Title
Novel mechanism for the generation of human xeno-autoantibodies against the nonhuman sialic acid N-glycolylneuraminic acid.

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Sialic acids (Sias) are monosaccharides with a shared 9-carbon backbone, typically found at the terminal ends of vertebrate cell surface and secreted glycoconjugates (Schauer, 1982; Varki, 1992; Traving and Schauer, 1998; Angata and Varki, 2002; Chen and Varki, 2010). Most mammals express two common Sias, N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc). However, humans are deficient in Neu5Gc synthesis because of a human-specific mutation inactivating the CMAH gene responsible for converting CMP-Neu5Ac to CMP-Neu5Gc (Chou et al., 1998; Irie et al., 1998). All human adults have varying levels of circulating IgM, IgG, and IgA antibodies against Neu5Gc (Zhu and Hurst, 2002; Tangvoranuntakul et al., 2003; Nguyen et al., 2005; Padler-Karavani et al., 2008; Tahara et al., 2010). At the same time, dietary Neu5Gc from foods such as red meat or milk products can be metabolically incorporated into human tissues, particularly epithelia and endothelia (Tangvoranuntakul et al., 2003; Hedlund et al., 2007; Hedlund et al., 2008), through a mechanism involving macropinocytosis and...
delivery of free Neu5Gc to the cytosol via a lysosomal transporter (Bardor et al., 2005; Yin et al., 2006). Evidently, although the human immune system can react to this xeno-antigen, human biochemical pathways do not see it as foreign. Thus, anti-Neu5Gc antibodies represent novel “xeno-autoantibodies,” which recognize a “non-self” animal-derived antigen in the context of “self.” Indeed, we have recently demonstrated that human anti-Neu5Gc antibodies interact with metabolically incorporated Neu5Gc to promote chronic inflammation, likely contributing to tumor progression (Hedlund et al., 2008) and vascular inflammation (Pham et al., 2009).

Given their potential contribution to the pathogenesis of dietary red meat–associated diseases, it is important to understand when and how anti-Neu5Gc antibodies emerge in humans. Here, we show that these antibodies emerge postnatally in humans during the first year of life. Other postnatally acquired human antibodies against foreign glycans, e.g., blood group antibodies and anti–α-Gal antibodies, are thought to be induced by commensal bacteria expressing these epitopes (Springer and Horton, 1969; Galili et al., 1988). However, although many bacteria can synthesize and express Neu5Ac (Vimr and Lichtensteiger, 2002; Vimr et al., 2004), none are known to synthesize Neu5Gc. Here, we demonstrate that dietary Neu5Gc can be incorporated by a common human commensal bacterium, providing a mechanism for generating anti-Neu5Gc antibodies during the first year of life. To our knowledge, this is the first example in which a diet-derived molecule is scavenged by resident bacteria from within the host and effectively expressed as an immunogenic antigen.

RESULTS
Human anti-Neu5Gc antibodies appear during the first year of life and correlate with the introduction of Neu5Gc in the diet
Sera from infants age 0–12 mo (cord, 3 mo, 6 mo, and 12 mo) were analyzed by ELISA for the presence of anti-Neu5Gc IgM and IgG antibodies against Neu5Gcα2–6Galβ1–4Glc (Gcα2–6Lac), an epitope against which most human adults possess high levels of IgM and IgG antibodies (Padler-Karavani et al., 2008). All sera were from infants who had been exclusively breastfed for the first 3 mo of life, and then switched to cow’s milk–based formula. Solid foods were also introduced starting at 3 mo of age and included both foods lacking Neu5Gc, such as fruits and vegetables, and Neu5Gc–rich foods, such as beef, pork, and lamb. Anti-Neu5Gc IgM antibodies were absent at birth (cord serum) and at 3 mo, appeared at 6 mo and achieved almost adult levels at 12 mo (Fig. 1 A). As expected because of transplacental transport of IgG, all cord sera contained anti-Neu5Gc IgG antibodies, at levels similar to maternal anti-Neu5Gc IgG. These anti-Neu5Gc IgG antibodies diminished at 3 mo, followed by increasing levels at 6 and 12 mo (Fig. 1 B). There was no difference between male and female anti-Neu5Gc IgM and IgG titers (unpublished data). The reactivity of 12 mo sera against Gcα2–6Lac was significantly reduced after truncation of the target epitope’s Neu5Gc side chain by mild periodate oxidation (Tangvoranuntakul et al., 2003), further demonstrating the specificity of the IgG antibodies for Neu5Gc-containing glycans (Fig. 1 C). The absence of anti-Neu5Gc IgM antibodies in cord sera suggests that anti-Neu5Gc antibodies are not germ-line encoded “natural antibodies” (Ochsenbein and Zinkernagel, 2000), but instead require a postnatal antigenic stimulus. And the early appearance and class switching of these antibodies indicate that humans are exposed to the Neu5Gc antigenic stimulus early in life. The nadir in anti-Neu5Gc IgG titer seen at 3 mo is also consistent with the half-life of maternally derived IgG (Morell et al., 1970), and suggests that these infants lack the production of endogenous anti-Neu5Gc IgG antibodies at 3 mo, when their diets were devoid of Neu5Gc. Interestingly, both infant IgM and IgG anti-Neu5Gc antibodies arise soon after the introduction of Neu5Gc in the diet in the form of cow’s milk formula and baby foods containing red meat.

