Diversity in specificity, abundance, and composition of anti-Neu5Gc antibodies in normal humans: Potential implications for disease

Vered Padler-Karavani[2], Hai Yu[3], Hongzhi Cao[3], Harshal Chokhawala[3], Felix Karp[4], Nissi Varki[4], Xi Chen[3], and Ajit Varki[12]

[2]Glycobiology Research and Training Center and Department of Medicine, University of California, San Diego, La Jolla, CA 92093; [3]Department of Chemistry, University of California-Davis, One Shields Avenue, Davis, CA 95616; and [4]Glycobiology Research and Training Center and Department of Pathology, University of California, San Diego, La Jolla, CA 92093, USA

Human heterophile antibodies that agglutinate animal erythrocytes are known to detect the nonhuman sialic acid *N*-glycolylneuraminic acid (Neu5Gc). This monosaccharide cannot by itself fill the binding site (paratope) of an antibody and can also be modified and presented in various linkages, on diverse underlying glycans. Thus, we hypothesized that the human anti-Neu5Gc antibody response is diverse and polyclonal. Here, we use a novel set of natural and chemoenzymatically synthesized glycans to show that normal humans have an abundant and diverse spectrum of such anti-Neu5Gc antibodies, directed against a variety of Neu5Gc-containing epitopes. High sensitivity and specificity assays were achieved by using *N*-acetylneuraminic acid (Neu5Ac)-containing probes (differing from Neu5Gc by one less oxygen atom) as optimal background controls. The commonest anti-Neu5Gc antibodies are of the IgG class. Moreover, the range of reactivity and Ig classes of antibodies vary greatly amongst normal humans, with some individuals having remarkably large amounts, even surpassing levels of some well-known natural blood group and xenoreactive antibodies. We purified these anti-Neu5Gc antibodies from individual human sera using a newly developed affinity method and showed that they bind to wild-type but not Neu5Gc-deficient mouse tissues. Moreover, they bind back to human carcinomas that have accumulated Neu5Gc in vivo. As dietary Neu5Gc is primarily found in red meat and milk products, we suggest that this ongoing antigen-antibody reaction may generate chronic inflammation, possibly contributing to the high frequency of diet-related carcinomas and other diseases in humans.

Keywords: antibodies/cell surface molecules/human/ Neu5Gc/sialic acids

[1]To whom correspondence should be addressed: Tel: +1-858-534-2214; Fax: +1-858-534-5611; e-mail: a1varki@ucsd.edu

Introduction

Human "heterophile" antibodies agglutinating animal erythrocytes were described in the 1920s in patients injected with animal serum, by Hanganutziu (1924) and Deicher (1926). These are since referred to as Hanganutziu–Deicher (HD) antibodies (Higashi et al. 1977). Such antibodies were later detected in patients who had never received animal sera, including those with melanomas and various carcinomas (reviewed in Malykh et al. 2001), as well as under certain inflammatory or infectious conditions (Beer 1936; Morito et al. 1982). These studies defined HD antibodies by hemagglutination of bovine, ovine, or equine erythrocytes, or by ELISA assays against crude "bovine high-molecular-weight glycoproteins" from such erythrocytes (Beer 1936; Nakarai et al. 1990; Malykh et al. 2001). It was subsequently noted that HD antibodies were directed against the common mammalian sialic acid (Sia) called *N*-glycolylneuraminic acid (Neu5Gc) (Higashi et al. 1977; Merrick et al. 1978; Nishimaki et al. 1979; Morito et al. 1982; Takiguchi et al. 1984; Mukuria et al. 1986) and to be of potential prognostic significance in melanomas (Nakarai et al. 1990). The heterophile "Paul–Bunnel" antibodies of infectious mononucleosis were also shown to bind Neu5Gc-containing glycoconjugates (Golaszewska et al. 2003).

Sialic acids are acidic sugars with a 9-carbon backbone, typically located at the outermost end of glycan chains of vertebrate cell surface and secreted glycoconjugates (Angata and Varki 2002). *N*-Acetylneuraminic acid (Neu5Ac) and its hydroxylated form, Neu5Gc, are the two major Sia forms in most mammals. The evolutionary lineage leading to humans suffered an inactivating mutation in the *CMAH* gene responsible for biosynthesis of CMP-Neu5Gc, the sialylation donor for biosynthesis of Neu5Gc-containing molecules (Varki 2001). Despite this irreversible human mutation, Neu5Gc accumulation has long been reported in many human tumors (Malykh et al. 2001) and has more recently been found in smaller amounts in normal adult human tissues (Tangvoranuntakul et al. 2003).

We recently generated a human-like *Cmah* mutation in mice and found no detectable Neu5Gc in normal or malignant tissues (Hedlund et al. 2007), effectively ruling out a second pathway for Neu5Gc biosynthesis. However, Neu5Gc is metabolically incorporated into human cells from animal-derived components of culture media (Tangvoranuntakul et al. 2003). A similar accumulation takes place in the human body, arising from dietary sources such as red meat and milk products (Tangvoranuntakul et al. 2003). There are mechanistic explanations for this metabolic incorporation

(Bardor et al. 2005) and for enhanced uptake and incorporation into malignant cells (Nguyen et al. 2005; Yin et al. 2006).

One defined cancer-associated HD antigen is the ganglioside GM3(Neu5Gc) (NeuGcα2-3Galβ1-4G1cβ1-1'Ceramide) (Higashi et al. 1977; Asaoka et al. 1992; Malykh et al. 2001). Further studies using this ganglioside as an ELISA target claimed a very low frequency of HD antibodies ($< 1–2\%$) in normal subjects (Merrick et al. 1978; Morito et al. 1982, 1986; Higashihara et al. 1991). However, arbitrary cutoffs for background subtraction were used, apparently assuming that normal humans *should* be negative (Halbert et al. 1982; Nakarai et al. 1990; Iznaga et al. 1996). Using a novel and more precise method, we recently reported that all normal humans actually have detectable circulating anti-Neu5Gc antibodies. Alpha-linked Neu5Ac and Neu5Gc (with a single oxygen atom difference) were used as targets for ELISA detection of antibodies in human serum (Tangvoranuntakul et al. 2003; Nguyen et al. 2005). The difference in binding to the two epitopes was designated as Neu5Gc-specific antibodies. Another group reached the same conclusion using different methods (Zhu and Hurst 2002). We then showed that these antibodies induce complement-mediated cytotoxicity on Neu5Gc-fed human leukemic cells (Nguyen et al. 2005).

All of these studies assumed that HD antibodies were solely detecting Neu5Gc. However, Neu5Gc-containing glycans are diverse and presented on many glycoconjugates, including glycolipids as well as N-linked and O-linked chains of glycoproteins. Also, this monosaccharide cannot by itself fill the binding site (paratope) of an antibody, which can accommodate several linked monosaccharides (Padlan and Kabat 1988; Sigurskjold and Bundle 1992; Lee et al. 2006; Houliston et al. 2007). Furthermore, structural diversity results from Neu5Gc modification such as 9-O-acetylation, sialyl linkage difference, and complexities of an underlying glycan structure (Angata and Varki 2002). Thus, there are actually many potential Neu5Gc-containing epitopes to be recognized by human anti-Neu5Gc antibodies. In this regard, there are a few monoclonal antibodies that selectively recognize epitopes including Neu5Gc. In every case, the single oxygen atom difference between Neu5Gc and Neu5Ac is crucial for antibody recognition, but the epitopes recognized are extended ones, involving the underlying glycan chain (Miyake et al. 1988; Tai et al. 1988). In contrast, monoclonal antibodies that recognize the same underlying glycan in the presence of either Neu5Ac or Neu5Gc are rare.

All these data led us to hypothesize that the human immunological response to Neu5Gc is actually polyclonal and diverse and involves recognition by multiple antibodies with different specificities. After almost 100 years since "HD" antibodies were discovered, we can now obtain or synthesize defined glycans in sufficient quantities to specifically address this hypothesis. Such data are also needed for better understanding their significance in human cancer and other diseases, and in evaluating their therapeutic potential. Here, we explore and characterize a diverse anti-Neu5Gc response in normal human sera, purify the antibodies using newly developed affinity methods, and show that they can bind back to human carcinomas that have accumulated Neu5Gc in vivo. Potential implications of these antibodies for pathogenesis of Neu5Gc-accumulating human cancers are discussed.

Results

Lack of correlation between anti-Neu5Gc-antibodies measured against two different ELISA target antigens

As normal human sera rarely contained heterophile hemagglutinating antibodies, it was originally assumed that such sera must not have anti-Neu5Gc antibodies. Thus, when ganglioside GM3(Neu5Gc) was identified as one of the "HD" antigens in cancer (Higashi et al. 1977; Asaoka et al. 1992; Malykh et al. 2001) and used as an epitope for ELISA assays, any antibody binding signal obtained using normal serum was presumed to be "nonspecific" and was subtracted as "background" (Iznaga et al. 1996). A more precise way to detect anti-Neu5Gc antibodies was to use otherwise identical ELISA targets bearing Neu5Ac instead of Neu5Gc as background, i.e., subtracting a control differing only by the single oxygen atom missing in humans. Using this criterion, we recently detected antibodies in normal humans against synthetic Neu5Gc α-linked to polyacrylamide (Neu5Gcα-PAA), subtracting Neu5Acα-PAA binding as the background (Tangvoranuntakul et al. 2003; Nguyen et al. 2005). To compare levels of such antibodies with those against GM3(Neu5Gc), we developed a sensitive ELISA method in which conditions were optimized to allow testing of both targets on the same plate, using matching Neu5Ac-containing glycoconjugates as controls, and serial dilutions of human IgG for quantification of the antibodies. All 16 random normal human sera tested had detectable levels of IgG antibodies against both epitopes. However, there was little correlation between the two sets of values (Figure 1A), indicating that they represent different and/or overlapping sets of antibodies.

Anti-Neu5Gc antibodies in normal humans are of broad and variable specificities

Antibody–glycan contacts tend to occur in shallow cavities, and the binding region can accommodate interaction with parts of several monosaccharide residues of a glycan (Padlan and Kabat 1988; Sigurskjold and Bundle 1992; Lee et al. 2006; Houliston et al. 2007). Furthermore, Neu5Gc is a terminal Sia of both glycolipids and glycoproteins, commonly attached to underlying sugars via an α2-3-linkage to Gal, an α2-6-linkage to Gal and GalNAc, or an α2-8 linkage to another sialic acid. Moreover, hydroxyl groups at positions C4, C7, C8, and C9 can be modified in nature, commonly by O-acetyl esters (Angata and Varki 2002). Furthermore, the underlying glycan and/or the attachment of the glycan to the protein or lipid could be an epitope determinant. Additional complexity occurs in density of such epitopes, e.g., while some glycoproteins contain only one glycosylation site, mucins contain numerous sialyl-O-glycans. Given the wide diversity in naturally occurring Neu5Gc-containing structures, we therefore hypothesized that the human antibody response against Neu5Gc is far more diverse than previously recognized and that the above assays might detect only a fraction of the antibody response against this nonhuman molecule.

