

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

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The nonhuman sialic acid *N*-glycolylneuraminic acid (Neu5Gc) is metabolically incorporated into human tissues from certain mammalian-derived foods, and this occurs in the face of an anti-Neu5Gc "xeno-autoantibody" response. Given evidence that this process contributes to chronic inflammation in some diseases, it is important to understand when and how these antibodies are generated in humans. We show here that human anti-Neu5Gc antibodies appear during infancy and correlate with weaning and exposure to dietary Neu5Gc. However, dietary Neu5Gc alone cannot elicit anti-Neu5Gc antibodies in mice with a humanlike Neu5Gc deficiency. Other postnatally appearing anti-carbohydrate antibodies are likely induced by bacteria expressing these epitopes; however, no microbe is known to synthesize Neu5Gc. Here, we show that trace exogenous Neu5Gc can be incorporated into cell surface lipooligosaccharides (LOS) of nontypeable *Haemophilus influenzae* (NTHi), a human-specific commensal/pathogen. Indeed, infant anti-Neu5Gc antibodies appear coincident with antibodies against NTHi. Furthermore, NTHi that express Neu5Gc-containing LOS induce anti-Neu5Gc antibodies in Neu5Gc-deficient mice, without added adjuvant. Finally, Neu5Gc from baby food is taken up and expressed by NTHi. As the flora residing in the nasopharynx of infants can be in contact with ingested food, we propose a novel model for how NTHi and dietary Neu5Gc cooperate to generate anti-Neu5Gc antibodies in humans.

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Abbreviations used: DMB, 1,2-diamino-4,5-methylene-dioxybenzene; LOS, lipooligosaccharides; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; NTHi, non-typeable *Haemophilus influenzae*; Sia, Sialic acid.

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

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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 Neu5Gc α 2–6Gal β 1–4Glc (Gc α 2–6Lac), 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 Gc α 2–6Lac 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

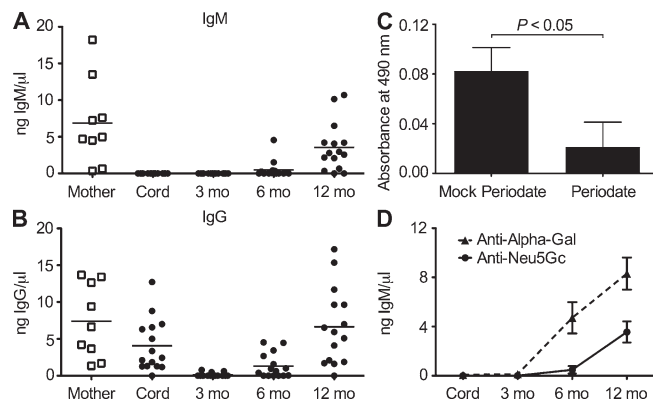


Figure 1. Anti-Neu5Gc antibodies in human infants. (A and B) Levels of anti-Neu5Gc IgM (A) and IgG (B) antibodies in infant sera ($n = 15$, filled circles) from birth (cord), 3, 6, at 12 mo (for each infant) and adult sera from the pregnant mothers ($n = 9$, open squares) were measured by ELISA against Neu5Gc α 2–6Lac β -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) Neu5Gc α 2–6Lac β -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 OD₄₉₀ \pm 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 \pm SEM.

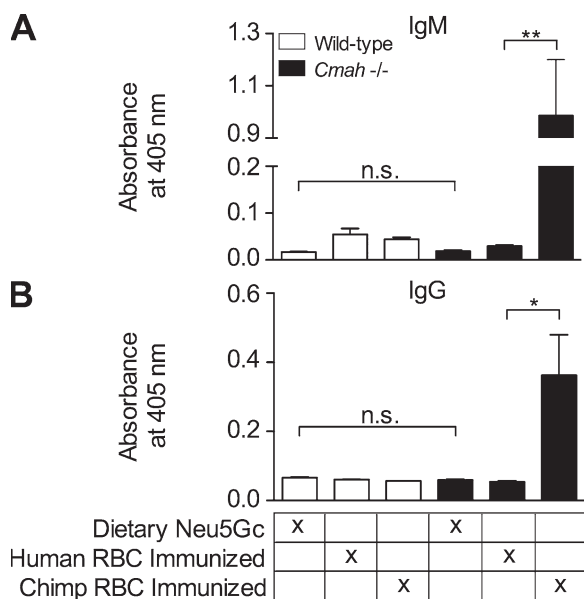


Figure 2. Dietary Neu5Gc does not elicit anti-Neu5Gc antibodies.

