodies used a Neu5Gc-containing glycolipid column, but did not rigorously define specificity (29). We developed a multistep approach to affinity chromatography that optimizes the specificity and avidity of the final preparation (see Fig. 7, which is published as supporting information on the PNAS web site, for details). The antibody preparation that bound to the fetuin column and then rebound to a modified fetuin column reacted with synthetic Neu5Gc-LacNAc-PAA but not with Neu5Ac-LacNAc-PAA, which differ by only a single oxygen atom (Fig. 1A). It also recognized many proteins in Neu5Gc containing bovine and chimpanzee sera, but not in human serum (Fig. 1B, note that chimpanzee proteins are  $\approx$ 99–100% identical to those of humans) (31). Sialidase pretreatment abrogated reactivity (Fig. 1B). Antibody specificity was confirmed for immunohistochemistry analyses (data not shown), where chimpanzee tissue sections stained intensely and human tissues showed only a limited staining (the latter was later shown to be specific, see below). In addition to the original immunogen (Neu5Gc $\alpha$ 2-3Gal $\beta$ 1-4Glc1-1'Ceramide), this preparation recognizes Neu5Gc in either  $\alpha$ 2-3 or  $\alpha$ 2-6 linkage to underlying Gal $\beta$ 1-4GlcNAc(LacNAc)-PAA (data not shown). This reactivity was also blocked by chimpanzee, but not human serum (data not shown). Thus, this polyclonal preparation contains antibodies recognizing Neu5Gc on both glycoproteins and glycolipids, regardless of the linkage ( $\alpha$ 2-3 or  $\alpha$ 2-6) and regardless of the underlying sugar chain. Binding to synthetic PAA-based probes confirms that the nature of the protein or the lipid tail of glycolipids are not important binding determinants.

**Antibody Reactivity in Malignant, Fetal, and Normal Human Tissues.** Staining with the antibody showed cell type-specific reactivity in adult human tissues. A summary of findings is presented in Table 1, which is published as supporting information on the PNAS web site, and examples are shown in Fig. 2. Breast carcinomas showed reactivity in the majority of tumor cells and some related blood vessels. In the fetus, staining was prominent in epithelial cells and secretions, as well as in the placental villus blood vessels. The overall pattern of staining in 12 normal human tissues can be summarized as prominent in secretory epithelia and associated secretions and present in many blood vessels. Secondary antibody alone with or without normal IgY gave no staining (data not shown). The ability

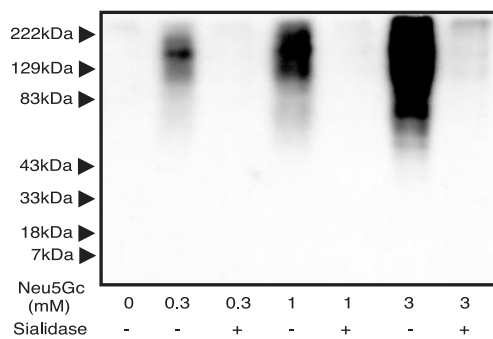


**Fig. 1.** Characterization of specificity of the anti-Neu5Gc antibody. (A) ELISA against Neu5Ac-LacNAc-PAA or Neu5Gc-LacNAc-PAA. Data show the mean of three determinations. (B) Bovine, chimpanzee, or human serum proteins (5  $\mu$ g each) were either treated or sham-treated with sialidase and separated by SDS/PAGE. Reactivity of the anti-Neu5Gc antibody was analyzed in a Western blot assay.

to specifically inhibit staining by chimpanzee serum or porcine mucin varied with individual human tissues (data not shown). However, in comparison to WT mice no clear staining was seen in tissues from *CMAH* null mice that are expected to be Neu5Gc-deficient (unpublished work). Thus, the variable inhibition by chimpanzee serum or porcine mucin in different human tissues may



**Fig. 2.** Examples of reactivity of anti-Neu5Gc antibody with human tissue sections. Reactivity of the antibody to tissue sections is indicated by the brown staining. (A) Blood vessels in placental villi. (B) Breast carcinoma, tumor cells, and blood vessel. (C) Skin and eccrine glands. (D) Kidney tubules and secretions. For a summary of staining patterns for all tissues studied see Table 1. (Magnification:  $\times 20$ .)



