

# Effects of Natural Human Antibodies against a Nonhuman Sialic Acid That Metabolically Incorporates into Activated and Malignant Immune Cells<sup>1</sup>

Dzung H. Nguyen, Pam Tangvoranuntakul,<sup>2</sup> and Ajit Varki<sup>3</sup>

Humans are genetically incapable of producing the mammalian sialic acid *N*-glycolylneuraminic acid (Neu5Gc), due to an inactivating mutation in the enzyme synthesizing it. Despite this, human cells and tissues appear capable of metabolically incorporating Neu5Gc from exogenous sources, including dietary red meat and dairy products. All normal humans studied are now shown to have circulating Abs against Neu5Gc, with marked differences in isotype levels. The question arises whether such Abs can adversely affect Neu5Gc-expressing human cells or tissues. In this study, we show that although normal human PBMC do not incorporate Neu5Gc during *in vitro* incubation, activated T cells do. Primary human leukemia cells and human leukemic cell lines are even more efficient at incorporation. Human sera containing naturally high levels of anti-Neu5Gc IgG Abs (hereafter abbreviated GcIg) deposited complement on Neu5Gc-expressing leukemic cells and activated T cells, but not on normal cells. The binding of GcIg resulted in complement-mediated cytotoxicity, which was inhibited by heat inactivation. Low anti-Neu5Gc IgG-containing human sera did not mediate any of these effects. Mixed killing assays confirmed the 15-fold selective killing of leukemic cells over PBMC by GcIg following Neu5Gc feeding. This approach could potentially serve as novel way to target malignant cells for death *in vivo* using either natural Abs or anti-Neu5Gc Abs prepared for this purpose. Further studies are needed to determine whether deposition of natural GcIg and complement can also target healthy proliferating immune cells for death *in vivo* following incorporation of dietary Neu5Gc. *The Journal of Immunology*, 2005, 175: 228–236.

Sialic acids (Sias)<sup>4</sup> are a family of nine-carbon acidic monosaccharides mostly derived from *N*-acetylneuraminic acid (Neu5Ac) that are typically found as terminal sugars of glycan chains attached to cell surface glycoproteins and glycolipids, as well as secreted glycoconjugates (1–4). Human cells express predominantly Neu5Ac but not the hydroxylated form, *N*-glycolylneuraminic acid (Neu5Gc), due to a homozygous deletion mutation in the human CMP-Neu5Ac hydroxylase (*CMAH*) gene (5). Other mammals, including our closest relatives, the chimpanzees, have an intact *CMAH* gene and express both Neu5Ac and Neu5Gc (6).

In the 1920s, Hanganutziu and Deicher independently described human immune reactions to therapeutic injections of animal antisera, resulting in the formation of “serum sickness antibodies” (7). Neu5Gc bound to horse serum glycoconjugates was later found to

be the dominant epitope recognized by Hanganutziu-Deicher (HD) Abs, which were typically detected by their ability to agglutinate animal erythrocytes (8, 9). HD Abs and HD Ags were then discovered in the sera and tissues of cancer patients (10). Various human tumors also have been reported previously to express Neu5Gc, including colon carcinomas, retinoblastomas, breast cancers, and melanomas (11–15). Although most of these studies used immunochemical techniques that could have generated false positives, a few used chemical methods to show that human tumors do indeed contain Neu5Gc (16–18). Curiously, Furukawa et al. (19) demonstrated that two Abs that recognize Neu5Gc-bearing gangliosides did not react to freshly isolated cancer tissues but did react to FBS-cultured melanoma and astrocytoma cells, suggesting that the Neu5Gc epitope is derived from the media. Because the human genetic mutation in *CMAH* is irreversible, and no alternate metabolic pathway for producing Neu5Gc has been found, we suggested recently that human tumor Neu5Gc may be derived from dietary sources (11).

We have previously detected Neu5Gc in various human tissues in small amounts (11). A possible explanation is that human cells take up and metabolically incorporate free or bound Neu5Gc. The presence of Neu5Gc in FCS could also explain prior reports of Neu5Gc in cultured human cell lines. Indeed, we have shown that a human colon carcinoma cell line (Caco-2) is incapable of synthesizing Neu5Gc when grown in Neu5Gc-free human serum, but became positive when free Neu5Gc was added to the media (11). More recently, we have also shown that human embryonic stem cells can incorporate Neu5Gc from the special medium and feeder layers used for their culture (20). We have also recently characterized the biochemical pathways by which human cells take up and incorporate Neu5Gc (21). Because Neu5Gc differs from Neu5Ac by only a single oxygen atom, the human enzymes, transporters, and transferases that normally process Neu5Ac apparently

Glycobiology Research and Training Center, and Departments of Medicine and Cellular and Molecular Medicine, University of California at San Diego, La Jolla, CA 92093

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<sup>2</sup> Current address: Silicon Genetics, Redwood City, CA 94063.

<sup>3</sup> Address correspondence and reprint requests to Dr. Ajit Varki, University of California at San Diego, La Jolla, CA 92093. E-mail address: varkiadmin@ucsd.edu

<sup>4</sup> Abbreviations used in this paper: Sia, sialic acid; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; *CMAH*, CMP-Neu5Ac hydroxylase; HD, Hanganutziu-Deicher; AF488, Alexa Fluor 488; GAH, goat anti-human; PAA, polyacrylamide; RT, room temperature; SEB, staphylococcus enterotoxin B; DMB, 1,2-diamino-4,5-methylenedioxybenzene; DiOC, 3,3'-dioctadecyloxycarbocyanine perchlorate; PI, propidium iodide; AML, acute myeloid leukemia; ADCC, Ab-dependent cell-mediated cytotoxicity.

handle exogenous Neu5Gc as if it were a molecule native to human cells. In addition, others have described the processing and incorporation of unnatural Sias into human and bacterial cells (22–25).

Although it was well known that humans develop Abs against Neu5Gc upon receiving injections of animal sera, the presence of such Abs in normal healthy humans was thought to be rare (26–28). However, recent studies by our laboratory and another group have shown that this is not the case. In our initial study, many normal human sera were positive for IgA, IgM, or IgG Abs against Neu5Gc (11). The total levels of IgG varied widely among individuals. Similarly, others found that 85% of healthy human subjects had anti-Neu5Gc Ig activity (29). The significance of such anti-Neu5Gc Abs in apparently healthy humans remains unknown, and their functional capabilities have not been demonstrated.

In this study, we show that normal resting PBMCs do not incorporate exogenous Neu5Gc in vitro. In contrast, activated T cells and leukemic cells efficiently incorporate free Neu5Gc, making them preferred targets for “natural” Neu5Gc-specific IgG. We further demonstrate that natural Neu5Gc Abs can mediate killing of leukemic cells. Such Ab effects demonstrate the possibility that these Abs could potentially be harnessed for antileukemic therapy.