To address this possibility, we analyzed the human immunological response to Neu5Gc using a variety of glycans with a terminal Neu5Gc, but otherwise similar in structure to natural human antigens bearing Neu5Ac. These included purified glycolipids, glycans conjugated to PAA, and α2-3- or α2-6-linked sialoside pairs that were chemoenzymatically synthesized using a one-pot three-enzyme system and conjugated to human serum albumin (HSA) (Yu, Chokhawala, et al. 2006; Yu et al. 2007).
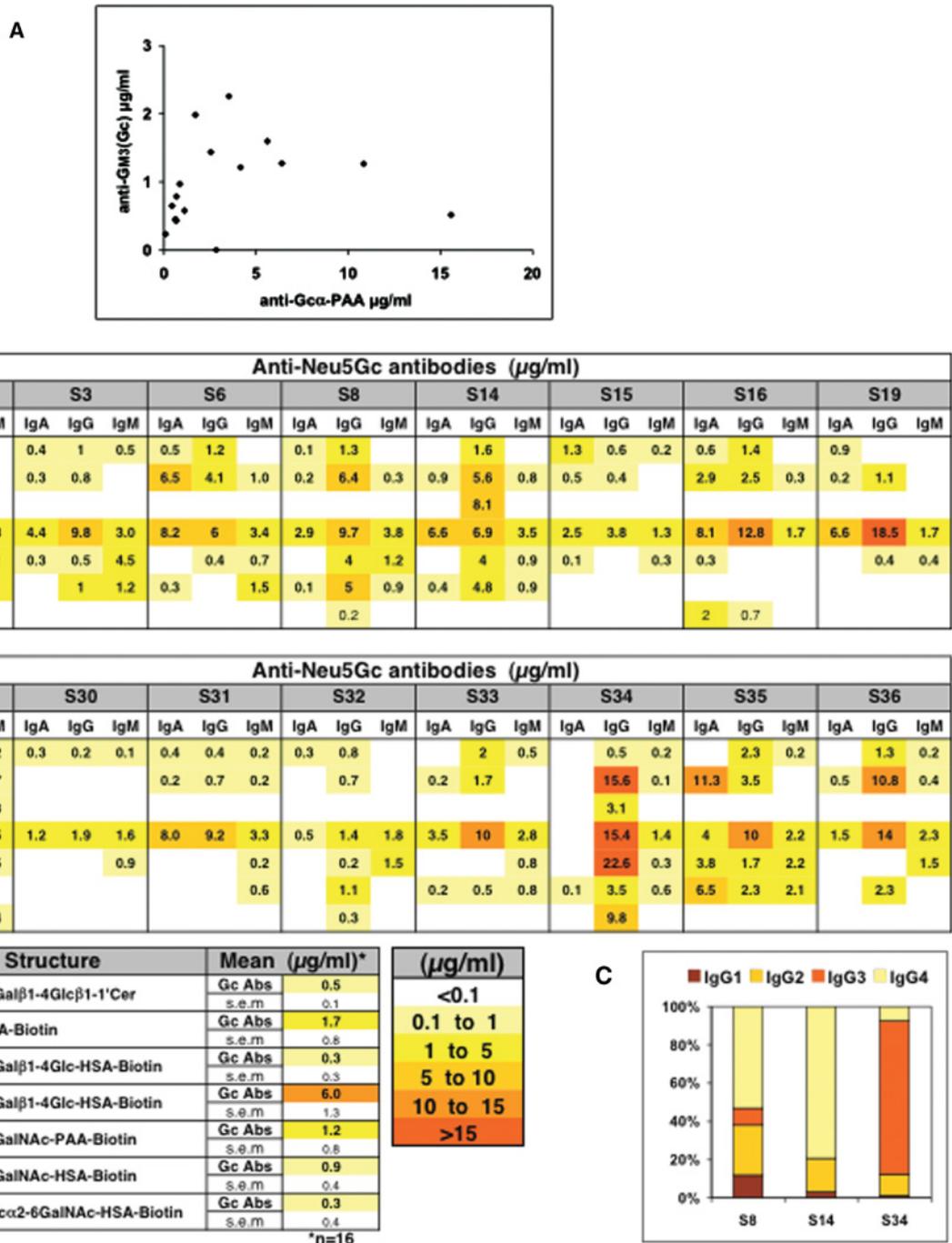
Fig. 1. Anti-Neu5Gc antibodies in normal humans are of broad and variable specificities. (**A**) Levels of anti-Neu5Gcα-PAA and anti-GM3(Neu5Gc) IgG antibodies in normal human sera ($n = 16$) were quantified in triplicates by ELISA using Neu5Ac-glycans for background subtraction. Each dot represents a pair of values for a single human. (**B**) Levels of anti-Neu5Gc IgA, IgG and IgM antibodies against various synthetic Neu5Gc-containing glycans were quantified as above. The lower panel shows the mean values and standard errors (SEM) for each glycoconjugate tested. (**C**) Levels of anti-Neu5Gc IgG-subclasses (IgG$_1$, IgG$_2$, IgG$_3$, or IgG$_4$) against Neu5Gcα-PAA in three of the human sera. Absorbance values were converted into percent. One representative experiment of three.

Panel B — Anti-Neu5Gc antibodies (µg/ml)

Human Serum	S2			S3			S6			S8			S14			S15			S16			S19		
Glycoconjugate	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM
GM3(Gc)	0.6	0.6		0.4	1	0.5	0.5	1.2		0.1	1.3			1.6		1.3	0.6	0.2	0.6	1.4		0.9		
Gcα-PAA		1.1		0.3	0.8		6.5	4.1	1.0	0.2	6.4	0.3	0.9	5.6	0.8	0.5	0.4		2.9	2.5	0.3	0.2	1.1	
Gc2-3LacHSA														8.1										
Gc2-6LacHSA	15.3	23	3.3	4.4	9.8	3.0	8.2	6	3.4	2.9	9.7	3.8	6.6	6.9	3.5	2.5	3.8	1.3	8.1	12.8	1.7	6.6	18.5	1.7
Gc2-6GalNAcPAA	0.2	0.7	2.1	0.3	0.5	4.5		0.4	0.7		4	1.2		4	0.9	0.1		0.3	0.3				0.4	0.4
Gc2-6GalNAcHSA	1.3	2.2	2		1	1.2	0.3		1.5	0.1	5	0.9	0.4	4.8	0.9									
9OAcGc2-6GalNAcHSA											0.2								2	0.7				

Human Serum	S26			S30			S31			S32			S33			S34			S35			S36		
Glycoconjugate	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM
GM3(Gc)		0.5	0.2	0.3	0.2	0.1	0.4	0.4	0.2	0.3	0.8			2	0.5		0.5	0.2		2.3	0.2		1.3	0.2
Gcα-PAA		0.6	0.7				0.2	0.7	0.2		0.7		0.2	1.7			15.6	0.1	11.3	3.5		0.5	10.8	0.4
Gc2-3LacHSA			0.8														3.1							
Gc2-6LacHSA	6.1	12.2	4.5	1.2	1.9	1.6	8.0	9.2	3.3	0.5	1.4	1.8	3.5	10	2.8	15.4	1.4		4	10	2.2	1.5	14	2.3
Gc2-6GalNAcPAA		0.9	0.5			0.9		0.2			0.2	1.5			0.8	22.6	0.3		3.8	1.7	2.2			1.5
Gc2-6GalNAcHSA								0.6			1.1		0.2	0.5	0.8		3.5	0.6	6.5	2.3	2.1		2.3	
9OAcGc2-6GalNAcHSA		0.4									0.3						9.8							

Glycoconjugate	Structure	Mean (µg/ml)*	
GM3(Gc)	Neu5Gcα2-3Galβ1-4Glcβ1-1'Cer	Gc Abs	0.5
		s.e.m	0.1
Gcα-PAA	Neu5Gcα-PAA-Biotin	Gc Abs	1.7
		s.e.m	0.8
Gc2-3LacHSA	Neu5Gcα2-3Galβ1-4Glc-HSA-Biotin	Gc Abs	0.3
		s.e.m	0.3
Gc2-6LacHSA	Neu5Gcα2-6Galβ1-4Glc-HSA-Biotin	Gc Abs	6.0
		s.e.m	1.3
Gc2-6GalNAcPAA	Neu5Gcα2-6GalNAc-PAA-Biotin	Gc Abs	1.2
		s.e.m	0.8
Gc2-6GalNAcHSA	Neu5Gcα2-6GalNAc-HSA-Biotin	Gc Abs	0.9
		s.e.m	0.4
9OAcGc2-6GalNAcHSA	9OAc-Neu5Gcα2-6GalNAc-HSA-Biotin	Gc Abs	0.3
		s.e.m	0.4

*n=16

Legend (µg/ml): <0.1 | 0.1 to 1 | 1 to 5 | 5 to 10 | 10 to 15 | >15

Matched Neu5Ac-containing glycans were used as background controls. After testing a range of serum dilutions, we chose 1:100 as optimal for quantitative detection of multiple Ig isotypes in all sera. Each assay plate also incorporated standards and internal controls.

The same normal human sera were tested against an array of seven such sialyl-glycan pairs on the same plate (Figure 1B). While anti-Neu5Gc IgD antibodies were not detected, variable and complex patterns of IgM, IgA, and IgG reactivities were seen. The most prominent reactivity was typically against the Neu5Gcα2-6Galβ1-4Glc-HSA-conjugate (Neu5Gcα2-6Lac-HSA) with generally lower or no reactivity with Neu5Gcα2-3Lac-HSA or 9-O-acetyl-Neu5Gcα2-6GalNAc-HSA (Figure 1B). Notably, while some sera showed

an overall high reactivity to most glycans (Figure 1B, IgG: S8, S14, S34; IgA: S35), others showed low or no reactivity at all (Figure 1B, S30).

The scaffold to which the glycan was attached also influenced reactivity. For example, with Neu5Gcα2-6GalNAc attached to either PAA or HSA, there was limited correlation between the levels detected (Figure 1B). Since the PAA is ~30 kDa and carries 7–9 glycans and the HSA is 66.5 kDa and contains 9–12 glycans, the latter may present the glycans in a more widely spaced fashion. This fits with previous studies, in which two mouse monoclonal antibodies against Sialyl-Tn (anti-Neu5Acα2-6GalNAc) differentially bound to the same antigen, depending upon the extent of clustering (Zhang et al. 1995). Anti-Neu5Gc antibodies could also be detected by ELISA using natural Neu5Gc-containing antigens, including porcine and bovine submaxillary mucins (PSM and BSM, respectively) that carry small *O*-glycans with different levels of Neu5Gc and different amounts of 9-*O*-acetylation (see *Materials and methods* for details). These natural molecules that contain Neu5Gc were also detected by anti-Neu5Gc antibodies in normal human sera and showed high interindividual variability (data not shown). Furthermore, reactivity was altered when BSM was pretreated with base to remove 9-*O*-acetyl groups from the Neu5Gc residues (data not shown).

Further complexity is reflected by variable IgG subclasses. IgG$_1$ and IgG$_3$ are the main human IgG subclasses involved in complement activation and opsonization, and in protein antigen recognition, while IgG$_2$ (IgG$_3$ in mice) is associated with glycan antigen recognition (Siber et al. 1980). While two tested sera (S8 and S14) had mainly IgG$_2$ and IgG$_4$ with lower levels of IgG$_1$, IgG$_3$ predominated in the third (S34, Figure 1C). Taken together, all data confirm our hypothesis of a diverse and polyclonal response against Neu5Gc, with marked variations in anti-Neu5Gc antibody profiles amongst normal individuals.