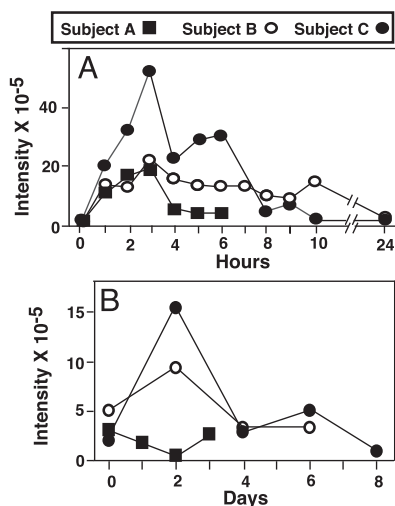
**Fig. 3.** Incorporation of free Neu5Gc into glycoproteins of cultured human epithelial cells. Western blot analysis of 7  $\mu$ g of total proteins extracted from Caco-2 cells not fed or fed with Neu5Gc. Proteins were treated or sham-treated with *A. ureafaciens* sialidase and separated by SDS/PAGE on a 12.5% gel. Reactivity with the anti-Neu5Gc antibody was analyzed in a Western blot assay.

reflect expression of different types of Neu5Gc-containing epitopes. Of course, we cannot rule out a novel human-specific epitope that is not Neu5Gc and still cross-reacts with this extensively characterized antibody. To be certain of our finding, we turned to MS analyses.

**MS Analysis of Human Tissues Confirms Neu5Gc.** Sias were released and purified from human heart, kidney, liver, and spleen glycopeptides and analyzed by DMB-HPLC plus MS (14). A Neu5Gc standard gave signals at  $m/z$  442 and 424, consistent with molecular ions of DMB-derivatized Neu5Gc and its dehydrated form (data not shown). As expected, selective secondary MS on the  $m/z$  442 ion peak gave the  $m/z$  424 dehydrated form (data not shown). MS on putative DMB-derivatized Neu5Gc from human kidney also gave ion peaks at  $m/z$  442 and 424 (Fig. 8, which is published as supporting information on the PNAS web site). Selective secondary MS of the  $m/z$  442 ion gave a single ion peak at  $m/z$  424, confirming the presence of Neu5Gc (Fig. 8). Analysis of glycopeptides and some glycolipid fractions from human heart, liver, and spleen tissues gave similar spectra. MS done in this manner is not truly quantitative. However, we had earlier noted small HPLC peaks from normal adult human tissues eluting in the position expected for Neu5Gc (8). These peaks (now confirmed to be Neu5Gc) represented  $<1\%$  of total Sias in liver, skin, and kidney,  $\approx 1\%$  in spleen and testes, and  $\approx 2\%$  in the heart (8).

**Human Epithelial Cells in Culture Can Metabolically Incorporate Neu5Gc.** Although human cells cultured in FCS express cell surface Neu5Gc in small amounts (8, 24), this expression could represent either metabolic incorporation or nonspecific absorption. Human epithelial cells were cultured in human serum until no traces of Neu5Gc derived from FCS could be detected by HPLC (typically  $\approx 4$  days). Culture was then continued for 3 days in the absence or presence of varying amounts of free Neu5Gc. HPLC analysis showed increasing incorporation of Neu5Gc into the cells over time, with the highest level reaching 85% of total Sia after incubation in 3 mM Neu5Gc for 3 days (data not shown). Metabolic incorporation of Neu5Gc into glycoproteins was confirmed by Western blotting using the anti-Neu5Gc antibody (Fig. 3). Staining was seen only when Neu5Gc was present in the medium, and reactivity was eliminated by sialidase treatment (further confirming the specificity of the antibody). These data suggested that the small amounts of Neu5Gc in normal human tissues could originate from dietary sources.

**Ingested Neu5Gc Is Absorbed and Excreted by Normal Adult Humans.** Purified Neu5Gc was orally administered, and its fate was followed by examination of blood, serum, urine, hair, and saliva. Considering



**Fig. 4.** MS detection of urinary excretion and salivary mucin incorporation of ingested Neu5Gc. Sias were purified from the urine (A) and salivary mucins (B) of the three subjects before Neu5Gc ingestion and at various subsequent time points and analyzed by DMB derivatization, HPLC, and MS. The sum of the intensities of ions at  $m/z$  424 and  $m/z$  442 at each time point is plotted for each subject.

the uncertain risks, three of the authors (A.V., E.M., and P.G.) volunteered as subjects. Initial studies on the first subject indicated no immediate ill effects from ingestion of up to 150 mg of porcine mucin Sias (5% Neu5Ac, 95% Neu5Gc). The other two subjects then also ingested the same amount. There were no changes in vital signs, basic hematology, and serum chemistry values. Free Sias were purified from urine samples and analyzed for Neu5Gc content by DMB-HPLC. Because overlapping fluorescent peaks prevented definitive identification and quantification, HPLC fractions eluting at the time expected for DMB-Neu5Gc were collected and subjected to MS, confirming an increase in ion peaks at  $m/z$  424 and  $m/z$  442. The sum of ion intensities of  $m/z$  424 and  $m/z$  442 at each time point provided an estimate of Neu5Gc quantity. Ingested Neu5Gc was rapidly absorbed and excreted into the urine (Fig. 4A). About 3–6% of the ingested dose was excreted within 4–6 h, with the peak excretion rate at 2–3 h and a return to baseline levels within 24 h. Notably, small amounts of Neu5Gc were also present at baseline.