## Materials and Methods

### Cells and reagents

CEM human T leukemic cells (originally isolated from peripheral blood of a 4-year-old female with acute lymphoblastic leukemia) (30) and U937 human histiocytic lymphoma cells (established from pleural effusion malignant cells of a patient with histiocytic lymphoma) (31) were obtained from the American Type Culture Collection and propagated in RPMI 1640 (Invitrogen Life Technologies), supplemented with 10% FCS. K562 human leukemia cells (established from the pleural effusion of a 53-year-old female with chronic myelogenous leukemia) (32) were purchased from American Type Culture Collection and grown in IMDM (Invitrogen Life Technologies), supplemented with 10% FCS. Human PBMCs were isolated from fresh blood of normal volunteers by Ficoll-Paque (Amersham Biosciences) and cultured in RPMI 1640 containing a lot of 5% human serum type AB (Irvine Scientific), tested to be free of natural anti-Neu5Gc Abs (hereafter called RPMI-5HS). Cryopreserved circulating acute leukemia cells from human patients were kindly provided by Dr. E. Ball (University of California at San Diego, La Jolla, CA), thawed, washed in RPMI 1640, and resuspended in RPMI-5HS for Neu5Gc-loading studies. Neu5Gc and Neu5Ac were purchased from Inalco Spa and Pfanstiehl Laboratories, respectively. Annexin V-Alexa Fluor 488 (AF488) conjugate, goat anti-human (GAH) IgG (H + L)-AF488, and the live/dead cell-mediated cytotoxicity kit were from Molecular Probes. FITC-goat anti-chicken IgY was purchased from Southern Biotechnology Associates.

### Neu5Gc cell feeding

Neu5Gc and Neu5Ac were dissolved into RPMI 1640 to give a 30 mM stock solution, and the pH was neutralized with 1 M NaOH before sterilizing with a 0.2- $\mu$ m pore size filter. Before feeding, cell lines were cultured in Neu5Gc-free Neu5Ac-rich RPMI-5HS for at least 4 days to “chase out” any Neu5Gc that may have been incorporated from FCS. As mentioned earlier, the lot of pooled human serum was selected to be very low in natural anti-Neu5Gc Abs. PBMCs were fed with Neu5Gc immediately after isolation from human blood or after several days of incubation in RPMI-5HS. Neu5Gc or Neu5Ac stock solution was added to cells to the desired concentration, usually from 0.3 to 6.0 mM and incubated for the desired amount of time, up to 3 days. Cells were washed twice with PBS to remove nonincorporated Sias before further studies.

### Detection of anti-Neu5Gc Ig in human serum by ELISA

Human serum IgG, IgA, and IgM were detected by ELISA, as described previously (11). Briefly, microtiter plates were coated with Neu5Ac-polyacrylamide (PAA) or Neu5Gc-PAA (GlycoTech) at 500 ng/well in 50 mM sodium carbonate-bicarbonate buffer (pH 9.5) at 4°C overnight. Plates were washed with TBS and blocked with TBST for 2 h at room temperature (RT). Dilutions of human serum (1/50 in TBST) were added in triplicate to the wells and incubated for 4 h at RT. Wells were washed with TBS, and then HRP-conjugated mouse anti-human IgA (Calbiochem), anti-human

IgG (Jackson ImmunoResearch Laboratories), or anti-human IgM (Kirkegaard and Perry Laboratories) diluted in TBST (1/20,000) was added to the wells for 1.5 h at RT. Wells were developed with an *o*-phenylenediamine-containing substrate buffer and measured at an OD<sub>490</sub> wavelength on a SpectraMax 250 (Molecular Devices). Neu5Gc-specific Ab levels were defined by subtracting the readings obtained with the Neu5Ac-PAA target from the readings obtained using a Neu5Gc-PAA target. OD levels were quantified into micrograms per milliliter using standard dilution curves of purified human IgG, IgA, and IgM (Jackson ImmunoResearch Laboratories).

### Activation of T cells

PHA-L or staphylococcus enterotoxin B (SEB) was added to PBMCs ( $2.5 \times 10^6$  cells/ml) at 10  $\mu$ g/ml in RPMI-5HS. Cells were cultured for 3 days at 37°C and then washed extensively with PBS before Sia feeding assays.

### Flow cytometry

Flow cytometry was used to detect cell surface Neu5Gc by Ab staining. Cells ( $0.1$ – $1.0 \times 10^6$ ) were incubated with 0.5–2  $\mu$ g of chicken anti-Neu5Gc IgY (ChGcAb) (11) in 100  $\mu$ l of PBS for 30 min on ice. For IgG binding from human sera with anti-Neu5Gc Abs, cells were incubated with 10% of the serum in question, on PBS for 30 min on ice. Chicken IgY was detected by goat anti-chicken IgY-FITC conjugate (1  $\mu$ g/100  $\mu$ l in PBS) and human IgG with 1  $\mu$ g/100  $\mu$ l GAH IgG-AF488 conjugate in PBS for 30 min on ice. Cells were either resuspended in PBS and analyzed immediately or resuspended in PBS with 1% paraformaldehyde for analysis at a later time. Cellular fluorescence was quantitated on a FACSCalibur (BD Biosciences) using CellQuest software.

### HPLC analysis of Sias

Total cell lysates were treated with 2 M acetic acid for 3 h at 80°C to release Sias from glycoconjugates. Free Sias were then derivatized with 1,2-diamino-4,5-methylenedioxybenzene (DMB) and analyzed by fluorescence detection by reverse-phase HPLC as described previously (33, 34).

### Serum toxicity assay

Measurements of anti-Neu5Gc Ab-induced cell toxicity were performed using live/dead cell-mediated cytotoxicity kit (Molecular Probes), which contains a stock solution of 3,3'-diocetadecyloxycarbocyanine perchlorate (DiOC) to label live cell membranes and propidium iodide (PI) to label dead cells. After Sia feeding, target cells ( $1$ – $5 \times 10^6$ ) were labeled with 1.5  $\mu$ M DiOC in 1 ml of PBS for 30 min at 37°C, washed, and resuspended in RPMI-5HS. Cells ( $1 \times 10^4$ ) were mixed with various concentrations of human serum from donors in a final volume of 100  $\mu$ l, and incubated for 2 h at 37°C. Fifty microliters of 0.3 mM PI in RPMI-5HS was then added, and cells were allowed to incubate an additional 30 min before dilution into PBS and analysis by double fluorescence flow cytometry on a FACSCalibur. Green DiOC fluorescence was measured on FL1 (530  $\pm$  15 nm-bandpass filter), and DNA-bound PI fluorescence was measured on FL3 (630  $\pm$  10 nm-bandpass filter).