Dietary Neu5Gc alone is insufficient to elicit anti-Neu5Gc antibodies in Neu5Gc-deficient mice
The temporal correlation between the appearance of anti-Neu5Gc antibodies and the introduction of animal-derived foods suggested that dietary Neu5Gc might represent the antigenic stimulus. To study this issue experimentally, we used Cmah-null mice that have a human-like deficiency in Neu5Gc

Figure 1. Anti-Neu5Gc antibodies in human infants. (A and B) Levels of anti-Neu5Gc IgM (A) and IgG (B) antibodies in infant sera (n = 15, filled circles) from birth (cord), 3, 6, and 12 mo (for each infant) and adult sera from the pregnant mothers (n = 9, open squares) were measured by ELISA against Neu5Gcα2–6Lac–HSA. Each data point represents the mean of triplicate values from one individual, quantified according to an IgM or IgG standard curve. Horizontal lines represent mean values for each group. (C) Neu5Gcα2–6Lac–HSA coated on an ELISA plate was treated with mild periodate (periodate) or inactivated periodate (mock periodate), and then analyzed for binding by IgG antibodies from infants at 12 mo of age (n = 15). Bars represent mean absorbance values at OD492 ± SEM. Statistical analysis was performed using an unpaired two-tailed Student’s t test. (D) Infant sera (same infants as in A and B) were analyzed by ELISA for IgM antibodies against α-Gal–PAA (dashed line). For comparison, anti-α-Gal IgM levels were plotted with anti-Neu5Gc IgM levels (solid line; same data as in Fig. 1 A). Values represent mean IgM levels ± SEM.

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Normal mouse chow diet provides 39.9 µg/g total Neu5Gc (Tangvoranuntakul et al., 2003). Gavaged 250 µl of human RBCs immunized with Neu5Gc (from normal chow) after weaning (4–6 wk total), immunized with human RBC ghosts (WT, n = 4; Cmah−/−, n = 4), or immunized with chimpanzee RBC ghosts (WT, n = 4; Cmah−/−, n = 4). Sera were analyzed by ELISA for IgM (A; note the broken y axis) and IgG (B) antibodies against Neu5Gc-PA-A and shown as mean absorbance values at OD405 ± SEM. Statistical analysis was performed using an unpaired two-tailed Student’s t test. n.s, not significant, *, P < 0.05; **, P < 0.01. Data are representative of greater than three independent experiments.

Table I. Unsuccessful attempts at generating anti-Neu5Gc antibodies in Neu5Gc-deficient mice

| Unsuccessful attempt | Data/comments |
|----------------------|--------------|
| Feeding regular mouse chow | Normal mouse chow diet provides ~1 mg/kg/day Neu5Gc (>5 times the amount found in Western diets; Tangvoranuntakul et al., 2003). |
| Adding free Neu5Gc to drinking water | Maximum exposure 1 mg/ml in drinking water for 12 wk. |
| Feeding cow’s milk | Cow’s milk contains ~8 µg/g total Neu5Gc (Tangvoranuntakul et al., 2003). Gavaged 250 µl 5 d/wk for 4 wk. |
| Feeding goat cheese | Rich in Neu5Gc-containing glycolipids, 39.9 µg/g total Neu5Gc (Tangvoranuntakul et al., 2003). |
| Feeding mucin | Very rich in Neu5Gc. Mixed in with regular mouse chow. |
| Breastfeeding/fostering by WT females | WT mouse breast milk contains Neu5Gc. Simulates infant exposure to cow’s milk or cow’s milk-based formula. |
| In utero exposure of Cmah-null pups to Neu5Gc positive Cmah+/+ mother | Cmah-null mice born loaded with Neu5Gc, which clears within weeks. Simulates infant/fetal exposure to Neu5Gc through the mother’s diet. |
pathogens evade, dampen, and/or inhibit host immune defenses (Estabrook et al., 1997; Ram et al., 1998; Carlin et al., 2009). Several bacterial species, including Escherichia coli K1, Campylobacter jejuni, most meningococcal strains, and Streptococcus agalactiae are able to synthesize Sia de novo (Vimr and Lichtensteiger, 2002). However, the parent Sia structure is always Neu5Ac, and no microbe has ever been shown to synthesize Neu5Gc. Indeed, the biosynthesis of the N-glycolyl group of Neu5Gc appears to be a singular event in evolution, being confined to the Deuterostome lineage of animals (Schauer, 1982).

On the other hand, certain sialylated bacteria including Neisseria gonorrhoeae, Haemophilus influenzae, Haemophilus ducreyi, and Corynebacterium diphtheriae lack the biosynthetic machinery necessary for Sia synthesis and instead scavenge host-derived Sias (Mandrell et al., 1990; Mattos-Guaraldi et al., 1998; Schilling et al., 2001; Bouchet et al., 2003). Non-typeable H. influenzae (NTHi) colonizes most humans and can transition from a commensal to a pathogenic state in diseases such as infantile otitis media. NTHi can efficiently scavenge minute amounts of environmental free Sias via a specific transporter (Allen et al., 2005; Severi et al., 2005), and then use an endogenous CMP-Sia synthetase (Hood et al., 1999) and sialyltransferases (Jones et al., 2002) to decorate its LOS. These sialylated LOS molecules are critical for NTHi human serum resistance (Hood et al., 1999; Allen et al., 2005) and virulence in a chinchilla otitis media model (Bouchet et al., 2003; Jurcisek et al., 2005).

Of the aforementioned bacteria, only H. influenzae has the potential to access dietary Neu5Gc while living as part of the human flora. We hypothesized that NTHi might scavenge exogenous dietary Neu5Gc and express it as an immunogenic epitope, providing a mechanism for the generation of anti-Neu5Gc antibodies in human infants. To pursue this hypothesis, we grew the NTHi strain 2019 (Campagnari et al., 1987) in a Sia-free defined medium and analyzed the bacteria by flow cytometry using a Neu5Gc-specific chicken IgY antibody (Fig. 3 A). However, when exogenous free Neu5Gc (0.1 µM–1 mM) was added to the defined media, there was a dose-dependent increase in anti-Neu5Gc binding, again, compared with no binding by the control IgY antibody (Fig. 3 A). Similar results were found (unpublished data) using two other NTHi strains, int1 (Nizet et al., 1996) and DH1 (Houliston et al., 2007), as well as one encapsulated type b strain, Eagan (Anderson et al., 1972). Thus, uptake and surface expression Neu5Gc is a common feature among different strains of H. influenzae.