Inhibition assays confirm the key role of Neu5Gc in the epitopes recognized

To affirm that Neu5Gc is the immunodominant epitope, we used an ELISA inhibition assay (EIA), inhibiting reactivity of anti-Neu5Gc antibodies against various Neu5Gc glycoconjugates precoated on ELISA plates, using free Neu5Gc or Neu5Gc-containing glycoproteins (Figure 2). To increase the effective concentration of free α-Neu5Gc, we synthesized 2-*O*-methyl-α-Neu5Gc (Neu5Gc2Me), in which the ring structure and the α2-linkage are fixed, thereby maintaining the ring form and preventing mutarotation of the α-D-sialic acid anomer to the favorable β-D-sialic acid (equilibrium of 20:1 in favor of the β-anomer) (Gottschalk 1960; Achyuthan KE and Achyuthan AM 2001). Testing human serum S34, which showed the highest and the most variable anti-Neu5Gc reactivities, we found that the binding of IgG to Neu5Gcα2-6Lac-HSA was completely inhibited by 2.5 mM Neu5Gc2Me, but not at all by Neu5Ac2Me, which differs by only one oxygen atom from Neu5Gc2Me (Figure 2A). Several other sera were similarly tested against various Neu5Gc-glycoconjugates for both IgG and IgA binding, yielding similar inhibition (data not shown). In general, low titer anti-Neu5Gc responses required low concentrations of Neu5Gc2Me for complete inhibition (e.g., human serum S35 anti-Neu5Gc IgA against Neu5Gcα2-6Lac-HSA was completely inhibited by 0.04 mM Neu5Gc2Me; data not shown). Likewise, when
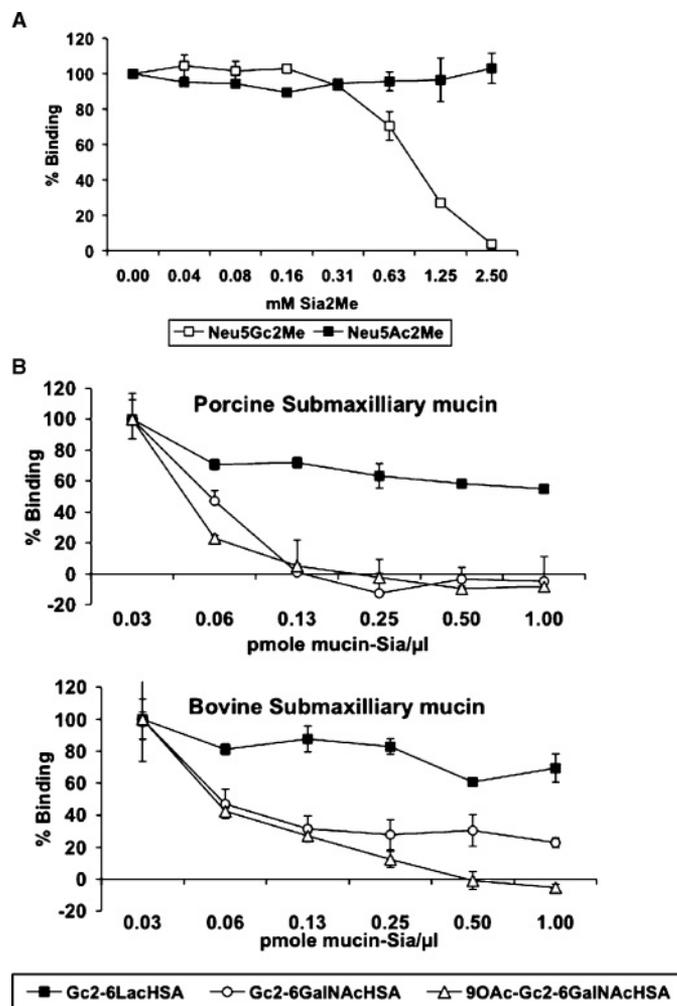
Fig. 2. Human serum anti-Neu5Gc reactivity is selectively inhibited by 2-*O*-methyl-α-Neu5Gc and by Neu5Gc-bound to glycoproteins. (**A**) ELISA inhibition assay (EIA) of anti-Neu5Gcα2-6Lac- IgG antibodies in human serum S34 with either 2-*O*-methyl-α-Neu5Gc (Neu5Gc2Me) or Neu5Ac2Me. Data are expressed in percent relative to binding of serum without inhibitor and represent mean ± SD of three independent experiments. (**B**) EIA of anti-Neu5Gc IgG antibodies in human serum S34 recognizing Neu5Gcα2-6Lac- or Neu5Gcα2-6GalNAc and 9-*O*-Ac-Neu5Gcα2-6GalNAc (Sialyl-Tn(Neu5Gc) and its *O*-acetylated derivative, respectively) with natural Sialyl-Tn(Neu5Gc)-containing mucins (sialo-glycoproteins). Data are expressed in percent relative to binding of serum without inhibitor to the respective Neu5Gc-glycoconjugate (100%) and show mean ± SD of triplicates representative of two (Neu5Gcα2-6Lac) or three (Neu5Gcα2-6GalNAc and 9-*O*-Ac-Neu5Gcα2-6GalNAc) independent experiments.

human serum was preincubated with the Neu5Gc-containing glycoproteins PSM or BSM (that carry small *O*-glycans with different levels of Neu5Gc and different amounts of 9-*O*-acetylation), binding to plates precoated with chemically synthesized Neu5Gcα2-6GalNAc or its *O*-acetylated derivative was completely inhibited (Figure 2B). In contrast, PSM and BSM only moderately (~20%) inhibited binding of anti-Neu5Gc antibodies specific for Neu5Gcα2-6Lac-HSA. This agrees with the fact that both mucins are rich with Sialyl-Tn structures but lack Siaα2-6Lac. Taken together, the inhibition studies confirm the key role of Neu5Gc in the epitopes recognized and further support our hypothesis of a diverse and polyclonal response against

Neu5Gc, with marked variations in anti-Neu5Gc antibody profiles amongst normal individuals.

Levels of anti-Neu5Gc antibodies in healthy human sera are high

There are some well-known examples of anti-glycan antibodies in normal human serum, e.g., the anti-blood group (A or B) and "anti-Gal" (anti-Galα1-3Galβ1-4GlcNAc) antibodies. In both instances, the antigens are not expressed on host cells and the antibodies are thought to result from antigenic stimulation by gastrointestinal bacteria (Springer and Horton 1969; Galili et al. 1988). These antibodies are a major cause for rejection of allotransplants and xenotransplants (Galili 2006; Milland and Sandrin 2006). Anti-Gal antibodies in human sera are considered the most abundant, produced by ∼1% of human circulating B-lymphocytes, while ∼0.25% of them produce anti-A or anti-B (Galili et al. 1993). In contrast to these antibodies that recognize highly defined glycan epitopes, we have shown that the anti-Neu5Gc response is against a spectrum of glycan antigens, with the most prominent being against Neu5Gcα2-6Lac and Neu5Gcα2-6GalNAc. Although these two structures share the same sialic acid linkage, their underlying structure makes them very different epitopes (Figure 1B).

We compared levels of IgA, IgG, and IgM antibodies against the most reactive epitope Neu5Gcα2-6Lac (Figure 1B), to the levels of anti-B blood group and anti-Gal antibodies. This was done by a similar ELISA assay using synthetic α-Gal and B-blood group linked to polyacrylamide (Galα1-3Galβ1-4GlcNAc-PAA and Fucα1-2(Galα1-3)Galβ-PAA, respectively) studying six of the previously tested human sera, three of blood type A (S6, S8 and S34) and three of blood type O (S14, S35, S36). This analysis revealed that anti-Neu5Gcα2-6Lac antibody response is quite high and similar in total amount to the anti-Gal response, representing a mean of ∼20 μg/mL antibodies in human sera (the sum of IgA, IgG, and IgM; Figure 3) and higher than that of the anti-B blood group antibodies (∼10 μg/mL, Figure 3). Thus, the anti-Neu5Gc antibody response in normal human sera can be high, in a range comparable to that of anti-Gal antibodies.

Anti-Neu5Gc antibodies can be affinity-purified from human serum

We next developed a sequential affinity column procedure for purification of anti-Neu5Gc antibodies from human serum. Aliquots of two human sera with high anti-Neu5Gc IgG or IgA reactivity (S34 and S35, respectively, Figure 1B) were precleared through a column of immobilized human serum sialoglycoproteins which carry ∼2.3 μmole/mL Neu5Ac but lack Neu5Gc. The flow-through was applied to a column of immobilized chimpanzee serum sialo-glycoproteins (which carry Neu5Ac and ∼0.3 μmole/mL Neu5Gc, but are otherwise very similar). Bound proteins were sequentially eluted with 5 mM glucuronic acid, 0.5 mM 2-O-methyl-α-Neu5Gc (Neu5Gc2Me), 2 mM Neu5Gc2Me, and finally, a weak acid. Almost all antibodies with anti-Neu5Gc reactivity bound to the chimpanzee serum column and were eluted by Neu5Gc2Me, with no more reactivity detected in the acid elution (Figure 4A). Western blot analysis confirmed the presence of IgA, IgG, as well as IgM in the mixture of purified anti-Neu5Gc antibodies (Figure 4B). The higher band in IgG (see human serum S34;
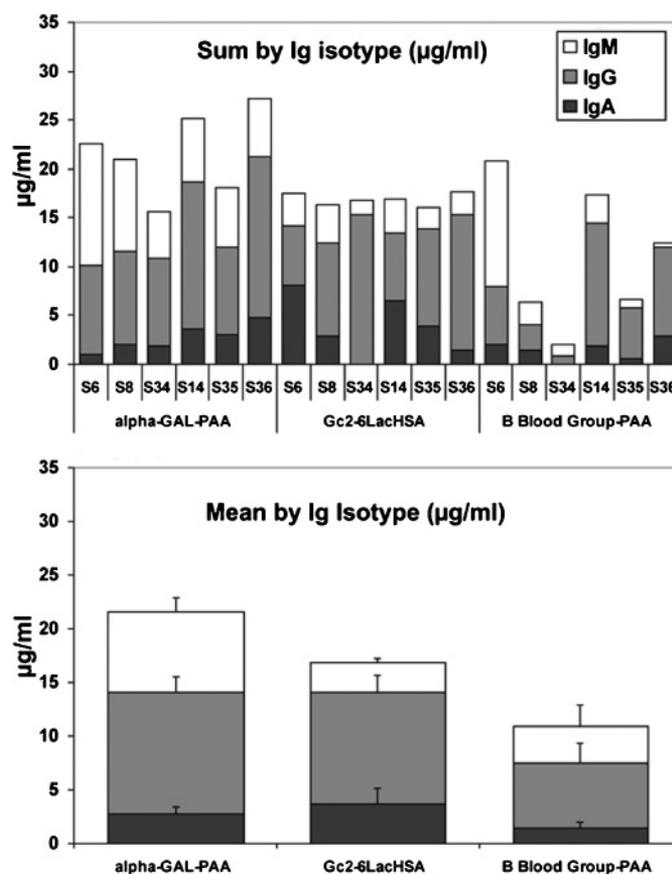

Fig. 3. Total amount of human anti-Neu5Gc antibodies can be quite high. Levels of two common anti-glycan antibodies, anti-Gal and anti-B Blood group, were determined in six of the previously tested human sera by ELISA and compared to the levels of anti-Neu5Gcα2-6Lac- antibodies (as described in Figure 1B). Three of the sera were of blood type A (human serum S6, S8 and S34) and three of blood type O (human serum S14, S35, S36). The lower panel shows the mean values and standard errors (SEM) for each Ig isotype tested against the various glycoconjugates.