### Mixed-target serum toxicity assay

A double-label flow cytometric technique was used to quantitate human serum anti-Neu5Gc Ab-mediated cell killing of mixed CEM cells and PBMCs. Unlabeled PBMCs were mixed with DiOC-labeled CEM cells (from a continuous culture in RPMI-5HS) at a ratio of 2:1 in RPMI-5HS. Free Neu5Gc or Neu5Ac, prepared in RPMI 1640, was added to the cells to a final concentration of 6 mM. Cells were incubated at 37°C for 4 h, washed twice with excess PBS, and then resuspended in RPMI-5HS to a concentration of  $1 \times 10^6$  total cells/ml. Human serum samples no. 14 or no. 2 (10  $\mu$ l) were then added to 90  $\mu$ l of Neu5Gc-fed cell mixtures and incubated for 2 h at 37°C, before the addition of 50  $\mu$ l of 0.3 mM PI in RPMI-5HS. Cells were incubated for an additional 30 min before being diluted into PBS and analyzed by flow cytometry.

### Statistical analysis

Results were analyzed using a paired Student's *t* test, with  $p < 0.05$  considered as significant.

## Results

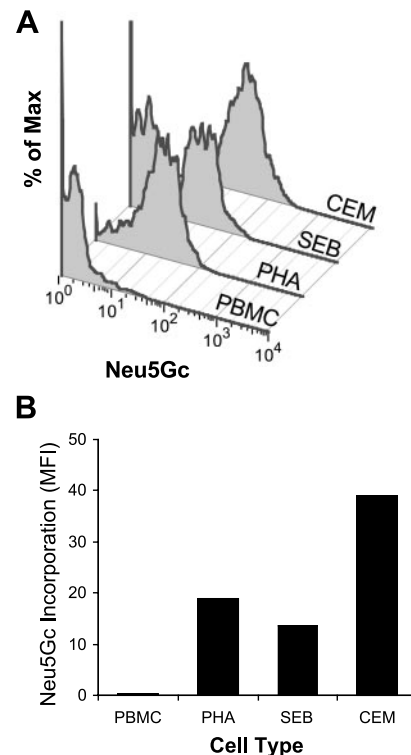
### All normal human sera contain Neu5Gc-specific Abs

We have reported recently that the majority of humans tested by a specific ELISA had detectable serum IgG, IgA, or IgM specific for

Neu5Gc (anti-Neu5Gc Abs) (11). Anti-Neu5Gc IgM is derived from B cells that have not yet undergone somatic hypermutation and isotype switching (35, 36). In this regard, it is interesting that all 21 healthy individuals tested had some detectable level of anti-Neu5Gc IgM (Table I). High levels of IgG and IgA, which are derived from B cells that have undergone isotype switching and affinity maturation (35, 36), suggest that previous exposure in the presence of Th cell-mediated response has occurred. In reanalyzing our original dataset along with additional samples, we have concluded that, in fact, all of our tested humans have some detectable Neu5Gc-specific reactivity in all three Ig subtypes (Table I). There was large variability in the Ab levels among individuals. Although the exact mechanisms for this variability are not known, the explanations are likely reflective of the variations in human immune responses as well as differences in lifetime exposure to dietary Neu5Gc. We also noted that retesting the same individuals at separate time points months apart showed the same rank order of IgG levels (data not shown), suggesting that these anti-Neu5Gc Abs levels are constitutive and stable in normal adult humans.

#### Activated PBMCs, but not resting PBMCs, incorporate Neu5Gc from media

To understand the potential significance of serum Abs against Neu5Gc in humans, we asked whether or not exogenous Neu5Gc could be incorporated into resting and activated human PBMCs. We used two distinct methods for T cell activation as follows: 1) PHA-L, a lectin that cross-links surface glycoproteins including TCR molecules (37); and 2) SEB, a superantigen that cross-links TCRs with MHC class II molecules (38). Using a 4-h incubation with 6 mM Neu5Gc, we observed no Neu5Gc incorporation into resting PBMCs, but a significant amount on PHA- and SEB-activated T cells, as detected by a previously characterized chicken polyclonal Ab (Fig. 1) (11). The levels of Neu5Gc incorporation were roughly half that seen with CEM cells (a T lymphoblastic leukemia cell line) incubated under the same conditions (Fig. 1B). Interestingly, we found that SEB-activated cells contained two distinct populations based on cell size. The larger cells, presumably



**FIGURE 1.** Activation enhances incorporation of Neu5Gc into human immune cells. PBMCs, PHA-activated T cells, SEB-activated T cells, and CEM cells were incubated with 6 mM Neu5Gc for 4 h. Cells were then washed, and the incorporated cell surface Neu5Gc was detected with ChGcAb and FITC-goat anti-chicken IgY by flow cytometry. The histograms of Neu5Gc staining are displayed (A), and the mean fluorescence intensity (MFI) of staining is also expressed (B). The results are representative of three independent experiments.

undergoing more rapid expansion and proliferation, incorporate Neu5Gc efficiently, whereas T cells that demonstrate no increase in cell size remained negative for Neu5Gc (Fig. 1A). The Neu5Gc incorporation profiles of these cells were also similar to feeding with 3 mM Neu5Gc for 24 h (data not shown). Very little Neu5Gc incorporation into activated T cells was observed with 0.3 mM Neu5Gc feeding for 24 h, suggesting that a high concentration of Neu5Gc is required for significant incorporation into cells (data not shown). We further evaluated resting PBMC incorporation of Neu5Gc using 3 mM for 48 h and found no significant incorporation of Neu5Gc (data not shown). These data suggest that human T cells can incorporate Neu5Gc under conditions of growth and proliferation, but poorly in the resting state.

#### Human leukemic cells incorporate free Neu5Gc

To further study Neu5Gc incorporation into human hemopoietic malignancy-derived lines, we also evaluated a line derived from chronic myelogenous leukemia in blast crisis (K562), and a histiocytic lymphoma line with monocytic properties (U937), in addition to the CEM cell line. After removal from FCS, the cells were subjected to continuous growth in a batch of pooled human serum (hereafter called HS) with very low natural anti-Neu5Gc Abs. Such cells showed little to no Neu5Gc expression by flow cytometry, as measured using the previously mentioned polyclonal chicken Ab specific for Neu5Gc (ChGcAb) (data not shown). However, subsequent addition of free Neu5Gc into media resulted in incorporation of Neu5Gc onto surface glycoconjugates, in a dose-dependent manner (Fig. 2A). Media supplemented with related sugars such as Neu5Ac or ketodeoxyoctulosonic acid did not

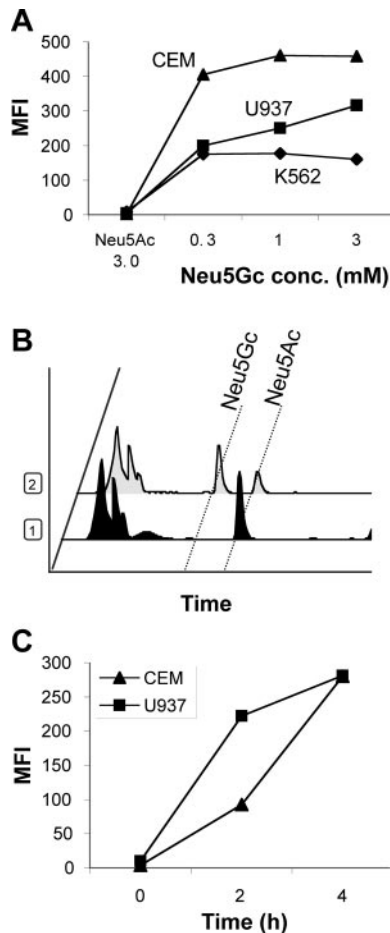
Table I. All human sera tested contain anti-Neu5Gc Igs