To further confirm Neu5Gc incorporation by NTHi, 2019 was grown in 1 mM Neu5Gc, treated with sialidase or heat-inactivated sialidase, and then probed in a whole-cell ELISA with the chicken anti-Neu5Gc antibody. Sialidase treatment decreased anti-Neu5Gc staining when compared with treatment with heat-inactivated sialidase, demonstrating release of Neu5Gc from NTHi surface glycans by active sialidase (Fig. 3 B). Finally, no Neu5Gc expression was seen in NTHi strain 2019 sialT (Allen et al., 2005), an isogenic mutant that lacks a critical Sia transporter (see Fig. 6 A, top left). Collectively, our data indicate that dietary Neu5Gc may be efficiently taken up and then expressed on cell surface molecules of NTHi.

**Infant antibodies against Neu5Gc-glycans appear coincident with the appearance of antibodies against NTHi**

Most humans are colonized by NTHi (Turk, 1984). We next asked whether there was a temporal correlation between the timing of initial NTHi colonization or infection and the appearance of anti-Neu5Gc antibodies. As neither nasopharyngeal nor middle ear cultures were available from the infants in our cohort, we used a whole-cell ELISA to screen for antibodies against NTHi as an indicator of colonization or infection. For these studies, NTHi was grown in Sia-free defined media to ensure absence of Neu5Gc on the LOS of the bacteria. In all infants analyzed, we could detect IgM antibodies

![Figure 3](image-url). NTHi can efficiently take up and incorporate Neu5Gc. (A) Dose-dependent uptake and expression of NTHi (2019) grown in a Sia-free defined media with 0.1 µM – 1 mM Neu5Gc. Neu5Gc was detected by flow cytometry analysis using a chicken anti-Neu5Gc IgY antibody. (B) 2019 grown in 1 mM Neu5Gc were treated with sialidase (sialidase) or heat-inactivated sialidase (mock sialidase) and probed with a chicken anti-Neu5Gc IgY antibody in a whole-cell ELISA. Data are representative of greater than three independent experiments and show the mean of triplicate absorbance values at OD405. Error bars represent SD.
against NTHi that increased significantly between birth, 3, 6, and 12 mo (Fig. 4). Because it is unlikely that all infants in our cohort have had NTHi otitis media by 3 mo of age, it is likely that these increased titers of antibody against whole-cell NTHi are a response to NTHi colonization. These results are consistent with those of others who have shown that most infants acquire NTHi within the first year of life (Vives et al., 1997), and that infants generate antibodies against outer membrane proteins on NTHi during the course of asymptomatic colonization (Faden, 2001). Thus, infants in our cohort generated an adaptive immune response against colonizing NTHi at about the same time that anti-Neu5Gc antibodies appear.

NTHi with Neu5Gc-containing LOS can induce IgM and IgG anti–Neu5Gc antibodies in Neu5Gc-deficient mice

Given the temporal relationship between the appearance of anti–Neu5Gc antibodies and colonization with NTHi, we next asked whether Neu5Gc-expressing NTHi could elicit an anti–Neu5Gc antibody response in Neu5Gc-deficient mice, which did not spontaneously express anti–Neu5Gc antibodies (Table I). To this end, Cmah−null mice were injected intraperitoneally with heat-killed NTHi, which had been grown in Sia-free media with Neu5Gc (generating Neu5Gc-expressing NTHi, Gc–NTHi) or without Neu5Gc (generating Sia-free NTHi, Sia-free NTHi). To assess the inherent immunogenicity of the Gc–NTHi, no adjuvant was used to enhance antibody responses. Sia-free NTHi represents an ideal negative control because, except for the absence of Neu5Gc, it is identical to Gc–NTHi. Indeed, only Cmah−null mice injected with Gc–NTHi generated anti–Neu5Gc IgM antibodies and underwent class switching to generate IgG antibodies after 2–3 injections (Fig. 5, A and B). In contrast, Cmah−null mice injected with Sia-free NTHi did not generate anti–Neu5Gc antibodies (Fig. 5, A and B). Multiple attempts to elicit anti–Neu5Gc antibodies in Cmah−null mice via intranasal administration of Neu5Gc-expressing NTHi have been unsuccessful (unpublished data). This is not surprising, given that mice show very rapid mucosal clearance of this human-specific microorganism and limited nasopharyngeal colonization (Zola et al., 2009). Furthermore, human infants are prone to major upper respiratory infections with NTHi, and the immune response we are studying would likely be enhanced by such inflammation in infants. The anti-Neu5Gc IgG antibodies generated in Cmah−null mice were also tested for reactivity against different Neu5Gc containing antigens and found to be of similar titer and specificity to those we have found in human infants (Fig. 5 C) and adults (Padler-Karavani et al., 2008).

Figure 4. Anti-NTHi antibodies in human infants. Infant sera (same infants analyzed in Fig. 1; n = 15) were analyzed by whole-cell ELISA for IgM antibodies against NTHi strain 2019 grown in Sia-free media (filled circles). Adult sera (n = 9) obtained from the pregnant mothers of the infants in this study were analyzed in parallel with infant sera for IgM anti-NTHi antibodies (open squares). Each circle or square represents the mean of triplicate values from one individual, quantified according to an IgM standard curve. Horizontal lines represent mean values for each group. Statistical analysis was performed using an unpaired two-tailed Student’s t test.