Sias could be converted into their corresponding acylmannosamines by sialate:pyruvate-lyases (32), derived from either intestinal cells or gut microbes. Such molecules could then enter cells and be reconverted back to the original Sias (33). To ascertain potential conversion of the ingested Neu5Gc into *N*-glycolylmannosamine (ManNGc), we looked for this neutral sugar in the urine. Small amounts of ManNGc were present at baseline in one subject (data not shown). There was no obvious increase over time in any subject (data not shown), indicating that the remaining ingested Neu5Gc was not absorbed and excreted as ManNGc. Thus, much of the uptake is apparently occurring as free Neu5Gc.

**Ingested Neu5Gc Can Be Incorporated into Newly Synthesized Glycoproteins.** Mucins from all baseline saliva samples showed very small amounts of Neu5Gc detectable by MS. An increase 2 days after ingestion occurred in two of three subjects, with a decline to baseline levels by 4 days (Fig. 4B). Although we failed to detect Neu5Gc in serum samples at all time points, small amounts were found in facial hair clippings at baseline for two subjects, with increases after ingestion. Overall the data show uptake and excretion of intact Neu5Gc and low-level metabolic incorporation into newly synthesized glycoconjugates. Although there were no immediate reactions to Neu5Gc ingestion, this does not rule out long-

term consequences. Because the basic findings were consistent, we did not risk further studies on ourselves, nor seek additional subjects.

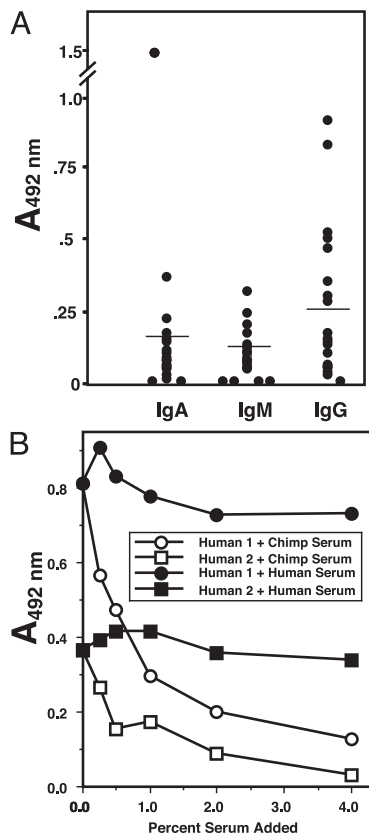
**Neu5Gc Is Enriched in Food Sources of Mammalian Origin.** Sias have never been detected in plants and are found in large amounts primarily in vertebrates and a few “higher” invertebrates (2, 3, 34). Although several pathogenic microbes can express Neu5Ac, none have been reported to express Neu5Gc. We determined the amounts of Neu5Gc in common dietary sources of animal origin (see Table 2, which is published as supporting information on the PNAS web site). The highest amount was found in lamb, pork, and beef (so-called “red meat”). In contrast, levels were very low or undetectable in poultry and fish. Hen eggs contain primarily Neu5Ac (35). Intermediate amounts were found in bovine milk and milk products, with higher levels in goat and sheep milk products. As summarized in Table 2, U.S. Department of Agriculture-recommended dietary intake would result in substantial intake of Neu5Gc from red meats and moderate amounts from dairy products.

**Circulating Anti-Neu5Gc Antibodies Are Present in Most Normal Humans.** A sensitive and specific assay for detecting anti-Neu5Gc antibodies in human sera was set up, comparing reactivity against Neu5Gc-PAA and Neu5Ac-PAA, which differ by only one oxygen atom. Anti-Neu5Gc antibodies of the IgA, IgM, and IgG classes were detected in the sera of all three subjects who ingested Neu5Gc at baseline, but no increases occurred after Neu5Gc ingestion (data not shown). Studies of additional normal adults showed that 16/18, 14/18, and 17/18 sera were positive for IgA, IgM, and IgG antibodies, respectively (Fig. 5A). Specificity was confirmed by showing that anti-Neu5Gc reactivity was blocked by chimpanzee serum in a concentration-dependent manner, but not by equivalent amounts of a Neu5Gc antibody-negative human serum (Fig. 5B). The chimpanzee serum used for inhibition was also negative for anti-Neu5Gc antibodies (Fig. 6). Further confirmation came from >70% inhibition of reactivity by free Neu5Gc but not by Neu5Ac (each at 2 mM, data not shown).