(A and B) WT (open bars, $n = 8$) and *Cmah*^{-/-} (filled bars, $n = 8$) were fed ~ 1 mg/kg/day 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 α -PAA and shown as mean absorbance values at OD₄₀₅ \pm 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.

synthesis (Hedlund et al., 2007). However, despite consumption of ~ 1 mg/kg/day of Neu5Gc in their regular chow (unpublished data; this represents approximately five times the amount present in the typical human Western diet; Tangvoranuntakul et al., 2003), *Cmah*-null mice did not spontaneously generate anti-Neu5Gc IgM or IgG antibodies after 6–9 mo (Fig. 2, A and B). In fact, as summarized in Table I, all attempts to induce anti-Neu5Gc antibodies in *Cmah*-null mice via dietary Neu5Gc exposure have been

unsuccessful to date. These include: providing free Neu5Gc in the drinking water at 1 mg/ml for 12 wk; cross-fostering *Cmah*-null pups with WT dams whose milk contains Neu5Gc (simulating cow's milk-based formula feeding in human infants); feeding with high-Neu5Gc content mucins; feeding with Neu5Gc-rich goat cheese; and oral gavage with cow's milk (unpublished data; see Table I for details). These negative results are also interesting because *Cmah*-null mice actually have relatively hyper-reactive B cells, apparently caused by loss of Neu5Gc-containing Siglec ligands (Naito et al., 2007; Cariappa et al., 2009).

In contrast, deliberate immunization with an artificial immunogen rich in Neu5Gc, such as chimpanzee RBCs, and complete Freund's adjuvant, did elicit anti-Neu5Gc IgM and IgG antibodies in *Cmah*-null, but not WT, mice (Hedlund et al., 2008). Thus, despite the lack of spontaneously generated anti-Neu5Gc antibodies, *Cmah*-null mice are capable of generating a humoral immune response against Neu5Gc upon active immunization (Fig. 2, A and B). Recently, another group also reported that a different strain of *Cmah*-null mice do not spontaneously express anti-Neu5Gc antibodies, and require artificial immunization to generate a humoral immune response (Tahara et al., 2010).

Trace amounts of Neu5Gc are taken up and expressed on LOS of NTHi, a human-specific commensal/pathogen

Some other postnatally appearing anticarbohydrate antibodies in humans, such as anti- α -Gal and antiblood group antibodies, have been shown to be elicited in response to resident gut bacteria expressing these epitopes (Springer and Horton, 1969; Galili et al., 1988; Posekany et al., 2002). Interestingly, we found that the appearance of anti-Neu5Gc antibodies in human infant sera coincides with the appearance of both anti- α -Gal (Fig. 1 D) and anti-blood group antibodies (not depicted), suggesting that anti-Neu5Gc antibodies may also be elicited by colonizing bacteria during infancy. In this regard, many human microbial pathogens/commensals express Sias on their surface glycans, creating a resemblance to "self" glycoproteins and glycolipids found in mammals (Vimr and Lichtensteiger, 2002; Vimr et al., 2004; Lewis et al., 2009). This molecular mimicry of host glycans can help

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 μ g/g total Neu5Gc (Tangvoranuntakul et al., 2003). Gavaged 250 μ l 5 d/wk for 4 wk.
Feeding goat cheese	Rich in Neu5Gc-containing glycolipids, 39.9 μ g/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 <i>Cmah</i> -null pups to Neu5Gc positive <i>Cmah</i> ^{+/-} mother	<i>Cmah</i> -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 (Diaz et al., 2009). When 2019 was grown in Sia-free defined media, the anti-Neu5Gc antibody background staining was similar to the control IgY, confirming the inability of NTHi to synthesize endogenous Neu5Gc (Fig. 3 A). However, when exogenous free Neu5Gc (0.1 μ M–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 2019siaT (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