Figure 5. Neu5Gc expressed on NTHi induces anti–Neu5Gc antibodies in Cmah−/− mice. (A and B) Cmah−/− mice were injected intraperitoneally with heat-killed NTHi (without adjuvant) which had been grown in sialic-acid-free media (Sia-free NTHi, n = 16) or with sialic-acid-free media with 1 mM Neu5Gc (Gc–NTHi, n = 17). All mice were injected a total of three times at 2-wk intervals. Sera collected after the third injection were analyzed by ELISA for IgM (A) and IgG (B) antibodies against Neu5Gcα-PAA. The highest and lowest value from each group was removed before graphing. Horizontal lines represent the mean values. Statistical analysis was performed using an unpaired one-tailed Student’s t test. (C) Pooled mouse serum from mice injected intraperitoneally with heat-killed NTHi grown in sialic-acid-free media with 1 mM Neu5Gc was analyzed in an ELISA for levels of anti–Neu5Gc IgG antibodies against Neu5Gcαx2–3Galβ1–4Glcβ–HSA (black bar), Neu5Gcαx2–6Galβ1–4Glcβ–HSA (gray bar), Neu5Gcαx2–3Galβ1–4GlcNαcβ–HSA (white hashed bar), and Neu5Gcαx2–6Galβ1–4GlcNαcβ–HSA (white bar). Values represent mean IgG levels, quantified according to a mouse IgG standard curve. Error bars represent SD. For comparison, serum from 12-mo-old infants (n = 15) was analyzed for levels of anti–Neu5Gc antibodies against Neu5Gcαx2–3Galβ1–4Glcβ–HSA (black bar), Neu5Gcαx2–6Galβ1–4Glcβ–HSA (gray bar; same data as shown in Fig. 1 B). Values represent mean IgG levels, quantified according to a human IgG standard curve. Error bars represent SEM. Data are representative of greater than three independent experiments.
Ham (5.13 ± 0.98 g/g) Turkey (<0.03 ± 0.07 g/g) Lamb (<0.03 ± 1.56 g/g) Red meat (<0.07 ± 2.68 g/g) Poultry 5.40 ± 10.19 g/g

Values from two independent experiments. Previous studies have shown that plants are devoid of sialic acids (Zeleny et al., 2006).

A, WT NTHi strain 2019 (WT, solid line) and Sia transporter mutant strain of 2019 (SiaT Mutant, dashed line) were grown in Sia-free media with or without the addition of commercially available baby foods and analyzed by flow cytometry for Neu5Gc staining using the chicken anti-Neu5Gc IgY antibody. Secondary antibody for WT and SiaT Mutant (not depicted) and IgY control for SiaT Mutant (not depicted) all showed similar shifts compared with the IgY control for WT (solid gray). [B] Anti-Neu5Gc IgG antibodies purified from serum from a single individual (S34) was used to probe NTHi in a whole-cell ELISA. NTHi was grown in Sia-free media (Sia-Free NTHi) or Sia-free media with 1 mM Neu5Gc (Gc-NTHi), and binding of human antibodies is shown the mean of triplicate values at OD405. Error bars represent SD. Statistical analysis was performed using an unpaired two-tailed Student’s t test. *** P < 0.0001. Data are representative of two (A) and three (B) independent experiments.

**DISCUSSION**

In this study, we propose a model for how NTHi and dietary Neu5Gc cooperate to generate anti-Neu5Gc antibodies in humans. Collectively, our data indicate a mechanism by which humans may generate anti-Neu5Gc antibodies in early life, by simultaneous exposure to Neu5Gc-containing foods and the incorporation and surface expression of the nonhuman Sia by colonizing NTHi. As a Gram-negative bacterium that expresses pathogen-associated molecular patterns such as LPS, Sia by colonizing NTHi. As a Gram-negative bacterium that expresses pathogen-associated molecular patterns such as LPS, NTHi that are colonizing the infant’s oropharynx and even upper airways can have direct access to dietary Neu5Gc, since reflux of ingested liquids into the infant upper respiratory tract is commonly observed (Cober and Johnson, 2005). To determine if NTHi can take up and express Neu5Gc from baby foods containing red meat, but not in those containing poultry or plants (Table II). Of course, many other oral and nasopharyngeal commensal bacteria express sialidases (Corfield, 1992), which could further increase the local concentrations of free Neu5Gc for use by NTHi in vivo.

**Purified human anti-Neu5Gc antibodies specifically recognize Neu5Gc-expressing NTHi**

To further corroborate our hypothesis, we asked if anti-Neu5Gc antibodies that were affinity purified from normal human serum (Padler-Karavani et al., 2008) could interact with Neu5Gc-expressing NTHi in a whole-cell ELISA. Indeed, human anti-Neu5Gc antibodies bound specifically to Neu5Gc-expressing NTHi (Gc-NTHi) and not to nonglycosidically bound Sia by colonizing NTHi (Sia-Free NTHi; Fig. 6 B).

NTHi can scavenge and express Neu5Gc from Neu5Gc-Containing Foods

NTHi that are colonizing the infant’s oropharynx and even upper airways can have direct access to dietary Neu5Gc, since reflux of ingested liquids into the infant upper respiratory tract is commonly observed (Cober and Johnson, 2005). To determine if NTHi can take up and express Neu5Gc from baby food, NTHi was grown in Sia-free media with or without various commercially available semi-solid baby foods and analyzed by flow cytometry for cell-surface Neu5Gc. As shown in Fig. 6 A, WT NTHi strain 2019 (WT) was found to express Neu5Gc when grown in the presence of baby foods consisting of red meat (beef, pork, and lamb) but not in poultry (chicken and turkey; unpublished data), vegetables or fruits. In contrast, there was no Neu5Gc expression seen in the siaT mutant of 2019, confirming that uptake through the Sia transporter is required for expression of dietary Neu5Gc by NTHi. Furthermore, an even greater shift in anti-Neu5Gc staining (an approximately fourfold increase in MFI compared with WT) was seen with a NTHi sialic acid lyase-deficient mutant (2019nanA; unpublished data), which develops a hyper-sialylated phenotype in the presence of exogenous Sia (Allen et al., 2005). The finding that Neu5Gc expression by NTHi was restricted to uptake from baby foods containing red meat is not surprising, as red meat is known to contain high levels of Neu5Gc, whereas poultry contains only Neu5Ac and plants contain no Sia (Tangvoranuntakul et al., 2003). NTHi do not produce a sialidase (Vimr and Lichtensteiger, 2002), and therefore require free (nonglycosidically bound) Sia for LOS sialylation. Indeed, when Sia levels were quantified in the baby food, considerable levels of free Neu5Gc (~2 µg/g; Table II) were detected in baby food containing red meat, but not in those containing poultry or plants (Table II). Of course, many other oral and nasopharyngeal commensal bacteria express sialidases (Corfield, 1992), which could further increase the local concentrations of free Neu5Gc for use by NTHi in vivo.