Some human sera required up to 3–4% chimpanzee serum to obtain complete blockade (Fig. 5B). Because chimpanzee serum glycoproteins carry  $\approx 0.1$  nmol of Neu5Gc per  $\mu\text{l}$ , the anti-Neu5Gc antibodies must be present at relatively high concentrations in such human sera. Indeed, these antibodies represented up to 0.25% of the total circulating IgG in some subjects (Fig. 6). This falls into the range of the well known xenoreactive antibodies against the non-human  $\alpha$ -Gal epitope (36, 37). We also compared levels of IgG anti-Neu5Gc and anti- $\alpha$ -Gal antibodies. As expected from prior literature, all of the human sera contained easily detectable anti- $\alpha$ -Gal antibodies. Anti-Neu5Gc levels were highly variable, and there was no direct correlation between the two antibody levels in any given individual. On the other hand, the relative levels of anti-Neu5Gc antibodies between individuals were reproducible on repeat sampling. Four chimpanzee sera showed no anti-Neu5Gc antibodies, but clear evidence of anti- $\alpha$ -Gal antibodies (Fig. 6).

## Discussion

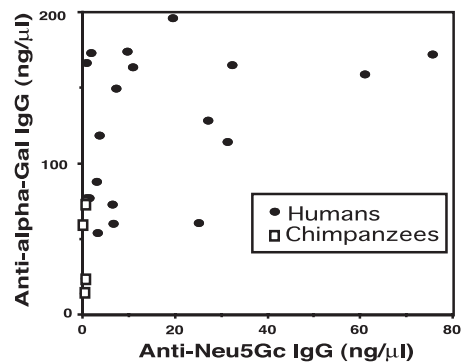
Existing data about Neu5Gc in human cells is controversial (16, 18, 19, 21, 22). Even in studies claiming its presence in malignant tissues, the cell type specificity of the presumed Neu5Gc was not defined. We have advanced understanding of this issue by using an affinity-purified polyclonal antibody against Neu5Gc. Our data support prior reports of Neu5Gc in human cancers and extends the finding to normal and fetal human tissues. Of course, despite our extensive efforts to define specificity, we cannot rule out the possibility of an unexpected epitope cross-reactive with this polyclonal antibody. Thus, we confirmed the presence of Neu5Gc in the normal human tissues with MS.



**Fig. 5.** ELISA detection of anti-Neu5Gc antibodies in normal human sera and demonstration of specificity. (A) Results are plotted as mean background values with PAA-Neu5Ac subtracted from the signal with PAA-Neu5Gc. The mean value for all positive sera in each subclass is represented by the horizontal bar. (B) Two of the human sera identified as medium or high positive for anti-Neu5Gc IgG antibodies to the target Neu5Gc-PAA were tested in the same ELISA with dilutions of chimpanzee serum added to inhibit binding. A human serum with undetectable anti-Neu5Gc antibodies was used as a control.

The inactivating mutation of *CMAH* in humans is genetically irreversible, no human genes have homology to *CMAH*, and evidence for an alternate biosynthetic pathway is lacking. Although human cells cultured in FCS appear to incorporate Neu5Gc (8, 24), this could be just passive adsorption. For example, glycolipids containing Neu5Gc are lipophilic molecules that can be directly integrated into plasma membranes. Although this could account for some of the Neu5Gc in gut epithelial cells, it cannot explain the enrichment of Neu5Gc in other internal human organs. Here we demonstrate that human cells in culture can metabolically incorporate free Neu5Gc into endogenous glycoproteins. Based on known cellular pathways (13), a possible explanation is that free Neu5Gc enters human cells via pinocytosis to reach the endosomal/lysosomal system. There it could be transported into the cytosolic compartment and get activated to CMP-Neu5Gc, which would eventually enter the Golgi apparatus, where sialyltransferases could catalyze transfer of Neu5Gc to newly synthesized glycoconjugates. Notably, all of the transporters and enzymes mentioned above can act on both Neu5Ac and Neu5Gc.

Regardless of the mechanisms of metabolic incorporation, we show that dietary sources could account for the small amount of Neu5Gc in normal adult human tissues. Intact humans absorbed a portion of ingested Neu5Gc, excreted it into the urine, and incorporated a small amount into newly synthesized glycoconjugates. The majority of ingested Neu5Gc was not absorbed, was eliminated from the body by means other than urinary excretion, or was possibly incorporated into tissues not analyzed in this study. These



**Fig. 6.** Comparison of levels of anti-Neu5Gc and anti- $\alpha$ -Gal antibodies in normal human sera. Serum samples were collected several months later from the same individuals studied in Fig. 4A. Levels of anti-Neu5Gc antibodies were studied identically, in comparison with parallel wells coated with Gal $\alpha$ 1-3Galbeta1-4GlcNAc-PAA, instead of Neu5Gc-PAA. The final readout of values was thus directly comparable between the two probes, after subtraction of the background readings with Neu5Ac-PAA. IgG levels were quantified as described.

findings differ from a report in rats, where  $\approx$ 98% of orally administered radioactive Sia was absorbed and excreted unaltered in urine within 6 h (38). This difference could reflect human-specific mechanisms to exclude the antigenic Neu5Gc either by decreasing absorption or eliminating Neu5Gc via other means. In this regard, we noted selective staining of the epithelia and the associated secretions in normal tissues (i.e., cells with a high turnover rate, and which secrete sialoglycoconjugates). The more prominent enrichment of Neu5Gc in carcinomas and fetuses could be caused by higher uptake by these rapidly growing tissues. The contrasting absence of Neu5Gc from plasma proteins and blood cells could be because the cells of origin (hepatocytes, plasma cells, and bone marrow cells) have a robust ability to generate their own Neu5Ac from endogenous pathways, which would block competition by exogenous Neu5Gc.