Figure 1C) represents IgG$_3$, which is consistent with earlier analysis. The eluted fractions were pooled, Neu5Gc2Me removed with simultaneous biotinylation, and then retested by ELISA against Neu5Gcα-PAA (Figure 4C). These purified antibodies could also bind various Neu5Gc-glycoconjugates, confirming the previously observed specificity and the efficiency of affinity purification (compare Figure 4D with Figure 1B).

Specificity of the purified antibodies confirmed by immunohistochemistry analysis of mouse tissues

We recently reported generation of a 'humanized' mouse model in which the CMP-Neu5Ac hydroxylase gene responsible for the generation of Neu5Gc was specifically inactivated ($Cmah^{-/-}$). In contrast to the wild-type mice that express Neu5Gc, Neu5Gc is completely absent from all tissues in these mice (Hedlund et al. 2007). Immunohistochemistry of mouse embryo tissues using the purified biotinylated human anti-Neu5Gc antibodies gave prominent staining in wild-type but not $Cmah^{-/-}$ mouse tissues (Figure 5A). This confirms our in vitro findings, showing that the purified human antibodies can recognize native Neu5Gc-containing antigens on tissues.
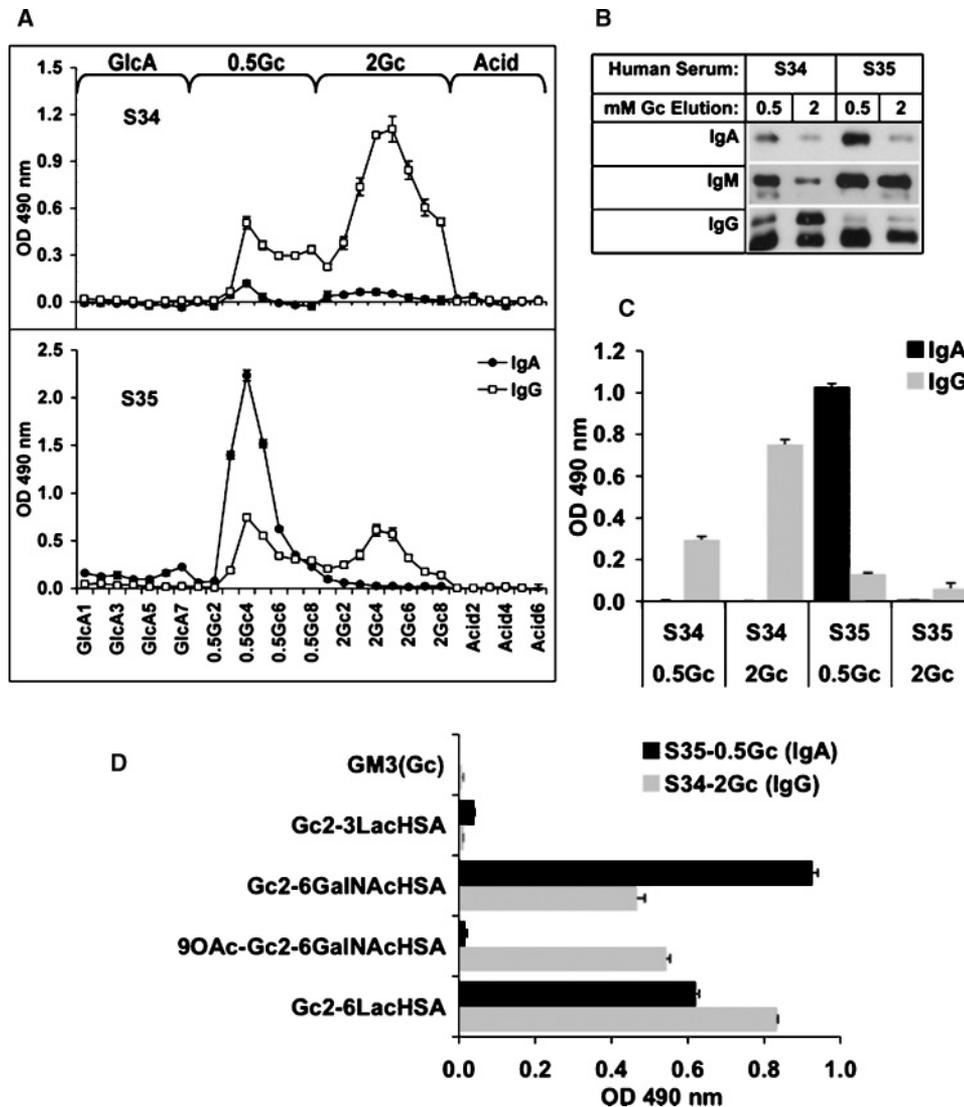
Fig. 4. Affinity purification of different isotypes of anti-Neu5Gc antibodies from human serum. (**A**) Anti-Neu5Gc antibodies were affinity-purified from human serum S34 and S35 over sequential columns of immobilized human and chimpanzee serum sialoglycoproteins. Antibodies binding to the latter column were eluted sequentially with 5 mM glucuronic acid (GlcA), 0.5 mM Neu5Gc2Me (0.5Gc), 2 mM Neu5Gc2Me (2Gc), and finally with a weak acid (acid). Fractions were collected and analyzed by ELISA against Neu5Gcα-PAA. Data are representative of two independent experiments with each human serum and show mean ± SD of triplicates. The 0.5Gc and 2Gc elution fractions of human sera S34 and S35 were pooled separately, and analyzed side by side by Western blot using HRP-conjugated anti-human IgA, IgG, or IgM (**B**) and by ELISA against Neu5Gcα-PAA (**C**) or complex-Neu5Gc-glycans (**D**). Data are representative of four (**B**) and two (**C**, **D**) independent experiments and show mean ± SD.

Purified human-anti-Neu5Gc antibodies react with human tumors

Neu5Gc is found in small amounts in normal adult human tissues including epithelia (Tangvoranuntakul et al. 2003) and many human epithelial tumors are reported to accumulate large amounts of Neu5Gc (Malykh et al. 2001). Having purified antibodies from human serum with confirmed specificity to various chemically synthesized or natural Neu5Gc-containing epitopes, we asked whether these antibodies could bind to human tumors containing Neu5Gc. Immunohistochemistry using these purified antibodies showed prominent staining of human breast carcinoma tissue sections (Figure 5B). The section is actually from a sample showing malignant as well as nonmalignant areas. While the human anti-Neu5Gc antibody binds well to malignant cells in the center, there is very little staining of the adjacent nonmalignant tissue. Staining was abolished upon pretreatment of tissues with sialidase and inhibited by absorption with Neu5Gc-containing chimpanzee serum glycoproteins (Figure 5B). Thus, the purified human antibodies bind to Neu5Gc-containing epitopes on native human cancer tissues. Implications for such specific recognition of human anti-Neu5Gc antibodies to human malignant tissues are further discussed below.

Discussion

Being foreign, Neu5Gc is immunogenic in humans (Tangvoranuntakul et al. 2003; Nguyen et al. 2005). Recent
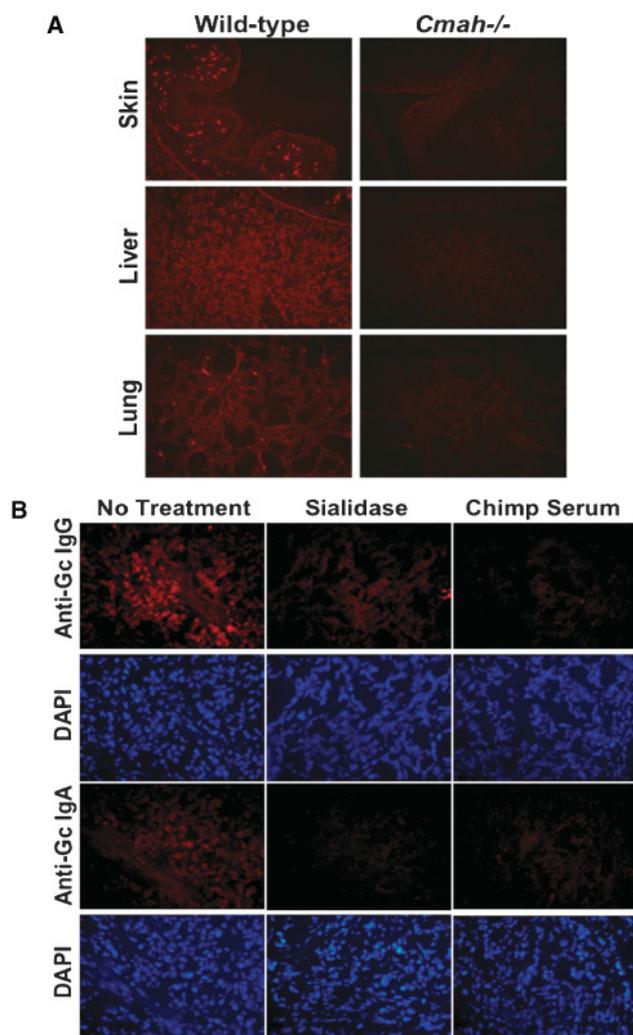
Fig. 5. Purified human-anti-Neu5Gc antibodies specifically bind to Neu5Gc-containing tissues from wild-type mice and to human tumors. (**A**) Purified human anti-Neu5Gc antibodies bind to wild type but not to 'humanized' mouse tissues. Immunohistochemistry analysis on representative frozen tissues of wild-type and $Cmah^{-/-}$ mouse embryos using biotinylated-anti-Neu5Gc antibodies purified from human serum (S34-2Gc). (**B**) Purified human anti-Neu5Gc IgA or IgG antibodies bind to human breast cancer tissues. Immunohistochemistry analysis on representative frozen tissues of human breast carcinoma using biotinylated-Neu5Gc specific IgA or IgG antibodies purified from human serum (S35-0.5Gc and S34-2Gc, respectively). Staining is prominent in tumor cells and is Neu5Gc-dependent, as it is decreased upon either treatment of cancer tissues with sialidase or by inhibition using chimpanzee serum, which contains multiple Neu5Gc-glycoproteins. Data are representative of at least three independent experiments using purified antibodies from S34 and S35.

advances in combinatorial carbohydrate synthesis allowed us to generate chemically defined oligosaccharides that could be used to discriminate the binding of antibodies that recognized either Neu5Gc- or Neu5Ac-terminated glycoconjugates. Here, we have shown that all normal humans studied have a variable range of circulating antibodies specific for Neu5Gc, spanning most of the human immunoglubulins isotypes, namely IgA, IgM, and IgG. Moreover, despite the fact that most anti-carbohydrate antibodies in humans are thought to be of the less reactive IgG_2 subclass (IgG_3 in mice) (Siber et al. 1980), we were able to

show that in some human sera anti-Neu5Gc antibodies can be of the IgG_3 subclass, which is normally involved in complement activation and opsonization (Siber et al. 1980).