**Table II.** Quantification of Neu5Gc in commercial baby food

| Baby foods | Red meat | Poultry |
|------------|----------|---------|
|            | Beef (n = 2) | Lamb (n = 1) | Ham (n = 1) | Chicken (n = 2) | Turkey (n = 2) |
| Total Neu5Gc µg/g | 10.19 | 5.40 | 5.13 | <0.07 | <0.07 |
| Free Neu5Gc µg/g | 2.68 | 1.56 | 0.98 | <0.03 | <0.03 |

*Total and free (nonglycosidically bound) Neu5Gc in commercial baby foods was determined by DMB-HPLC as described in Materials and methods. Data represent average values from two independent experiments. Previous studies have shown that plants are devoid of sialic acids (Zeleny et al., 2006).
this carbohydrate antigen elicits class switching to generate sometimes high levels of IgG antibodies. In this regard, direct engagement of LPS with TLR4 on B cells of mice is known to promote proliferation, class switching, and immunoglobulin secretion (Peng, 2005). Furthermore, LPS-induced secretion of BAFF and APRIL by dendritic cells and monocytes could contribute to T cell–independent induction of class switch recombination (Fagarasan and Honjo, 2000; Litinskiy et al., 2002), especially in the setting of inflammation (Ueda et al., 2007) that is commonly associated with NTHi-mediated otitis media. Future studies of mice and humans with various genetically defined immunodeficiencies should help to define the cellular and molecular pathways required for the generation of anti-Neu5Gc antibodies, and address the question of T cell dependence versus independence, and the role of Toll-like receptors. Identifying the specific B cell populations that produce anti-Neu5Gc antibodies and determining if these B cells undergo somatic hypermutation will also help to characterize the anti-Neu5Gc response in humans and mice. An additional contributing factor may be the relative variability of human B cells to stimulation (Soto et al., 2010).

We have shown here that NTHi is capable of scavenging Neu5Gc from the diet and expressing it as an immunogenic epitope, apparently contributing to the generation of anti-Neu5Gc antibodies in humans. Thus, our normal flora can act as “antigen-presenting cells” of bacterial rather than host origin, eliciting humoral immune responses that could contribute to inflammatory or autoimmune pathologies. Although NTHi is the first known commensal shown to express scavenged dietary Neu5Gc, it is possible that other as yet unknown commensal/pathogenic bacteria can do the same. In this regard, the wide variation in adult human anti-Neu5Gc titers and specificity between adult individuals (Padder-Karavana et al., 2008) may reflect multiple routes of xeno-autoimmunization.

**MATERIALS AND METHODS**

Mice. *C. albicans*–/– mice (Hedlund et al., 2007) were backcrossed onto a congenic C57BL/6 background. WT C57BL/6 mice were purchased from Harlan Laboratories. Mice were fed standard chow (PicoLab Rodent Diet 20; LabDiet) and water ad libitum and maintained on a 12-h light/dark cycle. All animal work was performed in accordance with The Association for Assessment and Accreditation of Laboratory Animal Care and under a protocol approved by the Institutional Animal Care and Use Committee of the University of California, San Diego, La Jolla, CA.

Human serum samples. Collection of maternal and infant blood samples for immunological studies was approved by the University of La Frontera, Temuco, Chile, Institutional Review Board and approved by the Regional Ethical Committee of the Chilean National Health Service for the Araucania Region in which the samples were collected. Written informed consent for study of infants was obtained from both parents. Serum was collected from 15 Chilean infants at birth (from cord blood), 3, 6, and 12 mo of age. Serum from 9 of the 15 mothers in the study was obtained during the third trimester of pregnancy, with consent.

Bacteria, growth conditions, and baby food. NTHi strains 2019 (Campagnari et al., 1987) and 2019aT (Allen et al., 2005) were a generous gift from Michael Apicella, Department of Microbiology, University of Iowa. Sialic acid–free bacterial stocks were prepared by passaging 2019 several times in sialic acid–free media: RPMI 1640 media (Sigma-Aldrich) supplemented with 1 µg/ml protoporphyrin IX (Sigma-Aldrich), 1 µg/ml l-lysine (Sigma-Aldrich), 10 µg/ml β-nicotinamide adenine dinucleotide (Sigma-Aldrich), 0.1 mg/ml hypoxanthine (Sigma-Aldrich), 0.1 mg/ml uracil (Sigma-Aldrich), and 0.8 mM sodium pyruvate (Invitrogen; Greiner et al., 2004; Allen et al., 2005). The absence of sialic acid was confirmed by HPLC and mass spectrometry. Commercial baby foods from Gerber Product Company and Beech-Nut Nutrition Company were purchased at a local grocery store.