Serum no.	Neu5Gc-specific Ab <sup>a</sup> ( $\mu\text{g/ml} \pm \text{SD}$ ) <sup>b</sup>		
	IgG	IgA	IgM
1	3.48 $\pm$ 0.07	0.06 $\pm$ 0.01	0.38 $\pm$ 0.03
2	0.19 $\pm$ 0.03	0.16 $\pm$ 0.01	1.03 $\pm$ 0.11
3	0.39 $\pm$ 0.03	0.18 $\pm$ 0.04	0.57 $\pm$ 0.16
4	1.94 $\pm$ 0.15	0.28 $\pm$ 0.02	0.42 $\pm$ 0.03
5	0.31 $\pm$ 0.05	0.11 $\pm$ 0.03	0.33 $\pm$ 0.15
6	3.35 $\pm$ 0.05	2.97 $\pm$ 0.14	0.70 $\pm$ 0.18
7	2.37 $\pm$ 0.09	0.46 $\pm$ 0.05	0.28 $\pm$ 0.15
8	5.42 $\pm$ 0.13	0.08 $\pm$ 0.05	0.59 $\pm$ 0.03
9	3.18 $\pm$ 0.08	0.68 $\pm$ 0.03	0.79 $\pm$ 0.08
10	3.48 $\pm$ 0.07	0.06 $\pm$ 0.00	0.38 $\pm$ 0.09
11	0.98 $\pm$ 0.05	0.24 $\pm$ 0.02	0.46 $\pm$ 0.03
12	2.08 $\pm$ 0.07	0.11 $\pm$ 0.02	0.37 $\pm$ 0.02
13	0.25 $\pm$ 0.05	0.03 $\pm$ 0.01	0.17 $\pm$ 0.06
14	6.35 $\pm$ 0.25	0.35 $\pm$ 0.04	0.66 $\pm$ 0.05
15	0.09 $\pm$ 0.03	0.13 $\pm$ 0.02	0.26 $\pm$ 0.01
16	0.54 $\pm$ 0.09	0.32 $\pm$ 0.07	0.38 $\pm$ 0.00
17	0.84 $\pm$ 0.05	0.16 $\pm$ 0.03	0.26 $\pm$ 0.01
18	0.42 $\pm$ 0.07	0.17 $\pm$ 0.02	0.18 $\pm$ 0.02
19	0.29 $\pm$ 0.05	0.11 $\pm$ 0.03	0.31 $\pm$ 0.06
20	1.16 $\pm$ 0.07	0.19 $\pm$ 0.03	0.14 $\pm$ 0.03
21	0.67 $\pm$ 0.03	0.09 $\pm$ 0.01	0.21 $\pm$ 0.03

<sup>a</sup> Neu5Gc-specific Ab in serum were quantified as described in *Materials and Methods*.

<sup>b</sup> SD was determined from triplicate wells.





**FIGURE 2.** Human leukemic cell lines incorporate free Neu5Gc from media. *A*, Cell lines were incubated with various concentrations of Neu5Gc or Neu5Ac for 3 days. Surface Neu5Gc expression was detected with ChGcAb and FITC-goat anti-chicken (GAC) IgY by flow cytometry. MFI, Mean fluorescence intensity. *B*, DMB-derivatized Sias from K562 cells fed for 3 days with 3 mM Neu5Ac (1) or Neu5Gc (2) were analyzed by DMB-HPLC. *C*, CEM and U937 cells were fed with 6 mM Neu5Gc for 0, 2, or 4 h and then probed with ChGcAb and FITC-GAC. The MFI for flow cytometry are presented. Results are representative of three independent experiments.

generate ChGcAb binding (Fig. 2A, and data not shown). Using a sensitive chemical method (DMB-derivatization followed by HPLC with fluorescent detection), we verified that K562 cells fed with 3 mM Neu5Gc for 3 days contained as much as 67% of their total Sias as Neu5Gc, compared with Neu5Ac-fed cells, which contained no detectable Neu5Gc (Fig. 2B). Further studies on the kinetics of Neu5Gc incorporation showed that 6 mM free Neu5Gc gave high levels of incorporation into CEM and U937 cells within 4 h of incubation (Fig. 2C). We could also detect significant amounts of Neu5Gc incorporation into peripheral blood blasts from nine cases of acute myeloid leukemia (AML) and one case of acute lymphocytic leukemia (Table II) after 24-h feeding with 3 mM Neu5Gc. Little to no Neu5Gc was detected on Neu5Ac-fed primary leukemia samples (Table II). Overall, these data demonstrate that although human leukemic cells do not synthesize their own Neu5Gc, they can readily incorporate it from the medium, achieving high levels on the cell surface. These data also support the concept that human malignancies that have been reported to express Neu5Gc in vivo likely incorporate it from exogenous sources.

Table II. Primary acute leukemias incorporate Neu5Gc

	Neu5Gc incorporation detected by ChGcAb	
	Neu5Ac-fed (MFI) <sup>a</sup>	Neu5Gc-fed (MFI)
AML-1 <sup>b</sup>	1.3	373
AML-2	6.3	407
AML-3	0.3	431
AML-4	0.0	723
AML-5	1.5	643
AML-6	0.2	450
AML-7	0.0	678
AML-8	0.0	361
AML-9	0.0	65
ALL	0.0	97
CEM <sup>c</sup>	0.0	906

<sup>a</sup> Median fluorescence intensity (MFI) was calculated by subtracting the MFI values for secondary alone control from the ChGcAb MFI. Samples where the background MFI was greater than the ChGcAb MFI are indicated as 0.

<sup>b</sup> Randomly selected primary myeloid leukemia (AML) or acute lymphocytic leukemia (ALL) samples were fed with 3 mM Neu5Ac or Neu5Gc for 24 h, and then probed with ChGcAb, followed by FITC-goat anti-chicken IgY.

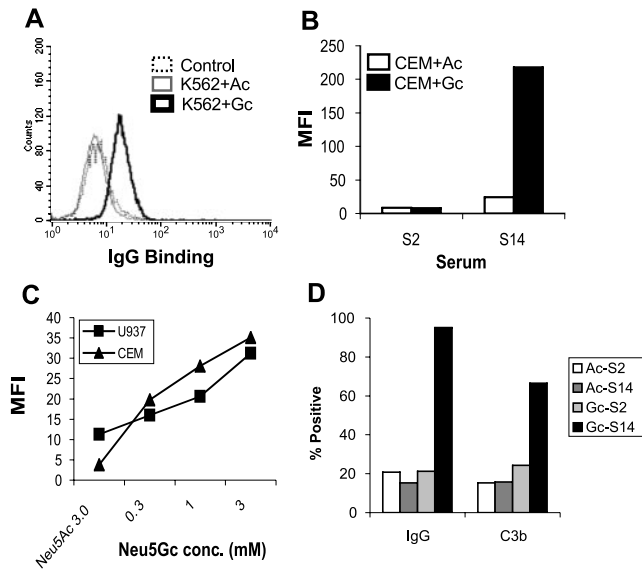
<sup>c</sup> CEM cells were used as a positive control for Neu5Gc incorporation.