We found that most normal humans have circulating anti-Neu5Gc antibodies. While this article was in preparation, another group independently reported a high prevalence of anti-Neu5Gc antibodies in normal human sera, using a different approach (39). The findings of both studies stand in contrast to previous reports of the prevalence of anti-Neu5Gc antibodies in normal humans, ranging only from 0–10% for the IgG class to 3.8–13.8% for the IgM class (40–42). This disparity can be explained by the greater sensitivity and lower background of the assays we used, the use of an optimal control target (identical except for Neu5Ac in place of Neu5Gc), and the checks for specificity (blockade by chimpanzee but not human serum, or by free Neu5Gc but not free Neu5Ac).

The anti-Neu5Gc antibodies could be a response to dietary Neu5Gc and/or to endogenously incorporated Neu5Gc. Although most normal human adults had anti-Neu5Gc antibodies, one-time ingestion of a large amount of Neu5Gc did not enhance baseline levels. This finding may reflect incorporation of the Neu5Gc into immune privileged cell types, rapid elimination of Neu5Gc via excretion by epithelial cells, or a preexisting partial tolerization to Neu5Gc. Another possibility is that induction of an immune response necessitates Neu5Gc accumulation and/or exposure over long time period. Other variables are the extent of maternal-fetal transfer of Neu5Gc and early childhood exposure to Neu5Gc in bovine milk or vaccines. Population studies are needed to pursue such issues.

Some humans had circulating anti-Neu5Gc IgG levels in the range seen for xenoreactive antibodies against the  $\alpha$ -Gal epitope that is deficient in all Old World primates, including humans (36, 37). Thus, anti-Neu5Gc antibodies comprise another potential barrier to xenotransplantation, especially from species such as the

pig, whose organs are rich in Neu5Gc. They also pose a potential limitation for the use of glycosylated therapeutic agents generated in the milk of animals or in nonhuman cells. There are differences between the two types of antibodies. First, although anti- $\alpha$ -Gal antibodies occur at relatively high levels in all humans, anti-Neu5Gc antibody levels vary greatly. Second, unlike Neu5Gc, the  $\alpha$ -Gal epitope itself is completely absent from normal humans and cannot be metabolically incorporated from dietary sources. Third, the anti- $\alpha$ -Gal response is apparently generated by postnatal exposure to exogenous antigens. In contrast, the anti-Neu5Gc response could be caused by exogenous antigens or endogenously incorporated molecules.

The remainder of this discussion contains speculation that is either directly testable or epidemiologically approachable. Consumption of red meat (the richest source of Neu5Gc) is associated with diseases such as cancer (43–48) and ischemic heart disease (44, 45, 49). In contrast, vegetarianism decreases the risk of cancer (44, 46) and heart disease (44, 45, 49). Although saturated fat in meat and dairy products is the usual explanation, population studies comparing anti-Neu5Gc antibodies and/or body burden of Neu5Gc with cancer and ischemic heart disease incidence may be worthwhile.

Some other diseases such as hepatitis, liver cirrhosis, infectious mononucleosis, rheumatoid arthritis, syphilis, and lepromatous leprosy are claimed to be associated with “Hanganutziu-Deicher” heterophile anti-Neu5Gc antibodies (40–42). Such antibodies could play a role in initiation or progression or simply arise from

immune dysregulation that allows preexisting memory B cells to expand. Alternatively, some of these diseases involve proliferation of vascularized tissues that could incorporate more dietary Neu5Gc, boosting a baseline immune response. The small amounts of Neu5Gc in normal tissues also raise the possibility that anti-Neu5Gc antibodies are involved in autoimmunity. In this regard, it is interesting that vegetarian diet has been suggested to improve rheumatoid arthritis (50).

The *CMAH* mutation occurred  $\approx 2.5$  million to 3 million years ago (14), before the first evidence for meat eating in the subsequently emerging *Homo* lineage (51). Of course, most diseases potentially associated with dietary Neu5Gc and/or Neu5Gc antibodies have an onset beyond the average age of reproduction, when natural selection is weak or nonexistent (52). Thus, there may not have been much selection initially against Neu5Gc intake and incorporation. However, in this era of increased longevity, the long-term consequences of constantly challenging our systems with this antigenic sugar and the implications of having anti-Neu5Gc antibodies in the circulation are provocative and should be further investigated.