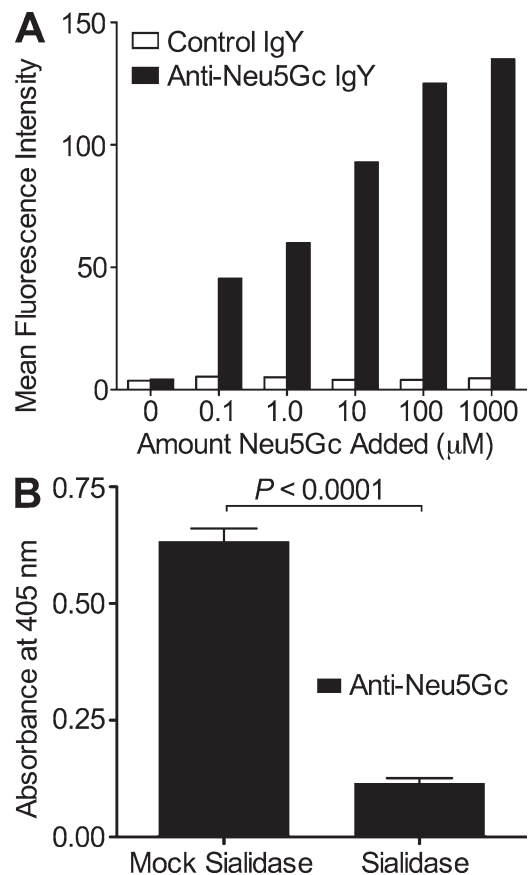


Figure 3. NTHi can efficiently take up and incorporate Neu5Gc. (A) Dose-dependent uptake and expression of NTHi (2019) grown in a Sia-free defined media with 0.1 μ M–1 mM Neu5Gc. Neu5Gc was detected by flow cytometry analysis using a chicken anti-Neu5Gc IgY antibody. (B) 2019 grown in 1 mM Neu5Gc were 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).

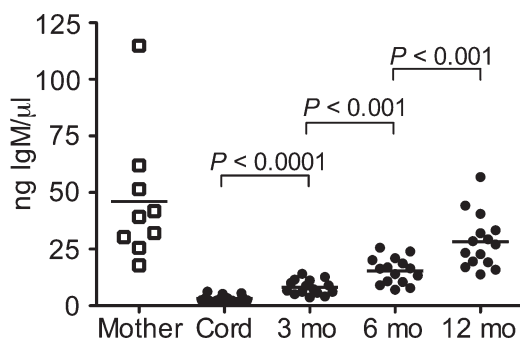


Figure 4. Anti-NTHi antibodies in human infants. Infant sera (same infants analyzed in Fig. 1; $n = 15$) were analyzed by whole-cell ELISA for IgM antibodies against NTHi strain 2019 grown in Sia-free media (filled circles). Adult sera ($n = 9$) obtained from the pregnant mothers of the infants in this 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.

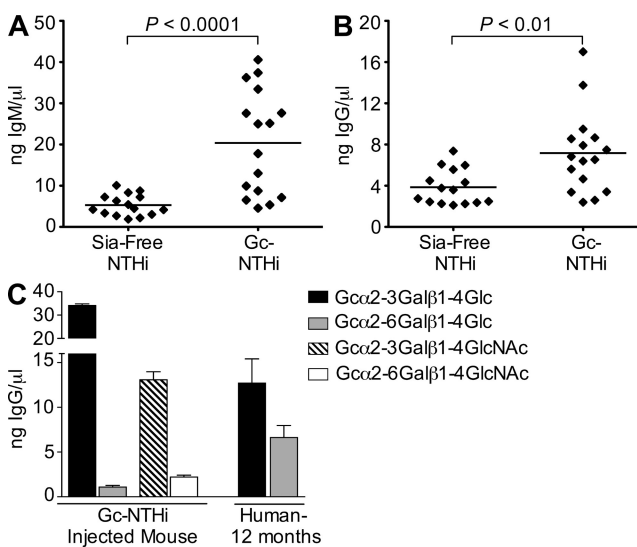


Figure 5. Neu5Gc expressed on NTHi induces anti-Neu5Gc antibodies in *Cmah*^{-/-} mice. (A and B) *Cmah*^{-/-} mice were injected intraperitoneally with heat-killed NTHi (without adjuvant) which had been grown in sialic-acid free media (Sia-free NTHi, $n = 16$) or with sialic-acid free media with 1 mM 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 Neu5Gc- α -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 Neu5Gc α 2-3Gal β 1-4Glc β -HSA (black bar), Neu5Gc α 2-6Gal β 1-4Glc β -HSA (gray bar), Neu5Gc α 2-3Gal β 1-4GlcNAc β -HSA (white hashed bar), and Neu5Gc α 2-6Gal β 1-4GlcNAc β -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 Neu5Gc α 2-3Gal β 1-4Glc β -HSA (black bar), Neu5Gc α 2-6Gal β 1-4Glc β -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.