#### Human serum IgG binds to Neu5Gc-fed leukemic cells and PHA-activated T cells

To verify that human serum IgG specifically recognizes Neu5Gc on cell surfaces, we determined the relative levels of IgG binding from human sera to Neu5Ac- and Neu5Gc-fed leukemic cells. As shown in the example in Fig. 3A, K562 cells fed with Neu5Gc (3 days, 3 mM) demonstrated a significant increase in IgG binding when incubated with serum no. 14, which had the highest level of anti-Neu5Gc IgG (hereafter called GcIg). Control K562 cells fed with Neu5Ac showed no increase in IgG binding over background levels (Fig. 3A). In contrast, serum no. 15, with low GcIg levels in the ELISA showed no binding of IgG to either Neu5Ac or Neu5Gc-fed K562 cells (data not shown). Similar results were obtained with CEM leukemic cells fed with Neu5Ac or Neu5Gc for a shorter period of time (1 day, 3 mM) (Fig. 3B). Binding of IgG from serum no. 14 was also observed in a Neu5Gc-feeding dose-dependent manner (ranging from 0.3 to 3.0 mM) in CEM and U937 cells (Fig. 3C). We also wanted to determine whether Neu5Gc incorporated into PHA-activated cells could serve as a target for human serum IgG and complement binding. After feeding PHA-activated cells with 3 mM Neu5Ac or Neu5Gc for 24 h, we incubated the cells in the presence of low (no. 2) or high (no. 14) Neu5Gc-reactive human serum for 2 h. We detected significantly increased binding of both human IgG and complement only on cells fed with Neu5Gc in the presence of high Neu5Gc-reactive serum (serum no. 14) (Fig. 3D). These results not only corroborate the ELISA findings, but also demonstrate that these natural human serum GcIg can bind to cell surface molecules of human cells carrying Neu5Gc.

#### Neu5Gc incorporation increases susceptibility to complement-mediated lytic cell killing by anti-Neu5Gc IgG-containing serum

The incorporation of Neu5Gc into cells makes them potential targets for Ab-mediated cell killing, by processes such as complement killing. To test sera for such anti-Neu5GcAb-mediated cytotoxicity, we fed K562 cells with 3 mM Neu5Ac or Neu5Gc for 3 days, and then exposed them to 10% serum no. 14 for 2 h, followed by the addition of PI to a final concentration of 0.1 mM for an additional 30 min. Before serum incubation, cells were labeled with a membrane dye, DiOC, to indicate the live cell population, because dead cells will poorly label with DiOC. The killing of DiOC-labeled cells results in the incorporation of PI into



**FIGURE 3.** Human serum IgG binds to Neu5Gc-fed cells. **A**, K562 cells were fed with 3 mM Neu5Ac or Neu5Gc for 3 days, and then incubated with 10% human serum (S) no. 14 on ice, followed by GAH IgG-AF488. The control histogram is the background fluorescence of cells incubated without serum no. 14 in the presence of secondary Ab only. **B**, CEM cells were fed with 3 mM Neu5Ac or Neu5Gc for 1 day, and then incubated with 10% serum no. 2 or serum no. 14 at 37°C, followed by GAH IgG-AF488 on ice. **C**, CEM and U937 cells were incubated with various concentrations of Neu5Ac or Neu5Gc for 3 days, and then incubated with 10% serum no. 14 on ice, followed by GAH IgG-AF488. Results are representative of three independent experiments. **D**, Three-day PHA-activated T cells were fed with 3 mM Neu5Ac or Neu5Gc for 24 h and then incubated with serum no. 2 or serum no. 14. IgG binding was detected with GAH IgG-AF488, and C3b binding was detected by a FITC-mouse anti-human C3c Ab that reacts with C3b.

these cells, which can then be quantitated by flow cytometric analysis. We found that K562 cells fed with Neu5Gc were susceptible to anti-Neu5Gc Ab-mediated killing within 2.5 h (Fig. 4A). The percentage of DiOC and PI double-positive Neu5Gc-fed cells was significantly greater than that of Neu5Ac-fed cells. We also evaluated cell killing of resting PBMCs, PHA-activated T cells, and CEM cells after Neu5Gc feeding using a dose titration curve of serum no. 14 (Fig. 4B). As expected, Neu5Ac-fed cells demonstrated no serum dose-dependent cytotoxicity in any cell type, indicating that the normal human Sia, Neu5Ac, is not a target of serum no. 14 (Fig. 4B). In contrast, we did find dose-dependent killing of all three cell types to widely different degrees when they were fed with Neu5Gc. Based on the calculated LD<sub>50</sub> values, Neu5Gc-fed CEM cells were ~10-fold more susceptible than PHA-activated T cells and ~30-fold more susceptible than PBMCs to killing by serum no. 14 (Fig. 4B). This demonstrates that the susceptibility of cells to killing by GcIg is reflective of their Neu5Gc incorporation levels. Whether there are other mechanisms to account for the differences in susceptibility remain to be determined. As complement inhibitory factors such as CD55 and CD59 play a role in susceptibility to complement-mediated killing, we confirmed that PHA-activated T cells did not express these proteins to greater levels than that of CEM cells (data not shown).

To test whether the human anti-Neu5GcAbs also induce killing of Neu5Gc-fed CEM cells in a complement-dependent manner, we compared serum no. 14 killing with and without prior heat inactivation (56°C for 30 min) of both serum no. 14 and the HS that was used in the media. The heat-inactivated serum demonstrated significantly reduced ability to kill CEM cells compared with con-

trol serum (Fig. 4C). We also confirmed that IgG binding was still retained after heat inactivation (Fig. 4D). We also evaluated GcIg binding at various input concentrations from 2 to 10% of heat-inactivated and control serum no. 14. We found a linear-positive correlation between killing and GcIg binding for control serum, but found no killing and no correlation with GcIg binding using heat-inactivated serum (Fig. 4E). These results suggest that the majority of cell killing is mediated by a complement-dependent mechanism.