A common problem with many commercially available anti-carbohydrate antibodies is low specificity and cross-reactivity with other glycans as was recently demonstrated by a high-throughput carbohydrate microarray on several such antibodies (Manimala et al. 2007). Here, we were able to affinity purify anti-Neu5Gc antibodies from the actual human sera that were analyzed using a novel method. The specificity of the purified antibodies was ensured by adding two additional steps before the antigen elution. In the first step, the human sera were precleared from most nonrelated antibodies by loading over a Neu5Ac-rich column (human sialoglycoproteins), followed by loading of the run-through over a Neu5Gc-rich column (chimpanzee sialoglycoproteins). In the second step, we used a carboxylate-containing monosaccharide (glucuronic acid) to remove antibodies that could bind to the Neu5Gc-rich column mostly due to charge. Finally, the effective concentration of the antigen that was used for the elution was increased by using 2-O-methyl-α-Neu5Gc (Neu5Gc2Me) rather than Neu5Gc. The specificity of the purified antibodies was then confirmed by testing them over the chemically defined Neu5Gc-glycoconjugates showing no cross-reactivity with their corresponding Neu5Ac-glycoconjugates. Some of these approaches could be adopted in the future to increase the specificity and reduce the cross-reactivity of other anti-carbohydrate antibodies, including those in which the antigen contains a sialic acid, for example, Sialyl-Tn and Sialyl-Le^x. Such reliable antibodies are necessary for various basic and clinical carbohydrate-based applications including cancer biopsies that allow the identification of patients with antigen-positive tumors (Manimala et al. 2007).

The highest levels of anti-Neu5Gc antibodies in all of the tested human sera are reactive to the structure Neu5Gcα2-6Lac and are comparable to the level of the anti-Gal antibodies, which are well known with respect to xenotransplantation and as a potential tool in cancer immunotherapy (Galili 2005). While the human immunological response to Neu5Gc and to α-Gal in humans shares some features, the former has some unique characteristics, as summarized in Table I. The α-Gal epitope is a single defined sequence (Galα1-3Galβ1-4GlcNAc-R) without any known structural varations and is specifically absent in cells of humans, apes, and Old World Monkeys, due to inactivation of the enzyme α1-3galactosyltransferase (*GGTA1* gene) (Galili 2005). Furthermore, all these species produce a natural "anti-Gal" antibody (thought to be ~1% of circulating immunoglobulins in humans) specific for the α-Gal epitope. While this sets up a major immunologic barrier to xenotransplantation (Galili 2005), it is not of known relevance to intrinsic human tumors, as metabolic incorporation of ingested α-Gal cannot occur. In contrast, we have shown that human anti-Neu5Gc antibodies are complex, polyclonal, and variable amongst individuals and have potential epitopes within the human body, due to metabolic incorporation of Neu5Gc from dietary sources, principally red meats and milk products. Therefore, Neu5Gc is in fact a unique novel type of antigen modifying the surface of human cells in many diverse ways. While it is recognized as "self" by the cellular biochemical machinery, it is recognized as "non-self" by the immune system and hence becomes a 'xeno-auto-antigen'. Here, we lay the foundation for studying the potential role of anti-Neu5Gc antibodies under human

Table I. Comparison between α-Gal and Neu5Gc in humans

Feature	α-Gal	Neu5Gc
Biosynthesis in human cells	Not synthesized due to inactivation of *GGTA1* gene (α1-3galactosyltransferase)	Not synthesized due to inactivation of *CMAH* gene (CMP-Neu5Ac hydroxylase)
Biosynthesis in other Old World primates	Absent, same as in humans	*CMAH* present and active
Metabolic incorporation	Not applicable. Ingested α-Gal would be released as free galactose in the gut	Can be metabolically incorporated from Neu5Gc-rich dietary sources
Presence of epitopes in human tissues	Not applicable, as metabolic incorporation cannot occur	Incorporated into normal human epithelial and endothelial cells, and enriched in human tumors
Target epitope(s) for human antibodies	Defined epitope (Galα1-3Galβ1-4GlcNAc-R)	Many epitopes, because of varied modifications, linkages, and underlying glycans, on glycoproteins or glycolipids
Humoral response in humans	Anti-Gal IgA, IgG, IgM	Anti-Neu5Gc IgA, IgG, IgM
Level of antibodies in humans	High in all individuals	Highly variable levels and Ig isotypes, can be at comparable levels to α-Gal antibodies
Potential role in diseases related to chronic inflammation	Not applicable, antigen not present in human tissues	Likely, antigens and antibodies can be present in the same human

See the text for discussion.

conditions associated with chronic inflammation, such as cancer, atherosclerosis, and autoimmune disease. In this regard, it is also interesting that such diseases appear to be rare in nonhuman primates (Olson and Varki 2003), which naturally express Neu5Gc, but not anti-Neu5Gc antibodies.

Altered glycosylation is a universal phenotype of cancer (Kobata and Amano 2005; Brockhausen 2006). One specific form of altered sialylation is accumulation of Neu5Gc in the face of an anti-Neu5Gc antibody response (Malykh et al. 2001). The presence of Neu5Gc in several human cancers has been documented by various immunological and chemical techniques (Malykh et al. 2001). Although earlier studies claimed the absence of Neu5Gc from normal human tissues, we showed that it is also present in smaller amounts in normal human epithelial and endothelial cells in vivo (Tangvoranuntakul et al. 2003). Furthermore, we recently demonstrated that mice with a human-like defect in the *CMAH* gene had no detectable Neu5Gc (Hedlund et al. 2007), effectively ruling out an alternate mammalian pathway for synthesis. This paradox is explained by our finding that humans can metabolically incorporate Neu5Gc via oral intake (Tangvoranuntakul et al. 2003). We have therefore suggested that the well-known epidemiological association of human cancers with consumption of red meat and milk (which happen to be the richest dietary sources of Neu5Gc) (Rose et al. 1986; Norat et al. 2002; Lewin et al. 2006) might be related to this unusual metabolic accumulation. Here, we have demonstrated another required component for this hypothesis – circulating antibodies that can recognize Neu5Gc on human tissues and can potentially generate chronic inflammation. To our knowledge, this is the first example wherein a nonhuman molecule becomes metabolically and covalently incorporated onto human cell surfaces, even in the face of an immune response against it. Further studies are needed to firmly establish a link between Neu5Gc expression in tumors and anti-Neu5Gc in the pathogenesis of carcinomas. In this regard, we have recently been able to successfully model this situation of Neu5Gc-positive syngeneic tumors in Cmah null mice (Hedlund et al. unpublished).

In work by others, an unnatural sialic acid was artificially loaded into tumor cells, and artificially produced antibodies were then used to kill it (Chefalo et al. 2006). However, in contrast to this experiment, Neu5Gc is naturally accumulating in human tumors at higher levels than in normal cells, in the face of circulating anti-Neu5Gc antibodies. This leads to the conclusion that the tumor cells that are best at accumulating Neu5Gc are the ones that survive the best inside the intact human, and thus have escaped immunosurveillance against those tumors.

There are many examples in which chronic inflammation seems to play a role in carcinoma incidence and progression, e.g., colon cancer in ulcerative colitis, pancreatic cancer following chronic pancreatitis, hepatocellular carcinomas following chronic hepatitis, and gastric cancer following *Helicobacter* infection (Mantovani 2005; De Visser et al. 2006). Recent work by others has demonstrated that antibody–antigen reactions in premalignant or malignant tissues can also set up chronic inflammation supporting tumor progression, even while further boosting antibody responses (Mantovani 2005; De Visser et al. 2006). In addition to that, others have suggested that a moderate anti-tumor immune reaction is optimal for tumor growth and that many de novo tumors, rather than being inhibited, are probably dependent, at least early in their progression, upon eliciting an immune reaction (Prehn and Prehn 1987; Prehn 2006). Here, we have shown that anti-Neu5Gc antibodies purified from normal human serum can specifically bind to human carcinoma sections. Additionally, we had previously shown that anti-Neu5Gc antibodies in normal human sera can deposit complement on Neu5Gc-expressing human cells in vitro (Nguyen et al. 2005). We suggest that while high levels of such antibodies could be cytotoxic, weak inflammation arising from the combination of Neu5Gc and anti-Neu5Gc antibodies could instead help promote carcinogenesis and/or facilitate tumor progression. Interestingly, increased α2-6-linked sialic acids on tumor cells are associated with cancer progression via other mechanisms, and the Neu5Gc version might further contribute by increasing expression of immunoreactive epitopes recognized by circulating antibodies specific for α2-6-linked Neu5Gc, which are of the highest levels (reactive to the structure Neu5Gcα2-6Lac). In further support of our hypothesis, the consumption of red meat (the richest known dietary source of Neu5Gc) is associated with higher risk of various cancers (Rose et al. 1986; Norat et al. 2002; Lewin et al. 2006)—in contrast to vegetarianism, which decreases risk (Key et al. 1999). As discussed earlier, enhanced incorporation of Neu5Gc in naturally occurring carcinomas suggests that there is a benefit to the tumor during its evolution.

While advanced cancer is most often fatal, early stage disease can have a good survival rate (Stewart et al. 2004). Thus, blood-based biomarkers have been aggressively sought (Bast et al. 2005; Ludwig and Weinstein 2005). We are currently studying some of these anti-Neu5Gc antibody responses as potential markers for cancer detection and prognosis.

In summary, our new findings have readdressed century-old ideas regarding the levels and roles of "HD"-(anti-Neu5Gc)-antibodies. Current developments have opened up new avenues for glycan synthesis, and we have used these tools to characterize the overall immunological response to Neu5Gc in normal humans. We showed that this response is polyclonal, with diverse and individualistic recognition patterns. In addition to their potential as novel serum markers for early detection of cancer and cancer risk, specific anti-Neu5Gc antibodies might be exploited for targeted-immunotherapy in the future. Further studies are needed to see if these antibodies indeed have a pathogenic role in cancer or in other unexplained human diseases associated with chronic inflammation and an apparent autoimmune component, such as atherosclerosis, rheumatoid arthritis, etc.

Materials and methods

Human serum samples

Normal human sera are obtained in this lab from apparently healthy adult male and female blood donors of various ethnic backgrounds from the University of California, San Diego School of Medicine students and personnel, with approval from the Institutional Review Board. Written, informed consent was obtained in advance from the volunteers. To assure confidentiality, samples were de-identified and coded with an S, then aliquoted and stored at $-80°C$. For this particular study, we randomly chose 16 of these 35 donors, based only on their current availability to re-donate samples for this study.

Antibodies

Purified human Immunoglobulins (Igs) of IgA, IgG, and IgM were from Jackson ImmunoResearch Laboratories (West Grove, CA) and purified human IgD from Bethyl Laboratories (Montgomery, TX). Horseradish peroxidase (HRP)-conjugated goat-anti-human Igs were from the sources indicated: HRP-anti-human IgA (Calbiochem, San Diego, CA), HRP-anti-human IgM (Kirkegaard and Perry Laboratories, Gaithersburg, MD), HRP-anti-human IgG (Bio-Rad, Hercules, CA), and HRP-anti-human IgD (Bethyl Laboratories, Montgomery, TX). HRP-mouse-anti-human IgG_1, IgG_2, IgG_3, or IgG_4 were from Zymed Laboratories (San Francisco, CA) and Cy3-Streptavidin from Jackson ImmunoResearch Laboratories).