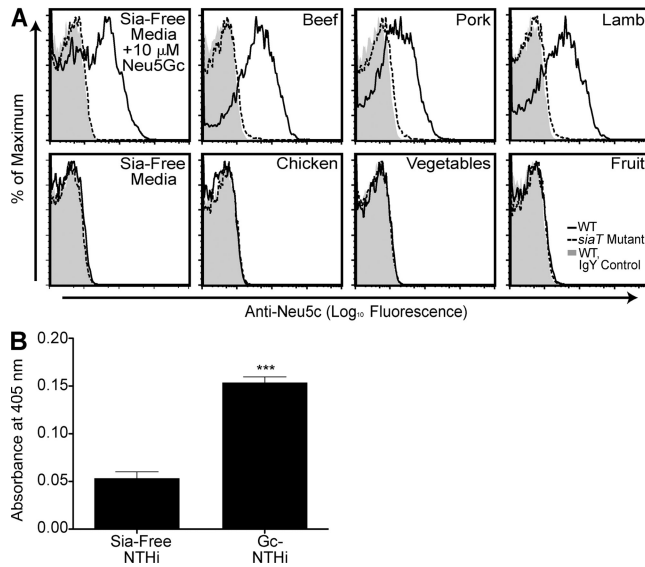


Figure 6. NTHi uptake and expression of Neu5Gc from baby food and its recognition by purified human anti-Neu5Gc antibodies.

(A) WT NTHi strain 2019 (WT, solid line) and *SiaT* 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.

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 (2019*nanA*; 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 μ g/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.

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 nonsialylated NTHi (Sia-Free NTHi; Fig. 6 B).

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, this Neu5Gc-expressing organism would simultaneously provide both immunogen and adjuvant. It is also interesting that

Table II. Quantification of Neu5Gc in commercial baby food

Baby foods	Red meat			Poultry	
	Beef (<i>n</i> = 2)	Lamb (<i>n</i> = 1)	Ham (<i>n</i> = 1)	Chicken (<i>n</i> = 2)	Turkey (<i>n</i> = 2)
Total Neu5Gc ^a μ g/g	10.19	5.40	5.13	<0.07	<0.07
Free Neu5Gc ^a μ g/g	2.68	1.56	0.98	<0.03	<0.03

^aTotal and free (non-glycosidically 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 overreactivity 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 (Padler-Karavani et al., 2008) may reflect multiple routes of xeno-autoimmunization.

MATERIALS AND METHODS

Mice. *Cmah*^{-/-} 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 2019*siaT* (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 $\mu\text{g}/\text{ml}$ protoporphyrin IX (Sigma-Aldrich), 1 $\mu\text{g}/\text{ml}$ L-histidine (Sigma-Aldrich), 10 $\mu\text{g}/\text{ml}$ 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. NTHi strain 2019 or 2019*siaT* grown to mid-log ($\text{OD}_{600} \sim 0.3-0.4$) 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 (FACSCalibur; 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, underwent incubated 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 ($\text{OD}_{600} 0.4$) in 150 μl PBS, pH 6.0 + 9 mM CaCl_2 with 10 mU active or heat-inactivated (10 min at 100°C) *Vibrio cholerae* sialidase (Sigma-Aldrich) for 3 h at 37°C. Neu5Gc was detected in a whole-cell ELISA by resuspending the bacteria in Milli-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 $\mu\text{l}/\text{well}$. Wells were then washed three times with 150 μl TBST, incubated with 100 $\mu\text{l}/\text{well}$ alkaline phosphatase (AP) donkey anti-chicken IgY (1:5,000; 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. NTHi 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 $\text{OD}_{600} 0.4$) intraperitoneally into *Cmah*-null and 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, *Cmah*-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 α -PAA (Glycotect) 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 α -PAA in 50 mM sodium carbonate-bicarbonate buffer, pH 9.5. Wells were emptied of coating solution and blocked with 200 $\mu\text{l}/\text{well}$ 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,

developed with *p*-nitrophenyl phosphate, and absorbance was measured at a 405-nm wavelength on a SpectraMax 250 (MDS Analytical Technologies). Alternatively, mouse anti-Neu5Gc antibodies against Neu5Gc α 2-6 or α 2-3 linked to Gal β 1-4Glc β -HSA, or Gal β 1-4GlcNAc β -HSA (synthesized as described previously; Yu et al., 2007) were detected by ELISA, as described above, using pooled sera from mice injected with NTHi strain 2019 grown in 1 mM Neu5Gc, as described above. Antibody levels were quantified after subtracting the readings obtained from coating with HSA alone.