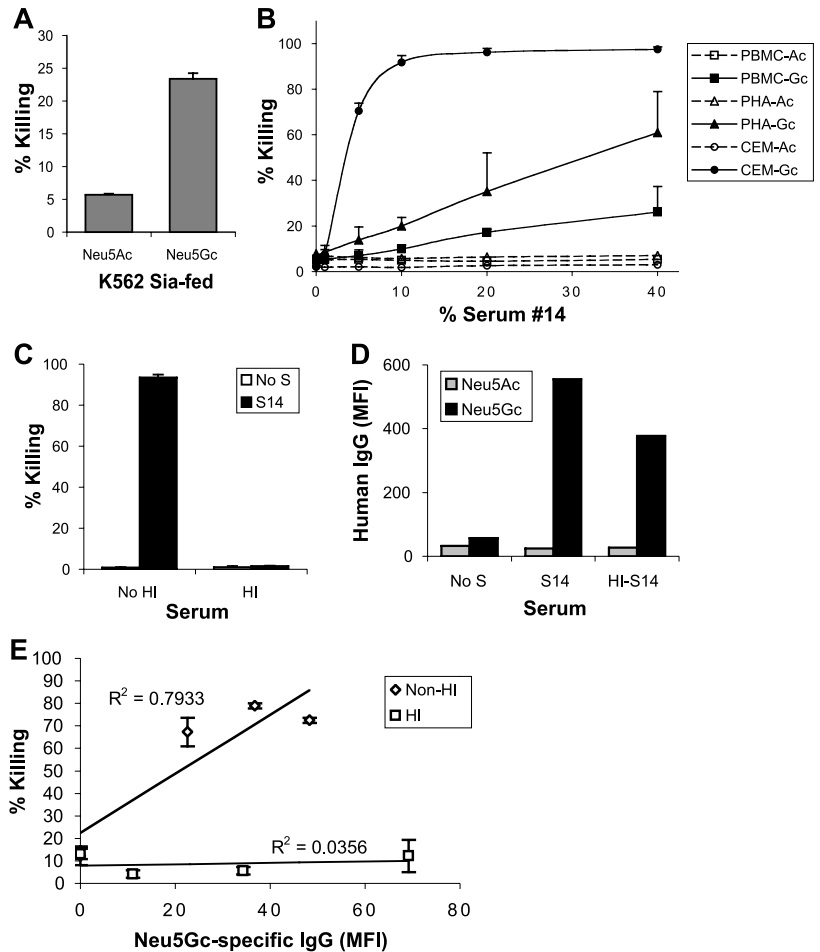
#### *Selective killing of leukemic cells mixed with PBMCs by human serum in a short-term Neu5Gc-feeding, mixed-target assay*

Neu5Gc incorporation is evident in CEM cells following a short-term 4-h feeding with 6 mM Neu5Gc, unlike resting PBMCs (Fig. 5A). Given this dramatic difference in the ability to incorporate Neu5Gc between human leukemic cells and normal PBMCs, we sought to determine whether leukemic cells could be specifically targeted for death by short-term Neu5Gc feeding. To simulate a leukemic condition in human blood, we mixed PBMCs and DiOC-labeled CEM cells, fed them with Neu5Gc for 4 h, and then incubated them with serum no. 14 or serum no. 2 to determine relative cell killing by GcIg. Indeed, we found that CEM cells were 15-fold more susceptible than PBMCs to GcIg-mediated killing as measured by PI incorporation (Fig. 5, D and E). In the presence of serum no. 14, PI-positive PBMCs increased by only 1.6% above the background. In contrast, toxicity increased by 25% in CEM cells above the background control with no serum added (Fig. 5E). Low GcIg-containing serum no. 2 did not induce increased cell death in PBMCs and only marginally increased it in CEM cells, a change that was not statistically significant ( $p = 0.13$ ) (Fig. 5, C and E). Very little killing of PBMCs or CEM cells was observed in the absence of additional serum (Fig. 5B). These results demonstrate that the ability to rapidly incorporate Neu5Gc greatly enhances cellular susceptibility to GcIg-mediated toxicity in a short-term feeding assay. Note that due to such a low E:T ratio (2:1), the majority of the toxicity observed here is not likely to be due to Ab-dependent cell-mediated cytotoxicity (ADCC) by the PBMCs, although some activity cannot be ruled out. In addition, ADCC experiments involving CEM targets and PBMC effectors demonstrated little increase in cell death by ADCC above the no-effector control (data not shown).

#### *Serum killing of Neu5Gc-fed primary leukemia cells corresponds to human IgG binding*

We further tested whether primary leukemia cells that incorporate Neu5Gc would be susceptible to killing by GcIg-containing serum. We found that four of eight AML samples had significant Neu5Gc-specific killing, as did the PHA-activated T cells and CEM cells (Fig. 6A). In this experiment, both the PHA-activated T cell samples demonstrated greater killing than any of the AML samples. Interestingly, when we also determined Neu5Gc-specific IgG binding, we found a linear relationship between GcIg binding and Neu5Gc-specific killing (Fig. 6A). In contrast, the levels of ChGcAb binding did not correlate well with killing for the cells used in Fig. 6A (data not shown). This suggests that there are likely to be epitopes recognized by the ChGcAb that do not correspond with Neu5Gc epitopes required for cell killing by GcIg. It may be that certain molecules such as glycolipids or certain Sia linkages to the underlying glycans are more capable of promoting cell killing by GcIg. Further studies will be needed to define such specificities. In any case, it appears that total GcIg binding correlates with cell killing to a better degree than ChGcAb binding and that this may be the main reason for the difference in killing between PHA-activated cells and CEM cells.

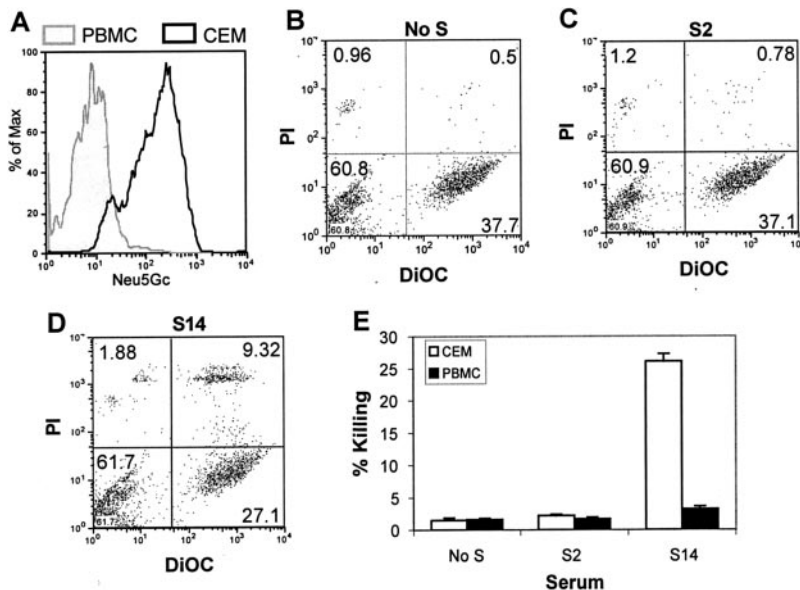
**FIGURE 4.** Human serum IgG induces lytic cell death of Neu5Gc-fed cells by a complement-dependent mechanism. *A*, K562 cells, fed with 3 mM Neu5Ac or Neu5Gc for 3 days, were labeled with DiOC, exposed to 10% serum no. 14 in RPMI-5HS, and then incubated with PI. Cells were analyzed by flow cytometry to determine the number of DiOC and PI double-positive cells. Error bars represent the SD of duplicate samples ( $p = 0.018$ ). *B*, PBMCs, PHA-activated T cells, and CEM cells were fed with 3 mM Neu5Ac or Neu5Gc for 24 h, washed, and then incubated for 2 h in the presence of the indicated concentrations of serum no. 14 before the addition of PI for 30 min. The indicated values are the average results for PBMCs and PHA-activated T cells from three different individuals, and the error bars represent the SD. CEM killing compared with PHA-activated T cell killing is statistically significant ( $p < 0.05$ ) for serum concentrations at 5% and greater. SDs for Neu5Ac-fed cells were all  $<2.0\%$  and not indicated on the graph for clarity. *C*, Neu5Gc-fed CEM cells (3 mM, 24 h) were exposed for 2 h to no serum or 10% serum no. 14 (S14) in RPMI-5HS that was previously heat inactivated (HI) or not, and subsequently exposed to PI. Error bars represent the SD of triplicate samples in a single experiment ( $p < 0.05$  for serum no. 14 comparisons with and without heat inactivation). *D*, Neu5Ac and Neu5Gc-fed CEM cells were incubated in the presence of no serum, 10% HI serum no. 14, or control serum no. 14 in RPMI-5HS for 30 min. IgG binding was quantitated by flow cytometry using GAH IgG-AF488. *E*, Neu5Gc-fed CEM cells were incubated with 0, 2, 5, or 10% control or HI serum no. 14 for 2 h. Cells were then labeled with GAH IgG-AF488 and PI. Error bars represent the SD of triplicates.