Glycoconjugates

All polyacrylamide (PAA)-glycoconjugates were from GlycoTech (Gaithersburg, MD); GM3(Neu5Ac) was purchased from Sigma (St. Louis, MO), and GM3(Neu5Gc) was purified from horse red blood cells as described (Kunihiko 1965). PSM or BSM were prepared as described (Tangvoranuntakul et al. 2003). The α2-3- and α2-6-linked sialyl-glycan pairs (in which the only difference is Neu5Ac versus Neu5Gc) were synthesized using an efficient one-pot three-enzyme chemoenzymatic synthetic system (Yu, Chokhawala, et al. 2006) containing an

E. coli K-12 sialic acid aldolase, an *N. meningitidis* CMP-sialic acid synthetase, and a sialyltransferase selected from a *Pasteurella multocida* multifunctional sialyltransferase (Yu et al. 2005) or a *Photobacterium damsela* α2-6-sialyltransferase (Yu, Huang et al. 2006). These sialosides were then conjugated to biotinylated HSA at pH 7.5 using a homobifunctional adipic acid *p*-nitrophenyl ester (Wu et al. 2004; Yu et al. 2007) as an efficient linker, then purified and confirmed by matrix assisted laser desorption ionization – time of flight (MALDI-TOF) (the biotin moiety was not used in the current studies). The sialic acid content and the purity of all glycoconjugates were further confirmed by 1,2-diamino-4,5-methylenedioxybenzene–high performance liquid chromatography (DMB–HPLC) as described herein.

DMB-HPLC analysis of sialo-glycoconjugates

The sialic acid (Sia) contents of all the glycoconjugates used were analyzed for their type (NeuAc/Neu5Gc +/− *O*-acetylation), quantity and purity. Sias were released from glycoconjugates by acid hydrolysis using 2 M acetic acid hydrolysis for 3 h at 80°C. Free Sias were then derivatized with 1,2-diamino-4,5-methylenedioxybenzene (DMB) and analyzed by fluorescence detection by reverse-phase HPLC (DMB-HPLC). Quantification of Sias was done by comparison with known quantities of DMB-derivatized Neu5Ac (Hara et al. 1986).

The natural sialoglycoproteins we used were PSM and BSM containing multiple small *O*-glycans with different levels of Neu5Gc, underlying structures and *O*-acetylation of Neu5Ac and Neu5Gc (Neu5Gc content, PSM > BSM; Sialyl-Tn (Neu5Ac/Neu5Gcα2-6GalNAc) content, BSM > PSM; and Sia *O*-acetylation content, BSM ≫ PSM). About 40% of PSM glycans consist of Siaα2-6GalNAc-Ser/Thr (Sialyl-Tn), along with other structures derived from the incomplete biosynthesis of a branched A blood group-like structure (GalNAcα1-3(Fucα1-2/3)Galβ1-3(Siaα2-6)GalNAc-Ser/Thr) (Gerken and Jentoft 1987). DMB-HPLC analysis showed that the sialic acid content was 84% Neu5Gc, 7.5% Neu5Ac, and 8.5% *O*-acetylated Sia (data not shown). The glycans of BSM contain 53% Sialyl-Tn and 22% Siaα2-6(GlcNAcβ1-3)GalNAc (as well as T antigen, Galβ1-3GalNAc-Ser/Thr, and Tn antigen) (Golubovic and Bojic-Trbojevic 2006). DMB-HPLC analysis showed that the Sia content is 20% Neu5Gc, 22% Neu5Ac, and 58% *O*-acetylated Sia, as analyzed by DMB-HPLC (data not shown).

Preparation of 2-O-methyl-α-Neu5Ac and 2-O-methyl-α-Neu5Gc

2-*O*-methyl-α-Neu5Ac and 2-*O*-methyl-α-Neu5Gc were prepared from Neu5Ac and Neu5Gc, respectively. In order to prepare 2-*O*-methyl-α-Neu5Ac, Neu5Ac was acetylated with acetic anhydride (Ac_2O) in pyridine and treated with diazomethane (CH_2N_2) to afford per-acetylated Neu5Ac methyl ester in 86% yield for two steps. The peracetylated Neu5Ac methyl ester was treated with acetyl chloride in acetic acid (AcCl/HOAc) to give acetochloroneuraminic methyl ester. The crude product was dissolved in anhydrous methanol and the mixture was kept at room temperature for 1 h (Kononov and Magnusson 1998). The crude acetylated methyl glycoside was treated with NaOMe/MeOH, 1 M NaOH, neutralized with H^+ resin, and purified with silica gel chromatography to give α-methyl Neu5Ac

in 42% yield and elimination product (Neu5Ac2en) in 28% yield in four steps. ^{1}H NMR (600 MHz, D$_2$O): 3.92–3.87 (m, 2 H), 3.82 (t, 1 H, J = 10.2 Hz), 3.74–3.68 (m, 2 H), 3.66 (dd, 1 H, J = 6.0 Hz and 12.0 Hz), 3.60 (dd, 1 H, J = 1.2 Hz and 9.0 Hz), 3.36 (s, 3 H), 2.73 (dd, 1 H, J = 4.8 Hz and 12.6 Hz), 2.05 (s, 3 H), 1.65 (t, 1 H, J = 12.6 Hz); ^{13}C NMR (150 MHz, D$_2$O): 175.25, 173.56, 100.88, 72.74, 71.84, 68.42, 68.36, 62.82, 52.11, 51.78, 40.25, 22.29.

The 2-O-methyl-α-Neu5Gc was obtained in 37% yield in six steps starting from Neu5Gc (Yu et al. 2004) using the similar method as described above for the preparation of α-methyl Neu5Ac. ^{1}H NMR (600 MHz, D$_2$O): 4.18 (s, 2 H), 3.96–3.82 (m, 5 H), 3.69 (dd, 1 H, J = 6.0 Hz and 11.4 Hz), 3.64 (d. 1 H, J = 9.0 Hz), 3.40 (s, 3 H), 2.78 (dd, 1 H, J = 4.8 Hz and 12.6 Hz), 1.70 (t, 1 H, J = 12.6 Hz); ^{13}C NMR (150 MHz, D$_2$O): 176.00, 173.41, 100.75, 75.52, 71.81, 68.36, 68.07, 62.83, 61.18, 59.54, 51.79, 40.20.

De-O-acetylation of bovine submaxillary gland mucins

Mucins were incubated with 0.1 M NaOH for 30 min at 37°C and subsequently neutralized with HCl. This removes base-labile O-acetyl esters but leaves the rest of the glycan intact (Diaz et al. 1989).

Detection of anti-Neu5Gc, anti-Gal, and anti-B blood group antibodies in human sera by ELISA

Human serum anti-Neu5Gc antibodies were detected by ELISA as previously described (Nguyen et al. 2005) with several modifications. We used 96-well microtiter plates (Costar, Cornning, NY) coated in triplicates with optimized buffers and saturating concentrations of various pairs of Neu5Ac- and Neu5Gc-glycoconjugates as follows: GM3 ganglioside in methanol (50 pmole Sia/well); Siaα-PAA-Biotin (250 ng/well) and Sia2-3/6Lac-HSA-Biotin (1 μg/well) in a 50 mM sodium carbonate-bicarbonate buffer, pH 9.5. Pairs of Siaα2-6GalNAc-HSA-Biotin (1 μg/well), 9-O-Ac-Siaα2-6GalNAc-HSA-Biotin (1 μg/well), PSM (100 pmole Sia/well), and BSM (190 pmole Sia/well) in a 50 mM sodium phosphate buffer, pH 7.5 (an optimal pH for preserving O-acetylation of Sia). Each plate was also coated with serial dilutions of purified human Igs (10–0.3 ng/well) of IgA, IgG, IgM, or IgD in the same buffer as the respective glycoconjugates that were coated to the plate (carbonate or phosphate buffers). Methanol was allowed to evaporate completely for 4 h at room temperature (RT) and plates were incubated overnight at 4°C. Wells were blocked for 2 h at RT with 1% ovalbumin (Grade V, Sigma, free of Neu5Gc) in PBS, followed by incubation with serum samples diluted 1:100 in the same blocking solution for 4 h at RT. The plates were washed three times with PBS containing 0.1% Tween (PBST) and subsequently incubated for 1 h at RT with HRP-conjugated goat-anti-human Igs diluted in PBS (anti-human IgA, 1:4000; anti-human IgM, 1:4000; anti-human IgG, 1:7000; anti-human IgD, 1:3000). After washing three times with PBST, wells were developed with an O-phenylenediamine in a citrate-PO$_4$ buffer, pH 5.5, and absorbance was measured at a 490 nm wavelength on a SpectraMax 250 (Molecular Devices, Sunnyvale, CA). Neu5Gc-specific antibody levels were defined by subtracting the readings obtained with the Neu5Ac-glycoconjugates from the readings obtained using the respective Neu5Gc-glycoconjugates (in the case of PSM and BSM, the background subtracted was that of triplicate wells containing only the respective buffer). Absorbance values were quantified into μg/mL using the standard dilution curves of the corresponding purified human Ig.

Several human sera were analyzed for the levels of the various anti-Neu5Gc IgG subclasses, which were detected by a similar ELISA. All human sera were tested for the four subclasses of IgG in triplicates on the same plate. Briefly, plates were coated with Neu5Acα-PAA or Neu5Gcα-PAA (250 ng/well) in a 50 mM sodium carbonate-bicarbonate buffer, pH 9.5. Human sera were used at 1:50 dilutions, antibodies were detected by HRP-conjugated mouse-anti-human IgG$_1$, IgG$_2$, IgG$_3$, or IgG$_4$ diluted 1:300 in PBS and the plate was developed as described above.

For detection of human serum anti-Gal or anti-B blood group antibodies, a similar ELISA method was used, except that the plates were coated with synthetic α-Gal or B-blood group antigens linked to polyacrylamide (Galα1-3Galβ1-4GlcNAc-PAA and Fucα1-2(Galα1-3)Galβ1-PAA, respectively) at 250 ng/well in a 50 mM sodium carbonate-bicarbonate buffer, pH 9.5).

ELISA inhibition assays (EIA) with free Neu5Gcα-methyl glycoside or natural Neu5Gc-containing glycocproteins

EIA of anti-Neu5Gcα2-6Lac IgG antibodies in human serum S34 was conducted as described above, except that the serum was diluted 1:100 in the blocking solution (1% ovalbumin in PBS) in the presence or absence of either Neu5Gcα-methyl glycoside (Neu5Gc2Me) or Neu5Ac2Me, and preincubated on ice for 2 h, then applied to plates coated in triplicates with Neu5Gcα2-6LacHSA (serum with/without inhibitor) and Neu5Acα2-6LacHSA (serum without inhibitor; used as the background control). The binding of anti-Neu5Gc antibodies was detected using HRP-conjugated anti-human IgG and developed as described. In addition, we conducted EIA of anti-Neu5Gc IgG antibodies in human serum S34 recognizing Neu5Gcα2-6Lac, Neu5Gcα2-6GalNAc, and 9-O-Ac-Neu5Gcα2-6GalNAc with natural Sialyl-Tn(Neu5Gc)-containing mucins (sialo-glycoproteins). Assays were conducted as described above, except that serum was preincubated in the presence or absence of either PSM or BSM and applied to plates coated with the various Neu5Gc-glycoconjugates. In every case, the corresponding Neu5Ac-glycoconjugate was used as the background control.