**Neu5Gc uptake by NTHi.** NTH strain 2019 or 2019aT grown to mid-log (OD$_{600}$ < 0.3–0.4) in sialic acid–free media, was grown for 2 h with or without various amounts of Neu5Gc (Inalco), washed twice with PBS, and then incubated for 1 h at RT with chicken anti-Neu5Gc IgY (1:2,000; Diaz et al., 2009), control chicken IgY (1:2,000; Jackson ImmunoResearch Laboratories), or PBS alone. After washing once with PBS, bacteria were resuspended in FITC donkey anti-chicken antibody (1:200; Jackson ImmunoResearch Laboratories) for 1 h at room temperature and analyzed by flow cytometry (FACS-Calibur; BD). For uptake of Neu5Gc from baby food, each food was diluted with an equal volume of sialic acid–free media and the particulate matter pelleted by centrifugation. After filter sterilization, the filtrate was added to an equal volume of mid-log bacteria in sialic acid–free media, undergoing incubation shaking (250 RPM) for 2 h at 37°C, washed twice with PBS, and stained for Neu5Gc by flow cytometry, as described above. Sialidase treatment after Neu5Gc loading of NTHi was performed by resuspending 500 µl (OD$_{600}$ 0.4) in 150 µl PBS, pH 6.0 + 9 mM CaCl$_2$, with 10 µM active or heat-inactivated (10 min at 100°C) *Fibrobacter succinogenes* sialidase (Sigma-Aldrich) for 3 h at 37°C. Neu5Gc was detected in a whole-cell ELISA by resuspending the bacteria in Mill-Q water, adding to a 96-well plate (Corning), and evaporating overnight. Wells were washed with Tris-buffered saline, pH 7.4, + 0.1% Tween (TBST), and then incubated with chicken anti-Neu5Gc IgY (1:1,000), isotype control chicken IgY (1:1,000), or TBST alone for 1 h at room temperature, 100 µl/well. Wells were then washed three times with 150 µl TBST, incubated with 100 µl/well alkaline phosphatase (AP) donkey anti-chicken IgY (1:1,000; Jackson ImmunoResearch Laboratories) for 1 h at room temperature, washed as before, and then developed with p-nitrophenyl phosphate, with product measured at 405-nm wavelength on a SpectraMax 250 (MDS Analytical Technologies).

**Generation of anti-Neu5Gc antibodies in mice.** NTH strain 2019 was grown to mid-log in sialic acid–free media with or without 1 mM Neu5Gc (Inalco), heat-killed, and injected (200 µl of OD$_{600}$ 0.4) intraperitoneally into *C. albicans*-null mice (WT C57BL/6 mice (age 5–8 wk, female). All mice were injected a total of three times at 2-wk intervals. No adjuvant was used with any of the bacterial injections. Erythrocyte immunizations were performed as described previously (Hedlund et al., 2008). In brief, *C. albicans*-null mice (age 6–9 wk, male and female) were injected intraperitoneally with 200 µg chimpanzee (Neu5Gc-rich; Yerkes National Primate Research Center, Emory University, Emory, GA) or human (Neu5Gc-free) erythrocyte membrane ghosts in 100 µl PBS with equal volume Freund’s complete adjuvant (Difco), and boosted twice (2 and 8 wk later) with the same amount of immunogen in Freund’s incomplete adjuvant (Difco). Serum for antibody analysis was collected 7 d after the second boost.

**Detection of mouse anti-Neu5Gc antibodies.** Mouse sera were analyzed for anti-Neu5Gc antibodies against Neu5Gc-PA (Glycotech) by ELISA, as previously described (Hedlund et al., 2008). In brief, 96-well plates (Costar 9018; Corning) were coated overnight at 4°C with 250 ng/well Neu5Gc-PA in 50 mM sodium carbonate-bicarbonate buffer, pH 9.5. Wells were emptied of coating solution and blocked with 200 µl/well TBS + 0.1% Tween, pH 7.4 (TBST) for 2 h at room temperature. Sera were added to the wells for 2 h at room temperature in triplicate, diluted 1:200 in 100 µl TBST. After washing three times with 150 µl TBST, wells were incubated with 100 µl alkaline phosphatase-conjugated goat anti-mouse IgM (Calbiochem) or IgG (Jackson ImmunoResearch Laboratories), diluted 1:5,000 in TBST for 1 h at RT. Wells were washed again, as described,
Quantification of Neu5Gc in commercial baby foods. For analysis of nonglycosidically bound (free) Neu5Gc in baby food, 50 mg of each baby food was resuspended in 200 µl Milli-Q water (for analysis of total Neu5Gc, samples were heated to 80°C in 2 M acetic acid for 3 h to release sialic acids) and centrifuged at 10,000 × g for 10 min at 4°C. The supernatant was then filtered through a 10,000 molecular weight cutoff filter, and sialic acids in the filtrate were derivatized with 1,2-diamino-4,5-methylenedioxybenzene (DMB; Sigma-Aldrich) as described previously (Manzi et al., 1990) and analyzed by reverse-phase HPLC using a C18 column (Phenomenex) at a flow rate of 0.9 ml/min, using 88% water, 5% acetonitrile, and 7% methanol. The excitation and emission were at 373 and 448 nm, respectively. The DMB-derivatized sialic acids were identified and quantified by comparing elution times and peak areas to known standards.

Statistics. Statistical analysis was performed using Prism v5.0a (GraphPad Software).

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A. Varki is a co-founder of, and shareholder in Sialix, Inc. (formerly Gc-Free, Inc.), a startup biotech company focused on solving problems arising from the pathological consequences of Neu5Gc and Neu5Gc-antibody interactions in humans. D. Ghaderi is currently an employee of Sialix, Inc. The authors have no additional competing financial interests.

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REFERENCES

Allen, S., A. Zaleski, J.W. Johnston, B.W. Gibson, and M.A. Apicella. 2005. Novel sialic acid transporter of Haemophilus influenzae. Infect. Immun. 73:5291–5300. doi:10.1128/IAI.73.5291-5300.2005

Anderson, P., R.B.J. Johnston Jr., and D.H. Smith. 1972. Human serum activities against Hemophilus influenzae, type b. J. Clin. Invest. 51:31–38. doi:10.1172/JCI106793

Angata, T., and A. Varki. 2002. Chemical diversity in the sialic acids and related alpha-keto acids: an evolutionary perspective. Chem. Rev. 102:439–469. doi:10.1021/cr000407m

Bardor, M., D.H. Nguyen, S. Diaz, and A. Varki. 2005. Mechanism of uptake and incorporation of the non-human sialic acid N-glycolytransferase acid into human cells. J. Biol. Chem. 280:4228–4237. doi:10.1074/jbc.M412040200

Bouchet, V., D.W. Hood, J. Li, J.R. Brinon, G.A. Randle, A. Martin, Z. Li, R. Goldstein, E.K. Schweda, S.I. Pelton, et al. 2003. Host-derived sialic acid is incorporated into Haemophilus influenzae lipopolysaccharide and is a major virulence factor in experimental otitis media. Proc. Natl. Acad. Sci. U.S.A. 100:8898–8903. doi:10.1073/pnas.1432026100

Campagnari, A.A., M.R. Gupta, K.C. Dudas, T.F. Murphy, and M.A. Apicella. 1987. Antigenic diversity of lipopolysaccharides of nontypable Haemophilus influenzae. Infect. Immun. 55:882–887.