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**Supplemental information****Human Uptake and Incorporation of an Immunogenic Non-human Dietary Sialic Acid****Methods**

*Purification of Neu5Gc from porcine submaxillary mucin* — Frozen porcine submaxillary glands (Pel-Freeze Biologicals, Rogers, AZ), were minced, homogenized in water, and total mucins precipitated from the water-soluble supernatant by slow acidification to 100mM acetic acid in the cold for a few hours. The precipitate was resuspended in water, the pH neutralized and dialyzed against water. Sia content was determined by DMB-HPLC analysis. Sias were released with mild acid, collected by dialysis (1000 molecular-weight-cut-off), de-O-acetylated and purified on ion exchange columns {000674}. Remaining contaminants were removed with a C18 cartridge. The yield was 563mg of Sia (95% Neu5Gc, 5% Neu5Ac) from 28.2g of mucin.

*Generation of a Monospecific Chicken Polyclonal Antibody against Neu5Gc* – Lipids were extracted from horse erythrocytes {Kunihiko S, 1965, #43365}, suspended in water, dialyzed against water, and fractionated on DEAE Sephadex A-25 {000623}. Eluted fractions containing  $G_{M3}$ (Neu5Gc) were detected by HPTLC, concentrated by evaporation, dialyzed against water and taken to dryness. Chickens were immunized and boosted with 1 mg each of  $G_{M3}$ (Neu5Gc) {003171}, except that BSA was not methylated. Egg yolks were collected, a total IgY fraction obtained by 40% ammonium sulfate precipitation, and dialyzed repeatedly against PBS, pH 7.4. Antibody titer was monitored by horse erythrocyte hemagglutination, and the pool stored at  $-20^{\circ}\text{C}$ .

*Columns for Affinity Chromatography*— The column for pre-clearing had 81.2mg of bovine asialofetuin (Sigma, St. Louis, MO) coupled to 5 ml Affi-Gel 15 beads (BioRad, Richmond, CA). The first affinity column contained 23.8mg native (fully sialylated) fetuin (Sigma) coupled to 5 ml of Affi-Gel 15. A modified fetuin-column with truncated Sia side chains was prepared by treating 5ml of an identical column with 25ml of 2mM sodium periodate in PBS, pH 7.4 for 30 min, rotating at  $4^{\circ}\text{C}$  in the dark. Ethylene glycol (250 $\mu\text{l}$  of 5M) was added for 5 min at  $4^{\circ}\text{C}$  to consume

excess periodate. After washing with 4x5ml PBS, pH 7.4 and 4x5ml 50mM sodium acetate buffer, pH 5.5, 20 ml of 20mM acetic hydrazide in 50mM sodium acetate buffer, pH 5.5 was added to block reactive aldehydes generated by periodate cleavage of the Sia side chain. After 2 hrs in the dark at room temperature (RT) with gentle shaking, the beads were washed with 2x20ml of PBS and resuspended in 0.01% sodium azide/PBS until use. Completeness of side chain truncation was confirmed by release of Sias using mild acid (2M acetic acid at 80°C for 3 hrs), derivatization with 1,2-diamino-4,5-methylene-dioxybenzene (DMB) (Sigma) and HPLC analysis {021635}.

*Affinity Purification of Chicken anti-Neu5Gc antibody*— The IgY fraction was thawed, clarified at 100,000g for 30 min, and 1 ml of supernatant pre-cleared by passage over the asialofetuin column. The flow-through fraction was pooled with column washes of 2x 5ml PBS, pH 7.4, and passed over an affinity column containing native (sialylated) fetuin (see Figure 7, Panel A). The flow-through and washes (2x5ml PBS, pH 7.4) were collected, and Neu5Gc-specific antibodies recovered by elution with 2x 5ml 0.1M citric acid, pH 3.0, into a vial containing 2.4ml of 1M Tris, pH 11. Eluted fractions were monitored by ELISA against Neu5Gc-containing bovine serum (see below). The flow-through fraction was reloaded onto the same fetuin column, washed and eluted again. This procedure was repeated until there was no further binding of antibodies.

*Removal of side-chain dependent anti-Neu5Gc antibodies*— The pooled fetuin-binding eluate fractions were concentrated to 3ml using a Millipore Ultrafree-15 centrifugal filter device with Biomax-30K membrane (Millipore, Bedford, MA), and then passed over the column of fetuin with truncated Sia side chains (Figure 7, Panel B). The flow-through fraction was collected along with washes of 2x 5ml PBS, pH 7.4. The antibodies that continued to bind to this modified column were then eluted with 2x5ml 0.1M citric acid, pH 3.0, into a vial containing 2.4ml of 1M Tris, pH 11. The column was then re-equilibrated in PBS, and the above procedure was repeated with the flow-through fraction until there was no further decrease in reactivity to bovine serum. The combined eluates from all of the runs were concentrated to 3ml using a Millipore Ultrafree-15 centrifugal filter device with Biomax-30K membrane.