Affinity purification of anti-Neu5Gc antibodies from human serum

Anti-Neu5Gc antibodies were purified from human serum on sequential affinity columns with immobilized human or chimpanzee sialo-glycoproteins (rich with Neu5Ac or Neu5Gc, respectively). All steps were conducted at 4°C. Briefly, 9 mL of 1:13 diluted human or chimpanzee serum in 0.1 M MOPS, pH 7.5 (∼5 mg protein/mL), was coupled overnight with gentle shaking to 6 mL of packed Affi-Gel 15 beads (BioRad; prewashed with 50 mM sodium acetate, pH 5.5). Remaining active esters were blocked for 1 h with 0.1 M ethanolamine–HCl, pH 8, the suspension was poured into a glass column, and washed with three column volumes of 50 mM sodium acetate, pH 5.5. Columns were calibrated by sequential washing with five column volumes of PBS, 0.1 M citric acid, pH 3, and finally with PBS containing 0.1% sodium azide, and held at 4°C until use. Antibody sources (5–8 mL of human sera S34 or S35) were incubated at 56°C for 30 min to inactivate complement, then

diluted 1:1 in PBS and passed through a 0.2 μm filter. The filtered human serum was passed over the column of immobilized human serum sialo-glycoproteins, and the flow-through material was applied to a column of immobilized chimpanzee serum sialo-glycoproteins. Subsequently, bound proteins were eluted from the chimpanzee sialo-glycoproteins column sequentially with 5 mM glucuronic acid (GlcA) in PBS (pH 7.4) followed by a two column volume wash with PBS, then eluted further with 0.5 mM Neu5Gc2Me in PBS, 2 mM Neu5Gc2Me in PBS, and finally with 0.1 M citric acid, pH 3. Fractions of 1 mL each were collected from the GlcA or Neu5Gc2Me elutions, while 2 mL fractions were collected from the acid elution, directly into vials containing 0.5 mL of 2 M Tris–HCl, pH 8. The collected fractions were analyzed by Western blot and ELISA. Subsequently, fractions 1 through 8 of either the 0.5 mM or 2 mM Neu5Gc2Me/PBS elutions were pooled, and filtered through Amicon Ultra-15 centrifugal filter unit (10 NMWL; Millipore, Billerica, MA) to remove free sialic acids; the retentate was collected and biotinylated using the EZ-Link Micro Sulfo-NHS-Biotinylation Kit (Pierce, Rockford, IL) according to the manufacturer's instructions.

Western blot for human Igs

Samples (purified anti-Neu5Gc antibodies) were denatured by a SDS sample buffer (×6), 5 μL/lane were loaded on 10% polyacrylamide mini gels (Bio-Rad, Hercules, CA), then electrophoresed and electrotransferred to nitrocellulose membranes. Membranes were then blocked for 20 min at RT with 5% nonfat dry milk (Bio-Rad) in Tris-buffered saline containing 0.1% Tween (TBST), next incubated for 1 h at RT with HRP-goat-anti-human Igs diluted in TBST containing 2% nonfat dry milk (anti-human IgA, 1:8000; anti-human IgM, 1:8000; anti-human IgG, 1:10,000), washed four times for 5 min with 2% nonfat dry milk in TBST, and then washed with TBST for 10 min. Subsequently, proteins were visualized by chemiluminescence detection (Pierce), followed by exposure to Kodak BioMax XAR film for 5–30 s.

Immunohistochemistry

Tissue samples of human breast carcinomas were obtained from the NCI funded the Cooperative Human Tissue Network, with approval of institutional review boards of the University of California, San Diego, CA. No patient identifier information accompanied the human samples. Human tissues and tissue samples from *Cmah⁻/⁻* or wild-type mice were frozen in optimum cutting temperature (OCT) compound (Sakura, Finetek, Sa Torrance, CA) and archived at −80°C. Prior to immunostaining, cryosections were air-dried, washed with PBST, and endogenous biotin and avidin activities sequentially quenched (Vector Laboratories, Burlingame, CA). Next, nonspecific binding sites were blocked with 0.5% fish gelatin (Sigma, St. Louis, MO) in PBST for 30 min, followed by fixation with 10% neutral buffered formalin (NBF; Fisher Scientific, Pittsburgh, PA) for 20 min. Sections were then incubated at RT for 1 h with optimally diluted biotinylated anti-Neu5Gc antibodies purified from human serum (S34 or S35) diluted 1:3 in the same blocking solution (0.5% fish gelatin in PBST). After washing with PBST, Cy3-streptavidin (Jackson, ImmunoResearch, West Grove, PA) diluted 1:500 in PBST was applied and following further washing, the slides were mounted with an aqueous mounting media

containing DAPI (Vector Laboratories, Burlingame, CA), and viewed with a Zeiss Axiolab (Zeiss, Germany) microscope. Images were captured with a Sony DKC-5000 digital system (Sony, Japan) and formatted using Adobe Photoshop (Adobe, San Jose, CA). Control human breast cancer tissues were treated with 250 milliunits of *Arthrobacter urafaciens* sialidase (AUS; EY Laboratories, San Mateo, CA) in 100 mM sodium acetate, pH 5.5, for 2.5 h at 37°C prior to fixation, then immunostained and viewed as above. As an additional control for human breast cancer staining, the purified and biotinylated human-anti-Neu5Gc antibodies were diluted 1:3 in the blocking solution containing 10% chimpanzee plasma (a rich source of Neu5Gc-containing glycoproteins; obtained as described (Nguyen et al. 2006)), incubated for 2 h at 4°C, then applied over tissue sections and incubated at RT for 1 h. Subsequently, tissues were immunostained and viewed as above.

Conflict of interest statement

None declared.

Abbreviations

α-Gal epitope, Galα1-3Galβ1-4GlcNAc-R; BSM, bovine submaxillary gland mucins; *CMAH*, CMP-Neu5Ac hydroxylase gene; DMB, 1,2-diamino-4,5-methylenedioxybenzene; GlcA, glucuronic acid; GM3(Neu5Gc), NeuGcα2-3Galβ1-4G1cβ1-1′Ceramide; HD, Hanganutziu—Deicher; HPLC, high performance liquid chromatography; HRP, Horseradish peroxidase; Igs, immunoglobulins; Neu5Ac, *N*-acetylneuraminic acid; Neu5Ac2Me, Neu5Ac α-methyl 2-*O*-glycoside; Neu5Gc, *N*-glycolylneuraminic acid; Neu5Gc2Me, Neu5Gc α-methyl 2-*O*-glycoside; PAA, polyacrylamide; PSM, porcine submaxillary gland mucins..

References

Achyuthan KE, Achyuthan AM. 2001. Comparative enzymology, biochemistry and pathophysiology of human exo-alpha-sialidases (neuraminidases). *Comp Biochem Physiol B Biochem Mol Biol*. 129:29–64.

Angata T, Varki A. 2002. Chemical diversity in the sialic acids and related alpha-keto acids: An evolutionary perspective. *Chem Rev*. 102:439–469.

Asaoka H, Nishinaka S, Wakamiya N, Matsuda H, Murata M. 1992. Two chicken monoclonal antibodies specific for heterophil Hanganutziu-Deicher antigens. *Immunol Lett*. 32:91–96.

Bardor M, Nguyen DH, Diaz S, Varki A. 2005. Mechanism of uptake and incorporation of the non-human sialic acid *N*-glycolylneuraminic acid into human cells. *J Biol Chem*. 280:4228–4237.

Bast RCJ, Lilja H, Urban N, Rimm DL, Fritsche H, Gray J, Veltri R, Klee G, Allen A, Kim N, et al. 2005. Translational crossroads for biomarkers. *Clin Cancer Res*. 11:6103–6108.

Beer P. 1936. The heterophile antibodies in infectious mononucloesis and after injection of serum. *J Clin Invest*. 15:591–599.

Brockhausen I. 2006. Mucin-type *O*-glycans in human colon and breast cancer: Glycodynamics and functions. *EMBO Rep*. 7:599–604.

Chefalo P, Pan Y, Nagy N, Guo Z, Harding CV. 2006. Efficient metabolic engineering of GM3 on tumor cells by *N*-phenylacetyl-D-mannosamine. *Biochemistry*. 45:3733–3739.

Deicher H. 1926. Über die Erzeugung heterospezifischer Hämagglutinine durch Injektion artfremden. *Serums Z Hyg*. 106:561–579.

De Visser KE, Eichten A, Coussens LM. 2006. Paradoxical roles of the immune system during cancer development. *Nat Rev Cancer*. 6:24–37.

Diaz S, Higa HH, Hayes BK, Varki A. 1989. *O*-Acetylation and de-*O*-acetylation of sialic acids. 7- and 9-*O*-acetylation of alpha2,6-linked sialic acids on endogenous *N*-linked glycans in rat liver Golgi vesicles. *J Biol Chem*. 264:19416–19426.

Galili U. 2005. The alpha-gal epitope and the anti-Gal antibody in xenotransplantation and in cancer immunotherapy. *Immunol Cell Biol*. 83:674–686.

Galili U. 2006. Xenotransplantation and ABO incompatible transplantation: The similarities they share. *Transfus Apher Sci*. 35:45–58.

Galili U, Anaraki F, Thall A, Hill-Black C, Radic M. 1993. One percent of human circulating B lymphocytes are capable of producing the natural anti-Gal antibody. *Blood*. 82:2485–2493.

Galili U, Mandrell RE, Hamadeh RM, Shohet SB, Griffiss JM. 1988. Interaction between human natural anti-alpha-galactosyl immunoglobulin G and bacteria of the human flora. *Infect Immun*. 56:1730–1737.

Gerken TA, Jentoft N. 1987. Structure and dynamics of porcine submaxillary mucin as determined by natural abundance carbon-13 NMR spectroscopy. *Biochemistry*. 26:4689–4699.

Golaszewska E, Kurowska E, Duk M, Koscielak J. 2003. Paul-Bunnell antigen and a possible mechanism of formation of heterophile antibodies in patients with infectious mononucleosis. *Acta Biochim Pol*. 50:1205–1211.

Golubovic SJ, Bojic-Trbojevic ZT. 2006. Human carcinoma-associated and salivary mucins detected by anti-bovine submaxillary mucin antibodies. *Biochemistry (Mosc)*. 71(Suppl 1):S9–S17.

Gottschalk A. 1960. *The Chemistry and Biology of Sialic Acids and Related Substances*. Cambridge: Cambridge University Press.

Halbert SP, Anken M, Henle W, Golubjatnikov R. 1982. Detection of infectious mononucleosis heterophil antibody by a rapid, standardized enzyme-linked immunosorbent assay procedure. *J Clin Microbiol*. 15:610–616.

Hanganutziu M. 1924. Hémagglutinines hétérogénétiques après injection de sérum de cheval. *C R Séances Soc Biol*. 91:1457–1459.

Hara S, Yamaguchi M, Takemori Y, Nakamura M, Ohkura Y. 1986. Highly sensitive determination of *N*-acetyl- and *N*-glycolylneuraminic acids in human serum and urine and rat serum by reversed-phase liquid chromatography with fluorescence detection. *J Chromatogr*. 377:111–119.