Carapella, A., H. Takematsu, H. Liu, S. Diaz, K. Haider, C. Boboila, G. Kalloo, M. Conolle, H.N. Shi, N. Varki, et al. 2009. B cell antigen receptor signal strength and peripheral B cell development are regulated by a 9-O-acetyl sialic acid esterase. J. Exp. Med. 206:125–138. doi:10.1084/jem.20081399

Carlin, A.F., S. Uchiyama, Y.C. Chang, A.L. Lewis, V. Nizet, and A. Varki. 2009. Molecular mimicry of host sialylated glycans allows a bacterial pathogen to engage neutrophil Siglec-9 and dampen the innate immune response. Blood. 113:3333–3336. doi:10.1182/blood-2008-11-187302

Chen, X., and A. Varki. 2010. Advances in the biology and chemistry of sialic acids. ACS Chem. Biol. 5:163–176. doi:10.1021/cb900236r

Chou, H.H., H. Takematsu, S. Diaz, J. Iber, E. Nickerson, K.L. Wright, E.A. Muchmore, D.L. Nelson, S.T. Warren, and A. Varki. 1998.
A mutation in human CMP-sialic acid hydroxylase occurred after the Homo-Pan divergence. Proc. Natl. Acad. Sci. USA. 95:11751–11756. doi:10.1073/pnas.95.20.11751

Cober, M.P., and C.E. Johnson. 1999. Optical resolution of bacterial sialic acid. Glycobiology. 9:47–56. doi:10.1093/glycob/9.1.47

Diaz, S.L., V. Padler-Karavani, D. Ghaden, N. Hurtado-Zoila, H. Yu, X. Chen, E.C. Brinkman-Van der Linden, A. Venkati, and N.M. Varki. 2009. Sialylation of the human salivary acid N-glycolylneuraminic acid in human tissues and biotherapeutic products. PLoS One. 4:e4241. doi:10.1371/journal.pone.0004241

Estabrook, M.M., J.M. Griffiss, and G.A. Jarvis. 1997. Sialylation of Neisseria meningitidis lipooligosaccharide inhibits serum bactericidal activity by masking lacto-N-neotetraose. Infect. Immun. 65:4436–4444.

Faden, H. 2001. The microbiologic and immunologic basis for recurrent otitis media. Adv. OtoRhinolaryngol. 56:1830–1841. doi:10.1007/s004310100754

Fagervan, S., and T. Honjo. 2000. T-Independent immune response: New aspects of B cell biology. Science. 290:89–92. doi:10.1126/science.290.5489.89

Galili, U., R.E. Mandrell, M. Shero, P. Casali, A.F. Ryan, and A.C. Schaffer. 1998. The microbiologic and immunologic basis for recurrent otitis media and in vivo modification of Neisseria meningitidis lipooligosaccharide inhibits serum bactericidal activity by masking lacto-N-neotetraose. Infect. Immun. 65:4436–4444.

Faden, H. 2001. The microbiologic and immunologic basis for recurrent otitis media in children. Eur. J. Pediatr. 160:407–413. doi:10.1007/s004310100754

Fagervan, S., and T. Honjo. 2000. T-Independent immune response: New aspects of B cell biology. Science. 290:89–92. doi:10.1126/science.290.5489.89

Galili, U., R.E. Mandrell, M. Shero, P. Casali, A.F. Ryan, and A.C. Schaffer. 1998. The microbiologic and immunologic basis for recurrent otitis media and in vivo modification of Neisseria meningitidis lipooligosaccharide inhibits serum bactericidal activity by masking lacto-N-neotetraose. Infect. Immun. 65:4436–4444.

Faden, H. 2001. The microbiologic and immunologic basis for recurrent otitis media in children. Eur. J. Pediatr. 160:407–413. doi:10.1007/s004310100754

Fagervan, S., and T. Honjo. 2000. T-Independent immune response: New aspects of B cell biology. Science. 290:89–92. doi:10.1126/science.290.5489.89

Galili, U., R.E. Mandrell, M. Shero, P. Casali, A.F. Ryan, and A.C. Schaffer. 1998. The microbiologic and immunologic basis for recurrent otitis media and in vivo modification of Neisseria meningitidis lipooligosaccharide inhibits serum bactericidal activity by masking lacto-N-neotetraose. Infect. Immun. 65:4436–4444.

Faden, H. 2001. The microbiologic and immunologic basis for recurrent otitis media in children. Eur. J. Pediatr. 160:407–413. doi:10.1007/s004310100754

Fagervan, S., and T. Honjo. 2000. T-Independent immune response: New aspects of B cell biology. Science. 290:89–92. doi:10.1126/science.290.5489.89

Galili, U., R.E. Mandrell, M. Shero, P. Casali, A.F. Ryan, and A.C. Schaffer. 1998. The microbiologic and immunologic basis for recurrent otitis media and in vivo modification of Neisseria meningitidis lipooligosaccharide inhibits serum bactericidal activity by masking lacto-N-neotetraose. Infect. Immun. 65:4436–4444.

Faden, H. 2001. The microbiologic and immunologic basis for recurrent otitis media in children. Eur. J. Pediatr. 160:407–413. doi:10.1007/s004310100754

Fagervan, S., and T. Honjo. 2000. T-Independent immune response: New aspects of B cell biology. Science. 290:89–92. doi:10.1126/science.290.5489.89

Galili, U., R.E. Mandrell, M. Shero, P. Casali, A.F. Ryan, and A.C. Schaffer. 1998. The microbiologic and immunologic basis for recurrent otitis media and in vivo modification of Neisseria meningitidis lipooligosaccharide inhibits serum bactericidal activity by masking lacto-N-neotetraose. Infect. Immun. 65:4436–4444.