Detection of anti-Neu5Gc, anti-Gal, and anti-NTHi antibodies in human sera.

Human anti-Neu5Gc antibodies were detected against Neu5Gc α 2-6 or α 2-3 linked to Gal β 1-4Glc β -HSA (Yu et al., 2007) and Gal α 1-3Gal-Polyacrylamide (α -Gal-PAA; GlycoTech) by ELISA, as previously described (Padler-Karavani et al., 2008). In brief, 96-well microtiter plates (Costar 9018; Corning) were coated overnight at 4°C, in triplicate, with saturating concentrations of Neu5Gc containing glycoconjugates α 2-6 or α 2-3 linked to Gal β 1-4Glc β -HSA, HSA (Sigma-Aldrich) alone or with saturating concentrations of α -Gal-PAA or PAA alone, (GlycoTech) in 50 mM sodium carbonate-bicarbonate buffer, pH 9.5. To standardize anti-glycan Ig signals, each plate was also coated with serial dilutions of human IgG and IgM, (Jackson ImmunoResearch Laboratories). Wells were blocked for 2 h at RT with 1% ovalbumin (Grade V; Sigma-Aldrich; free of Neu5Gc) in PBS, followed by incubation with serum samples diluted 1:100 in the same blocking solution for 2 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 IgM (Kirkegaard and Perry Laboratories), 1:4,000 diluted in PBS, or goat anti-human IgG (Bio-Rad Laboratories) 1:7000-1:6000 diluted in PBS. After washing three times with PBST, wells were developed with an *O*-phenylenediamine in a citrate-PO₄ buffer, pH 5.5, and absorbance was measured at 490 nm on a SpectraMax 250 (MDS Analytical Technologies). Anti-glycan Ig values were defined by subtracting readings obtained from HSA or PAA alone from those obtained from the Neu5Gc-glycoconjugate-HSA or α -Gal PAA antigens, respectively, and were quantified into nanograms/microliter using the standard dilution curves of the corresponding purified human IgG or IgM.

The Neu5Gc specificity of serum antibodies from 12-month-old infants was confirmed by mild periodate oxidation followed by borohydride reduction of the Neu5Gc α 2-6Gal β 1-4Glc β -HSA glycoconjugate, which selectively truncates the side chain of sialic acids while maintaining their negatively charged carboxyl group (Van Lenten and Ashwell, 1971). Neu5Gc α 2-6Gal β 1-4Glc β -HSA glycans were coated onto ELISA plates as described above, except 384-well microtiter plates (MaxiSorp; Nunc) were used to conserve reagents. After overnight coating of Neu5Gc α 2-6Gal β 1-4Glc β -HSA or HSA alone, wells were washed twice with 60 μ l PBS, pH 6.5. Wells were periodate treated with 80 μ l fresh 2 mM sodium metaperiodate (NaIO₄; Thermo Fisher Scientific) in PBS, pH 6.5, at 4°C in the dark for 20 min, and then quenched with 20 μ l 100 mM sodium borohydride (NaBH₄; EMD Chemicals; 20 mM final concentration) in PBS, pH 6.5, for 10 min at room temperature in the dark. Mock treatment was performed in parallel by premixing NaIO₄ with NaBH₄, which renders the NaIO₄ inactive. Borates formed during the borohydride treatment were removed by washing wells three times with 60 μ l of 50 mM NaOAc/100 mM NaCl, pH 5.5. Wells were then washed twice with 60 μ l PBS, pH 7.4. Blocking and detection of human serum IgG antibody binding was performed as described above.

Detection of human infant antibodies binding to NTHi was performed by whole-cell ELISA as described above, except the 96-well plates were coated with saturating amounts of Sia-free NTHi strain 2019, as described above.

Anti-Neu5Gc antibodies were affinity purified from the serum of an adult human (S34) and biotinylated as previously described (Padler-Karavani et al., 2008). Detection of purified human anti-Neu5Gc antibodies binding to Neu5Gc on NTHi was performed by whole-cell ELISA, as described above, using NTHi strain 2019 grown in Sia-free media with or without 1 mM Neu5Gc (Inalco).

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 μ l 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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