*Monitoring the Purification and Binding Specificity of the anti-Neu5Gc Antibody*—Microtiter plate (Costar, Corning, NY) wells were coated either with bovine serum (containing both Neu5Ac and Neu5Gc), or human serum (containing only Neu5Ac) at 115 µg protein/well, in 50mM sodium carbonate-bicarbonate buffer, pH 9.5, at 4°C overnight. Wells were washed with Tris buffered saline (TBS), pH7.5, and subsequently blocked with TBS+0.1% Tween (TBST) for 1 hour at RT. The wells were again washed 5X with TBS, and incubated with serial dilutions of the antibody in TBST for 2 h at RT, washed 5X again with TBS, and then incubated with a donkey anti-chicken IgY antibody conjugated with horse radish peroxidase (Jackson ImmunoResearch) at a 1:10,000 dilution in TBST for 1 hour at RT. After 5 washes in TBS, antibody binding was measured using 2.75mM O-phenylene-diamine (OPD) (Sigma) and 0.075% hydrogen peroxide in citrate phosphate buffer, pH 5.5; quenching with 40 µl of 4M sulfuric acid, and reading absorbance at 492nm with a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA).

### Figure Legends

**Figure 7. Affinity-purification of a monospecific Anti-Neu5Gc antibody.** The total IgY pool from eggs of chickens immunized with ganglioside G<sub>M3</sub>(Neu5Gc) was pre-cleared over a column of immobilized bovine asialofetuin (fetuin with sialic acids removed, not shown). The flow-through fraction was passed over a column of immobilized bovine fetuin (Panel A) with a very low density of bound Neu5Gc (2.7% of total Sias). This allowed only the highest affinity antibodies to bind. These were eluted with mild acid and dialyzed against buffer. The flow-through fraction was repeatedly reapplied, until no more antibodies bound. The pooled bound fractions were then reapplied to a similar column of immobilized fetuin (Panel B), which differed only in that it had been pretreated with mild periodate oxidation to selectively truncate the side chain of Sias. Repeated rounds of this process yielded a pool of antibodies that had originally bound to the unmodified fetuin column, and continued to bind to the modified column.

The starting IgY fraction showed three times higher reactivity against bovine serum glycoproteins (which carry both Neu5Ac and Neu5Gc), than against human serum glycoproteins (which carry only Neu5Ac). The two-step affinity purification process enriched for a subset of the

high affinity antibodies against Neu5Gc. Non-specific reactivity of the resulting antibody to human serum was reduced to background levels (not shown, see Figure 1 in the main text for further confirmation that the antibody has high specificity and avidity for Neu5Gc).

**Figure 8. Mass spectrometric detection of Neu5Gc in normal human kidney**

**glycopeptides.** Sias were released and purified from human kidney tissue, derivatized with DMB and fractionated by HPLC. (A) Mass spectrometry analysis showing ions at  $m/z$  442 and 424, consistent with the molecular ion of DMB-derivatized Neu5Gc and its dehydrated form. (B) Selective MS/MS on the  $m/z$  442 ion gives a fragment ion of  $m/z$  424, confirming that the original  $m/z$  442 ion was DMB-derivatized Neu5Gc.

**Table 1. Reactivity of normal human tissue sections with the anti-Neu5Gc antibody.**

<b>Tissue</b>	<b>Staining Pattern*</b>
Lung	Luminal edges of bronchiolar epithelial cells and associated secretions. Endothelium of blood vessels.
Skin	Luminal edges of epithelial cells of eccrine glands and associated ducts. Endothelium of blood vessels.
Colon	Luminal edges of colonic epithelial cells and associated secretions. Endothelium of blood vessels.
Prostate	Cytoplasmic staining of glandular epithelial cells. Endothelium of blood vessels.
Uterus	Epithelial cells of Endometrial glands (non-secretory phase studied).
Kidney	Edges of collecting duct epithelium and associated secretions. No Glomerular staining.
Spleen	Periarteriolar lymphoid sheath lymphocytes. Endothelium of blood vessels.
Pancreas	Epithelial cells of acini. Endothelium of blood vessels.
Testis	Basal cell layer (spermatogonia) of the seminiferous tubules. Endothelium of blood vessels.
Liver	Luminal edges of some bile duct epithelial cells. Endothelium of blood vessels.
Fetal Stomach	Luminal edges of epithelial cells of the villi and associated secretions. Endothelium of blood vessels.
Placenta	Endothelium.
Breast Carcinoma	Majority of carcinoma cells and and some blood vessels.

\*See Figure 2 for some examples of the staining pattern.