Faden, H. 2001. The microbiologic and immunologic basis for recurrent otitis media in children. Eur. J. Pediatr. 160:407–413. doi:10.1007/s004310100754

Fagervan, S., and T. Honjo. 2000. T-Independent immune response: New aspects of B cell biology. Science. 290:89–92. doi:10.1126/science.290.5489.89

Galili, U., R.E. Mandrell, M. Shero, P. Casali, A.F. Ryan, and A.C. Schaffer. 1998. The microbiologic and immunologic basis for recurrent otitis media and in vivo modification of Neisseria meningitidis lipooligosaccharide inhibits serum bactericidal activity by masking lacto-N-neotetraose. Infect. Immun. 65:4436–4444.

Faden, H. 2001. The microbiologic and immunologic basis for recurrent otitis media in children. Eur. J. Pediatr. 160:407–413. doi:10.1007/s004310100754

Fagervan, S., and T. Honjo. 2000. T-Independent immune response: New aspects of B cell biology. Science. 290:89–92. doi:10.1126/science.290.5489.89

Galili, U., R.E. Mandrell, M. Shero, P. Casali, A.F. Ryan, and A.C. Schaffer. 1998. The microbiologic and immunologic basis for recurrent otitis media and in vivo modification of Neisseria meningitidis lipooligosaccharide inhibits serum bactericidal activity by masking lacto-N-neotetraose. Infect. Immun. 65:4436–4444.
Soto, P.C., L.L. Stein, N. Hurtado-Zaza, S.M. Hedrick, and A. Varki. 2010. Relative over-reactivity of human versus chimpanzee lymphocytes: implications for the human diseases associated with immune activation. *J. Immunol.* 184:4185–4195. doi:10.4049/jimmunol.0903420

Springer, G.F., and R.E. Horton. 1969. Blood group isoantibody stimulation in man by feeding blood group-active bacteria. *J. Clin. Invest.* 48:1280–1291. doi:10.1172/JCI106094

Tahara, H., K. Ide, N.B. Basnet, Y. Tanaka, H. Matsuda, H. Takematsu, Y. Kozutsumi, and H. Ohdan. 2010. Immunological property of antibodies against N-glycolyneuraminic acid epitopes in cytidine monophospho-N-acetylneuraminic acid hydroxylase-deficient mice. *J. Immunol.* 184:3269–3275. doi:10.4049/jimmunol.090285

Tangvoranuntakul, P., P. Gagneux, S. Diaz, M. Bardor, N. Varki, A. Varki, and E. Muchmore. 2003. Human uptake and incorporation of an immunogenic nonhuman dietary sialic acid. *Proc. Natl. Acad. Sci. USA.* 100:12045–12050. doi:10.1073/pnas.2131556100

Traving, C., and R. Schauer. 1998. Structure, function and metabolism of sialic acids. *Cell. Mol. Life Sci.* 54:1330–1349. doi:10.1007/s000180050258

Turk, D.C. 1984. The pathogenicity of *Haemophilus influenzae*. *J. Med. Microbiol.* 18:1–16. doi:10.1099/00222615-18-1-1

Ueda, Y., D. Liao, K. Yang, A. Patel, and G. Kelsoe. 2007. T-independent activation-induced cytokine deaminase expression, class-switch recombination, and antibody production by immature/transitional 1 B cells. *J. Immunol.* 178:3593–3601.

Van Lenten, L., and G. Ashwell. 1971. Studies on the chemical and enzymatic modification of glycoproteins. A general method for the tritiation of sialic acid-containing glycoproteins. *J. Biol. Chem.* 246:1889–1894.

Varki, A. 1992. Diversity in the sialic acids. *Glycobiology.* 2:25–40. doi:10.1093/glycob/2.1.25

Vimr, E., and C. Lichtensteiger. 2002. To sialylate, or not to sialylate: that is the question. *Trends Microbiol.* 10:254–257. doi:10.1016/S0966-842X(02)02361-2

Vimr, E.R., K.A. Kalivyda, E.L. Deseo, and S.M. Steenbergen. 2004. Diversity of microbial sialic acid metabolism. *Microbiol. Mol. Biol. Rev.* 68:132–153. doi:10.1128/MMBR.68.1.132-153.2004

Vives, M., M.E. Garcia, P. Saenz, M.A. Mora, L. Mata, H. Sabharwal, and C. Svanborg. 1997. Nasopharyngeal colonization in Costa Rican children during the first year of life. *Pediatr. Infect. Dis. J.* 16:852–858. doi:10.1097/00006454-199709000-00007

Yin, J., A. Hashimoto, M. Izawa, K. Miyazaki, G.Y. Chen, H. Takematsu, Y. Kozutsumi, A. Suzuki, K. Futuhata, F.L. Cheng, 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. doi:10.1158/0008-5472.CAN-05-2615

Yu, H., H.A. Chokhawala, A. Varki, and X. Chen. 2007. Efficient chemoenzymatic synthesis of biotinylated human serum albumin–sialoglycoside conjugates containing O-acetylated sialic acids. *Org. Biomol. Chem.* 5:2458–2463. doi:10.1039/b706507h

Zeleny, R., D. Kolarich, R. Strasser, and F. Altmann. 2006. Sialic acid concentrations in plants are in the range of inadvertent contamination. *Planta.* 224:222–227. doi:10.1007/s00425-005-0206-8

Zhu, A., and R. Hurst. 2002. Anti-N-glycolyneuraminic acid antibodies identified in healthy human serum. *Xenotransplantation.* 9:376–381. doi:10.1034/j.1399-3089.2002.02138.x

Zola, T.A., E.S. Lysenko, and J.N. Weiser. 2009. Natural antibody to conserved targets of *Haemophilus influenzae* limits colonization of the murine nasopharynx. *Infect. Immun.* 77:3458–3463. doi:10.1128/IAI.01564-08