To further evaluate the correlation between GcIg binding and cell killing, we examined CEM cell cytotoxicity by serum no. 14 at increasing concentrations of Neu5Gc feeding. Once again, we found a positive relationship between GcIg binding and Neu5Gc-specific killing that approaches saturation below our standard experimental feeding concentration of 3 mM (Fig. 6*B*). This further supports the concept that the difference in cell susceptibility to

killing is directly related to the ability of GcIg to bind cellular targets.

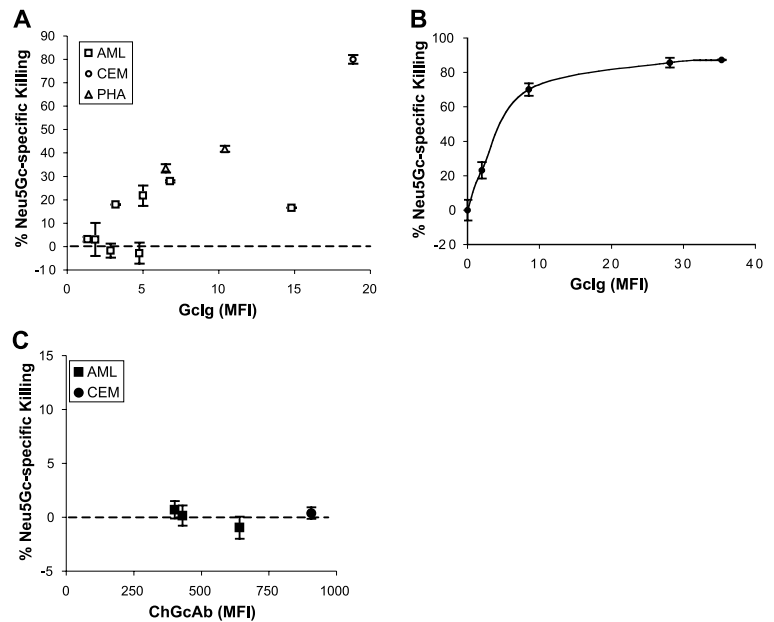
The cross-linking of Neu5Gc by Abs could potentially be a mechanism for cell death, similar to lectin-mediated activation-induced cell death (39). To rule out this possibility, we incubated Neu5Ac and Neu5Gc-fed CEM cells and three AML samples with our ChGcAb for 4 h, which is twice as long as the other serum



**FIGURE 5.** Neu5Gc-fed leukemic cells in the presence of PBMCs are selectively targeted for killing by human serum IgG. *A*, PBMCs and CEM cells were incubated with 6 mM Neu5Gc for 4 h and then labeled with ChGcAb and FITC-goat anti-chicken (GAC). *B–D*, PBMCs mixed with DiOC-labeled CEM cells (2:1) were fed with 6 mM Neu5Gc for 4 h, then incubated with no serum (*B*), 10% serum no. 2 (*C*), or serum no. 14 (*D*). PI incorporation was quantitated by flow cytometry. Live PBMCs appear in the lower left quadrant, live DiOC-labeled CEM cells appear in the lower right quadrant, and PI-positive dead cells appear in the upper right and left quadrants. *E*, Percentage killing was calculated from the number of cells in the upper quadrants compared with total DiOC<sup>+</sup> cells (CEM) or DiOC<sup>-</sup> cells (PBMC). Error bars represent the SD of triplicate samples from a single experiment. The experiment was reproduced three times with similar results. The specificity for CEM killing is statistically significant with serum no. 14 ( $p = 0.0004$ ), but not serum no. 2 ( $p = 0.18$ ).



**FIGURE 6.** Serum killing of Neu5Gc-fed primary leukemia cells corresponds to human IgG binding. *A*, Eight AML samples ( $\square$ ), two PHA-activated T cell samples ( $\triangle$ ), and CEM cells ( $\circ$ ) were incubated with 3 mM Neu5Ac or Neu5Gc for 24 h, washed, and then incubated for 2 h in the presence of 10% serum no. 14. Cells were then washed, labeled with GAH IgG-AF488 and PI for 30 min, and then analyzed by flow cytometry. Neu5Gc-specific killing was calculated by subtracting the percentage of PI<sup>+</sup> Neu5Ac-fed cells. Error bars represent the SD of duplicate samples. Neu5Gc-specific IgG binding was similarly calculated by subtracting the MFI of Neu5Ac-fed cells. *B*, CEM cells were fed with 3 mM Neu5Ac or Neu5Gc at 0, 0.375, 0.75, 1.5, and 3.0 mM. Cells were incubated with 10% serum no. 14 for 2 h, then labeled with GAH IgG-AF488 or PI, and analyzed by flow cytometry. Error bars represent the SD of triplicate samples. *C*, Neu5Ac and Neu5Gc-fed CEM cells ( $\bullet$ ) and AML cells from 3 donors ( $\blacksquare$ ) were incubated with ChGcAb (1/400 dilution) for 4 h and then incubated with PI for 30 min. Parallel experiments were performed to detect ChGcAb binding using FITC-goat anti-chicken (GAC) IgY, which is expressed as MFI. Error bars represent the SD of triplicate samples.



cytotoxicity assays. We found no measurable amount of Neu5Gc-specific cell death with any of our cells tested (Fig. 6C). Extending the incubation time to 24 h did not result in any increase in cell death for CEM cells (data not shown). Thus, we can rule out that cross-linking of Neu5Gc-bearing epitopes alone is responsible for cell killing.