**Table 2. Preliminary Survey of Total Sialic Acid and Neu5Gc Content of Common Food Items**

Food item	% Neu5Gc*	Total Sia* (ug/g)	Neu5Gc Content (ug/g)	Serving Size** (g)	Servings per day**	Total weight if eaten daily (g/day)	Neu5Gc intake if eaten daily*** (ug)
Cod	<0.1	40	<0.04	90	3	270	<11
Salmon	<0.1 –3.5	49	1.47	90	3	270	<11 - 397
Tuna	<0.1	32	<0.032	90	3	270	<9
Chicken	<0.1	76	<0.076	90	3	270	<21
Turkey	<0.1- 0.6	46	<0.046	90	3	270	<12 - 75
Duck	<0.1	20	<0.02	90	3	270	<5
Cow Milk (2%)	3	258	7.74	237	2	474	3668
Cow Milk (raw)	3	262	7.86	237	2	474	3726
Butter	3	40	1.2	5	3	15	18
Cow Cheese	4	160	6.4	50	3	150	960
Goat Cheese	42	95	39.9	44	3	132	5267
Lamb	18	101	18.2	90	3	270	4914
Pork	19	134	25.5	90	3	270	6885
Beef ****	31-43	70	30.1	90	3	270	5859 - 8127

\*Total Sia content and percent of Neu5Gc in each food sample was quantified as described in “Materials and Methods”. Range of values obtained are shown for food items that were studied more than once. Oral intake calculations include the highest values in such cases.

\*\*Recommended daily servings and serving sizes of each item are based upon guidelines set by the U.S. Department of Agriculture (Home Garden Bulletin No. 323. Nutrition and Your Health – Dietary Guidelines for Americans. Fifth Edition. 2000).

\*\*\*Oral intake calculation only. Actual bioavailability is unknown.

\*\*\*\*Lean beef and beef fat portions had similar Neu5Gc contents (data not shown).

**References for Supplemental information.**

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Figure 7 (Supplemental Information) Tangvoranuntakul et al.

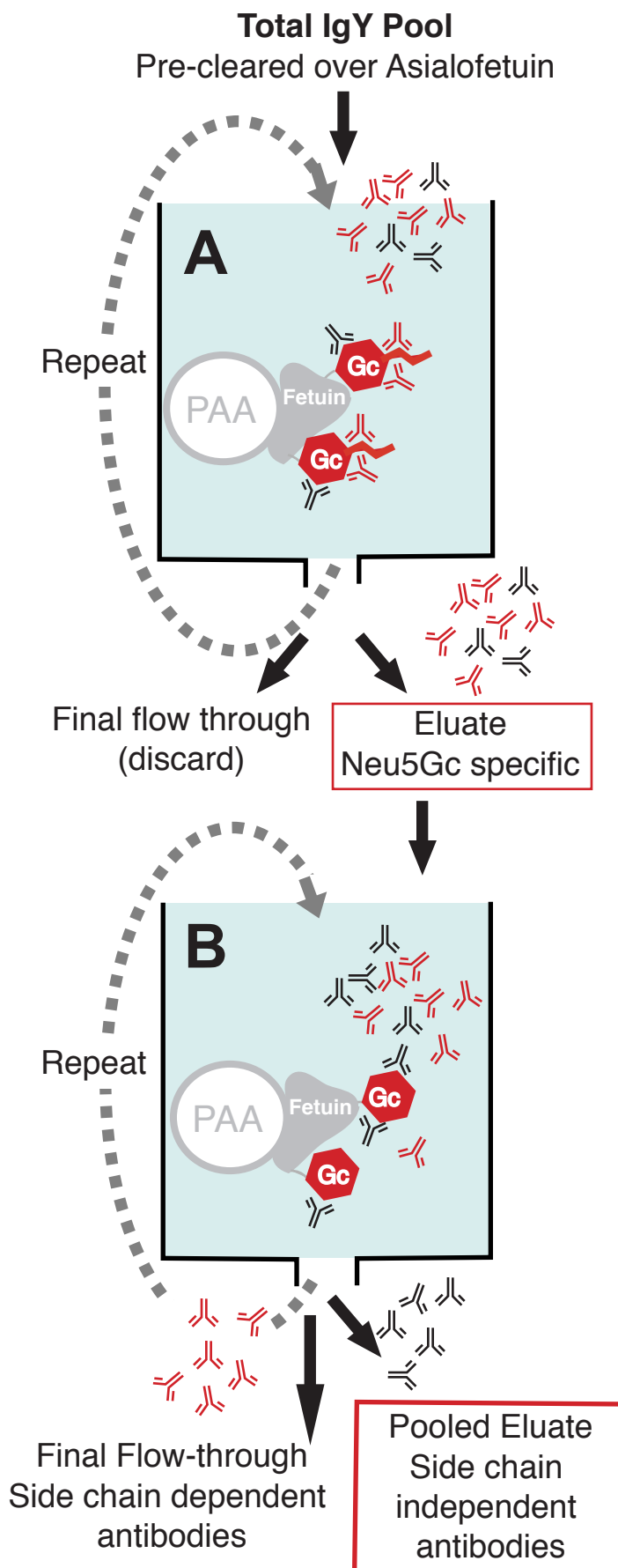


Figure 8. Supplemental Information  
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