Hedlund M, Tangvoranuntakul P, Takematsu H, Long JM, Housley GD, Kozutsumi Y, Suzuki A, Wynshaw-Boris A, Ryan AF, Gallo RL, et al. 2007. *N*-Glycolylneuraminic acid deficiency in mice: Implications for human biology and evolution. *Mol Cell Biol*. 27:4340–4346.

Higashi H, Naiki M, Matuo S, Okouchi K. 1977. Antigen of "serum sickness" type of heterophile antibodies in human sera: Indentification as gangliosides with *N*-glycolylneuraminic acid. *Biochem Biophys Res Commun*. 79:388–395.

Higashihara T, Takeshima T, Anzai M, Tomioka M, Matsumoto K, Nishida K, Kitamura Y, Okinaga K, Naiki M. 1991. Survey of Hanganutziu and Deicher antibodies in operated patients. *Int Arch Allergy Appl Immunol*. 95:231–235.

Houliston RS, Yuki N, Hirama T, Khieu NH, Brisson JR, Gilbert M, Jarrell HC. 2007. Recognition characteristics of monoclonal antibodies that are cross-reactive with gangliosides and lipooligosaccharide from *Campylobacter jejuni* strains associated with Guillain–Barre and Fisher syndromes. *Biochemistry*. 46:36–44.

Iznaga N, Carr A, Fernández LE, Solozabal J, Núñez G, Perdomo Y, Morales A. 1996. Amplified ELISA to detect autoantibodies to *N*-glycolyl-GM3 ganglioside. *J Clin Lab Immunol*. 48:75–85.

Key TJ, Fraser GE, Thorogood M, Appleby PN, Beral V, Reeves G, Burr ML, Chang-Claude J, Frentzel-Beyme R, Kuzma JW, et al. 1999. Mortality in vegetarians and nonvegetarians: Detailed findings from a collaborative analysis of 5 prospective studies. *Am J Clin Nutr*. 70:516S–524S.

Kobata A, Amano J. 2005. Altered glycosylation of proteins produced by malignant cells, and application for the diagnosis and immunotherapy of tumours. *Immunol Cell Biol*. 83:429–439.

Kononov LO, Magnusson G. 1998. Synthesis of methyl and allyl alpha-glycosides of *N*-acetylneuraminic acid in the absence of added promoter. *Acta Chem Scand*. 52:141–144.

Kunihiko S. 1965. The pattern of mammalian brain gangliosides III: Regional and developmental differences. *J Neurochem*. 12:969–979.

Lee M, Lloyd P, Zhang X, Schallhorn JM, Sugimoto K, Leach AG, Sapiro G, Houk KN. 2006. Shapes of antibody binding sites: Qualitative and quantitative analyses based on a geomorphic classification scheme. *J Org Chem*. 71:5082–5092.

Lewin MH, Bailey N, Bandaletova T, Bowman R, Cross AJ, Pollock J, Shuker DE, Bingham SA. 2006. Red meat enhances the colonic formation of the DNA adduct O6-carboxymethyl guanine: Implications for colorectal cancer risk. *Cancer Res*. 66:1859–1865.

Ludwig JA, Weinstein JN. 2005. Biomarkers in cancer staging, prognosis and treatment selection. *Nat Rev Cancer*. 5:845–856.

Malykh YN, Schauer R, Shaw, L. 2001. *N*-Glycolylneuraminic acid in human tumours. *Biochimie* 83:623–634.

Manimala JC, Roach TA, Li Z, Gildersleeve JC. 2007. High-throughput carbohydrate microarray profiling of 27 antibodies demonstrates widespread specificity problems. *Glycobiology*. 17:17C–23C.

Mantovani A. 2005. Cancer: Inflammation by remote control. *Nature*. 435:752–753.

Merrick JM, Zadarlik K, Milgrom F. 1978. Characterization of the Hanganutziu-Deicher (serum-sickness) antigen as gangliosides containing *N*-glycolylneuraminic acid. *Int Arch Allergy Appl Immunol*. 57:477–480.

Milland J, Sandrin MS. 2006. ABO blood group and related antigens, natural antibodies and transplantation. *Tissue Antigens*. 68:459–466.

Miyake M, Ito M, Hitomi S, Ikeda S, Taki T, Kurata M, Hino A, Miyake N, Kannagi R. 1988. Generation of two murine monoclonal antibodies that can discriminate *N*-glycolyl and *N*-acetyl neuraminic acid residues of GM2 gangliosides. *Cancer Res*. 48:6154–6160.

Morito T, Kano K, Milgrom F. 1982. Hanganutziu-Deicher antibodies in infectious mononucleosis and other diseases. *J Immunol*. 129:2524–2528.

Morito T, Nishimaki T, Masaki M, Yoshida H, Kasukawa R, Nakarai H, Kano K. 1986. Studies on Hanganutziu-Deicher antigens-antibodies: I. Hanganutziu-Deicher antibodies of IgG class in liver diseases. *Int Arch Allergy Appl Immunol*. 81:204–208.

Mukuria CJ, Fujii Y, Kato S, Naiki M. 1986. Specificities of human heterophile Hanganutziu and Deicher (HD) antibodies to glycosphingolipids and a glycoprotein. *J Biochem (Tokyo)*. 100:469–475.

Nakarai H, Chandler PJ, Kano K, Morton DL, Irie RF. 1990. Hanganutziu-Deicher antigen as a possible target for immunotherapy of melanoma. *Int Arch Allergy Appl Immunol*. 91:323–328.

Nguyen DH, Hurtado-Ziola N, Gagneux P, Varki A. 2006. Loss of Siglec expression on T lymphocytes during human evolution. *Proc Natl Acad Sci USA*. 103:7765–7770.

Nguyen DH, Tangvoranuntakul P, Varki A. 2005. Effects of natural human antibodies against a nonhuman sialic acid that metabolically incorporates into activated and malignant immune cells. *J Immunol*. 175:228–236.

Nishimaki T, Kano K, Milgrom F. 1979. Hanganutziu-Deicher antigen and antibody in pathologic sera and tissues. *J Immunol*. 122:2314–2318.

Norat T, Lukanova A, Ferrari P, Riboli E. 2002. Meat consumption and colorectal cancer risk: Dose-response meta-analysis of epidemiological studies. *Int J Cancer*. 98:241–256.

Olson MV, Varki A. 2003. Sequencing the chimpanzee genome: Insights into human evolution and disease. *Nat Rev Genet*. 4:20–28.

Padlan EA, Kabat EA. 1988. Model-building study of the combining sites of two antibodies to alpha(1→6)dextran. *Proc Natl Acad Sci USA*. 85:6885–6889.

Prehn RT. 2006. An adaptive immune reaction may be necessary for cancer development. *Theor Biol Med Model*. 3:6.

Prehn RT, Prehn LM. 1987. The autoimmune nature of cancer. *Cancer Res*. 47:927–932.

Rose DP, Boyar AP, Wynder EL. 1986. International comparisons of mortality rates for cancer of the breast, ovary, prostate, and colon, and per capita food consumption. *Cancer*. 58:2363–2371.

Siber GR, Schur PH, Aisenberg AC, Weitzman SA, Schiffman G. 1980. Correlation between serum IgG-2 concentrations and the antibody response to bacterial polysaccharide antigens. *N Engl J Med*. 303:178–182.

Sigurskjold BW, Bundle DR. 1992. Thermodynamics of oligosaccharide binding to a monoclonal antibody specific for a *Salmonella O*-antigen point to hydrophobic interactions in the binding site. *J Biol Chem*. 267:8371–8376.

Springer GF, Horton RE. 1969. Blood group isoantibody stimulation in man by feeding blood group-active bacteria. *J Clin Invest*. 48:1280–1291.

Stewart SL, King JB, Thompson TD, Friedman C, Wingo PA. 2004. Cancer mortality surveillance—United States, 1990–2000. *Morb Mortal Wkly Rep Surveill Summ*. 53:1–108.

Tai T, Kawashima I, Furukawa K, Lloyd KO. 1988. Monoclonal antibody R24 distinguishes between different *N*-acetyl- and *N*-glycolylneuraminic acid derivatives of ganglioside GD3. *Arch Biochem Biophys*. 260:51–55.

Takiguchi M, Tamura T, Goto M, Kusakawa S, Milgrom F, Kano K. 1984. Immunological studies on Kawasaki disease I: Appearance of Hanganutziu-Deicher antibodies. *Clin Exp Immunol*. 56:345–352.

Tangvoranuntakul P, Gagneux P, Diaz S, Bardor M, Varki N, Varki A, Muchmore E. 2003. Human uptake and incorporation of an immunogenic nonhuman dietary sialic acid. *Proc Natl Acad Sci USA*. 100:12045–12050.

Varki A. 2001. Loss of *N*-glycolylneuraminic acid in humans: Mechanisms, consequences and implications for hominid evolution. *Am J Phys Anthropol*. 44(Suppl 33):54–69.

Wu X, Ling CC, Bundle DR. 2004. A new homobifunctional *p*-nitro phenyl ester coupling reagent for the preparation of neoglycoproteins. *Org Lett*. 6:4407–4410.

Yin J, Hashimoto A, Izawa M, Miyazaki K, Chen GY, Takematsu H, Kozutsumi Y, Suzuki A, Furuhata K, Cheng FL, et al. 2006. Hypoxic culture induces expression of sialin, a sialic acid transporter, and cancer-associated gangliosides containing non-human sialic acid on human cancer cells. *Cancer Res*. 66:2937–2945.

Yu H, Chokhawala HA, Huang S, Chen X. 2006. One-pot three-enzyme chemoenzymatic approach to the synthesis of sialosides containing natural and non-natural functionalities. *Nat Protoc*. 1:2485–2492.

Yu H, Chokhawala H, Karpel R, Yu H, Wu B, Zhang J, Zhang Y, Jia Q, Chen X. 2005. A multifunctional *Pasteurella multocida* sialyltransferase: A powerful tool for the synthesis of sialoside libraries. *J Am Chem Soc*. 127:17618–17619.

Yu H, Chokhawala HA, Varki A, Chen X. 2007. Efficient chemoenzymatic synthesis of biotinylated human serum albumin-sialoglycoside conjugates containing *O*-acetylated sialic acids. *Org Biomol Chem*. 5:2458–2463.

Yu H, Huang S, Chokhawala H, Sun M, Zheng H, Chen X. 2006. Highly efficient chemoenzymatic synthesis of naturally occurring and non-natural alpha-2,6-linked sialosides: A *P. damsela* alpha-2,6-sialyltransferase with extremely flexible donor-substrate specificity. *Angew Chem Int Ed Engl*. 45:3938–3944.

Yu H, Yu H, Karpel R, Chen X. 2004. Chemoenzymatic synthesis of CMP-sialic acid derivatives by a one-pot two-enzyme system: Comparison of substrate flexibility of three microbial CMP-sialic acid synthetases. *Bioorg Med Chem*. 12:6427–6435.

Zhang S, Walberg LA, Ogata S, Itzkowitz SH, Koganty RR, Reddish M, Gandhi SS, Longenecker BM, Lloyd KO, Livingston PO. 1995. Immune sera and monoclonal antibodies define two configurations for the sialyl Tn tumor antigen. *Cancer Res*. 55:3364–3368.

Zhu A, Hurst R. 2002. Anti-*N*-glycolylneuraminic acid antibodies identified in healthy human serum. *Xenotransplantation*. 9:376–381.