## Discussion

In this study, we have demonstrated that a nonhuman Sia, Neu5Gc, is readily incorporated into human leukemic cells and activated T cells, but poorly into resting PBMCs. Although Neu5Gc is not membrane permeable, the mechanism for such uptake by leukemic cells likely occurs through fluid-phase pinocytosis followed by the action of a lysosomal Sia transporter, which normally transports Neu5Ac from the lysosome into the cytoplasm (21). The incorporation into leukemic cells and activated cells was quite rapid, demonstrating high surface levels within 4 h of incubation. Additionally, we have established that leukemic cell lines, as well as primary cell lines, cultured in human serum do not produce their own Neu5Gc, but can readily incorporate it, suggesting that the expression of Neu5Gc reported previously in cancer cells *in vivo* also likely originates from exogenous sources.

This study has shown for the first time that natural circulating anti-Neu5Gc Abs can be functionally relevant, binding to and eliciting cytotoxicity on leukemic cells and activated T cells that express Neu5Gc. This cytotoxicity occurred quite rapidly, with near-maximal killing achieved within 1 h of incubation with serum no. 14 (data not shown). Overall, our data suggest that the majority of cell killing was mediated by complement rather than ADCC, apoptosis, or activation-induced cell death, because these processes normally occur at much slower rates than complement-mediated killing. We also demonstrate a relationship between GcIg binding and cell killing, indicating that the ability of GcIg to bind to Neu5Gc on target cells directly relates to cell susceptibility to killing. The presence of Neu5Gc-reactive Abs has also been reported in patients with seemingly unrelated disease states, including cancer, hepatitis, infectious mononucleosis, syphilis, and leprosy, as well as in patients with rheumatoid arthritis (27). Additionally, anti-GM1 Abs in sera from patients with motor neuron disease and neuropathy recognize Neu5Gc-GM1 as well as Neu5Ac-GM1, despite the fact that mammalian brains contain almost exclusively

Neu5Ac (40, 41). The explanations for such anti-Neu5Gc Ab production under these conditions is not well understood. Investigation of the effects of Neu5Gc incorporation in an animal model is inherently difficult due to the fact that most mammals express Neu5Gc. We are currently generating a *Cmah*-deficient mouse model to pursue such studies.

The rapid incorporation of Neu5Gc into leukemic cells and their sensitivity to cytotoxicity makes them preferred targets for anti-Neu5Gc Abs over resting PBMCs. We propose that this specificity could be used for leukemia therapy to target and eliminate rapidly growing cancer cells from the blood. We have shown already that oral ingestion of a large amount of Neu5Gc (150 mg in a single dose) in normal humans was associated with evidence of absorption and clearance in the urine, but not with any obvious toxicity (11). Importantly, incorporation of Neu5Gc into normal peripheral blood leukocytes could not be detected in this intact human study. This was true even several days after the initial oral ingestion, when bone marrow cells exposed to the initial bolus would have matured into peripheral blood cells (11). Thus, *i.v.* delivery of high concentrations of Neu5Gc would likely be necessary for incorporation at high levels.

Although current combination chemotherapy for acute leukemias are effective at inducing complete remissions for most patients, the development of novel approaches to leukemia therapy is essential due to high rates of relapse and outgrowth of drug-resistant tumors, especially in adult leukemias (42–44). Thus, we suggest the possibility that delivery of a large *i.v.* pulse or short-term infusion of free Neu5Gc, followed shortly after by the delivery of a humanized IgG against Neu5Gc could provide a specific method of cell killing. Alternatively, some humans may already have pre-existing natural anti-human Abs and/or could be induced to express them by immunization with Neu5Gc-containing Ags (it should be noted that free unbound Neu5Gc itself would not be immunogenic, because it is a monosaccharide and also exists mostly in the  $\beta$ -configuration in solution). Regardless, there is obviously some risk that some other cell type within the body might also have some degree of Neu5Gc incorporation and be susceptible to Ab recognition. These concepts also support the potential for immunizing cancer patients with unnatural modified Sias followed by loading of cancer cells with the same immunogenic Sia, as

proposed by Guo and colleagues (45). If these modified Sias incorporate into leukemic cells as efficiently as Neu5Gc, they may provide an advantage over our proposed Neu5Gc targeting, which could damage other normal cells that already have low levels of Neu5Gc.

In our recent work, we have also reported that some normal autopsy human tissues can have small levels of Neu5Gc (primarily in endothelial cells and in certain epithelia and their secretions, and presumably derived from dietary sources) (11). Thus, there is also a potential risk that such cell types already expressing small amounts of Neu5Gc in the patient may be innocent bystanders in the proposed therapeutic approach. However, with all forms of effective therapy for acute leukemia, one has to tolerate a certain amount of toxicity to such normal cell types. Also, any normal T cells that happen to be concurrently activated could incorporate Neu5Gc and be targeted for death. However, such short-term side effects would be no worse from those currently experienced by patients with leukemia undergoing conventional chemotherapy. Moreover, given the extremely efficient incorporation of Neu5Gc by leukemic cells, there is a reasonable probability of defining a "therapeutic window" in which incorporation of Neu5Gc and the subsequent cytotoxicity is relatively specific for the leukemia cells. Such approaches could be first tested in a mouse model of the Neu5Gc-free human condition. The effectiveness of such a therapy would also rely on the specificity of Neu5Gc incorporation into bone marrow leukemic stem cells that serve as the progenitors of leukemic cells in the blood. Future studies will have to address whether such cells can incorporate Neu5Gc and be targeted by Abs, while sparing normal developing stem cells.

The incorporation of Neu5Gc into T cells during activation could potentially impact on the immune response mounted against a bacterial or viral infection in individuals with high GcIg. We found that activated T cells were much more efficient than resting PBMCs at incorporating Neu5Gc from the media. To fully characterize whether this could occur in vivo in humans, further studies will be needed to determine the levels of Neu5Gc in blood after consumption of high Neu5Gc-containing meals, the clearance rate of Neu5Gc from blood, and the incorporation into proliferating cells. In addition to complement-mediated cytotoxicity, GcIg could potentially mediate ADCC and phagocytosis. If capable of inducing such cell killing events in vivo, one's immune response to pathogens could be limited by self-targeting of Neu5Gc.

In this study, we have defined the functional significance of natural human Abs against a nonhuman Sia, Neu5Gc. Human serum GcIg was capable of inducing cytotoxic effects on leukemia cells and activated T cells. Based on our findings that leukemic cells can incorporate Neu5Gc much more efficiently than resting PBMCs, we propose that this disparity could allow for the preferential targeting of leukemic cells by GcIg. However, deposition of natural GcIg could also potentially target healthy proliferating immune cells for death following the incorporation of dietary Neu5Gc.

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## Disclosures

The authors have no financial conflict of interest.

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