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Origins and Implications of Human Xeno-Autoantibodies Against a Non-Human Sialic Acid

A dissertation submitted in partial satisfaction of
the requirements for the degree of Doctor of Philosophy
in

Biomedical Sciences

by

Rachel Elizabeth Taylor

Committee in Charge:
Professor Ajit Varki, Chair
Professor Victor Nizet
Professor Sharon L. Reed
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Professor Joseph L. Witztum

2010

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The dissertation of Rachel Elizabeth Taylor is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2010

DEDICATION

To my family

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Chapter 3, in part, is a reprint of the material as it appears in *Nature Biotechnology*, 28(8):863-7, 2010. The dissertation author was a co-author of this paper and Dr. Ajit Varki directed and supervised the research that forms the basis of this chapter.

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Robinson, J.L., **Taylor, R.E.**, Liotta, L.A., Bolla, M.L., Azevedo, E.V., Medina, I., and McAlpine, S.R. A progressive synthetic strategy for class B synergimycins. *Tetrahedron Letters*. 45(10):2147-50, 2004

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"A non-human sialic acid contaminates biotherapeutic products: implications for immunogenicity and efficacy, and a proposed solution" Ghaderi, D., **Taylor, R.E.**, Padler-Karavani, V. Diaz, S., and Varki, A. Meeting of the Society for Glycobiology, San Diego, CA. Abstract #83. November, 2009.

"Synthesis of antibiotics: synergimycin derivatives and Holliday junction inhibitors" Bolla, M.L.,**Taylor, R.E.**, Smith, J.M., Segall, A.M., and McAlpine, S.R. American Chemical Society, 224nd National Meeting, Boston, MA. Poster #92. August, 2002.

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ABSTRACT OF THE DISSERTATION

Origins and Implications of Human Xeno-Autoantibodies Against a Non-Human Sialic Acid

by

Rachel Elizabeth Taylor

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2010

Professor Ajit Varki, Chair

All human adults have circulating antibodies against *N*-glycolylneuraminic acid (Neu5Gc), a sialic acid absent in humans. Neu5Gc from foods of mammalian origin can be metabolically incorporated into human tissues, especially vascular endothelia and carcinomas. Thus, the potential exists for circulating anti-Neu5Gc "xeno-autoantibodies" to react against this exogenously incorporated "self" Neu5Gc. Indeed, recent studies have demonstrated that anti-Neu5Gc antibodies can contribute to tumor progression and vascular inflammation. This dissertation focuses on investigating the origins and other implications of these anti-Neu5Gc antibodies.

Chapter 2 of this dissertation describes a novel model in which resident bacteria cooperate with dietary Neu5Gc to generate anti-Neu5Gc antibodies in humans. We show that anti-Neu5Gc antibodies appear during infancy and correlate with the introduction of Neu5Gc in the diet. However, dietary Neu5Gc alone is insufficient to elicit anti-Neu5Gc antibodies in human-like Neu5Gc

deficient (*Cmah*-null) mice. While other postnatally-appearing anti-carbohydrate antibodies are likely induced by colonizing bacteria, no microbe is known to synthesize Neu5Gc. Here we show that anti-Neu5Gc antibodies appear coincident with antibodies against non-typeable *Haemophilus influenzae* (NTHi), an obligate human commensal and pathogen. We show that trace exogenous Neu5Gc can be incorporated into cell surface LOS molecules, which induce anti-Neu5Gc antibodies in *Cmah*-null mice. Furthermore, purified human anti-Neu5Gc antibodies specifically recognize Neu5Gc-expressing NTHi. Finally, Neu5Gc from infant foods is taken up and expressed by NTHi. We propose that incorporation of dietary Neu5Gc by NTHi residing in the nasopharynx can induce the production of anti-Neu5Gc antibodies in humans.

Cmah-null mice immunized with Neu5Gc-expressing NTHi generate anti-Neu5Gc antibodies with similar titer and specificity to humans. Chapters 3 and 4 describe two projects that use these mice to study the significance of anti-Neu5Gc antibodies. These two projects demonstrate that anti-Neu5Gc antibodies can contribute to enhanced clearance of Neu5Gc-containing biotherapeutics and reproductive incompatibility, respectively.

Chapter 5 details preliminary studies investigating the differential utilization of human and non-human sialic acids by NTHi. Here we investigate sialic acid expression preferences of NTHi and analyze sialic acid catabolism preferences of the NanA lyase. Finally, we present evidence that Neu5Gc utilization by NTHi has intriguing consequences for growth and survival in human serum.

CHAPTER 1

Introduction: Sialobiology of Humans and a Human-Specific Commensal/Pathogen, Non-Typeable Haemophilus influenzae (NTHi)

Sialic Acids: Structure, Diversity and Distribution in Nature

Sialic acids belong to a family of alpha-keto acidic monosaccharides whose 9-carbon backbone structures are derivatives of neuraminic acid (Neu) or deaminoneuraminic acid (Kdn). There are over 50 different sialic acids found in nature. These structurally diverse monosaccharides are commonly found in α2–3, α2–6, or α2–8 linkages to underlying galactose or *N*-acetylgalactosamine residues on glycosphingolipids, *N*-and *O*-linked glycans on glycoproteins, free glycans, and GPI-anchors (1-5).

Sialic acids are expressed on all vertebrate cells and are required for embryonic development (6). They are not ubiquitously expressed in nature but found mainly in the *Deuterostome* lineage of animals (including vertebrates and "higher" invertebrates) and occasionally in pathogenic/commensal bacteria (4, 7). Rarely, sialic acid can also be found in other taxa such as pathogenic protozoans and fungi (7), as well as *Drosophila* (8). Plants, however, are devoid of sialic acid (9).

The two most common sialic acids in mammals are *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc). Neu5Gc differs from Neu5Ac by the addition of a single oxygen atom forming an *N*-glycolyl moiety attached to the amino group in the C-5 position. The enzyme responsible for the synthesis of Neu5Gc is the CMP-*N*-acetylneuraminic acid hydroxylase (CMAH) which converts CMP-Neu5Ac to CMP-Neu5Gc. Humans do not express a functional CMAH enzyme and therefore are deficient in Neu5Gc synthesis (10).

Biological Roles of Sialic Acids

Sialic acids are typically located at the terminal ends of glycans. This terminal location allows them to act as ligands for both self and pathogen receptor binding.

Self receptors for sialic acids include cell surface proteins such as selectins and siglecs, which facilitate leukocyte/endothelial adhesion and extravasation and modulate innate immune responses, respectively, and Factor H, a soluble plasma protein which down-regulates alternative complement activation (11).

Human pathogens that bind to sialic acid during the initial stages of cell entry and infection include influenza A and B viruses (12) and *Plasmodium falciparum* (13). Additionally, sialic acids can serve as targets for binding by secreted microbial toxins such as cholera toxin and SubAB subtilase cytotoxin (14).

Loss of Neu5Gc Synthesis in Humans

While the great apes and most other mammals express both Neu5Ac and Neu5Gc, humans are deficient in Neu5Gc biosynthesis and have an excess of Neu5Ac on their cell surfaces. As mentioned earlier, the loss of Neu5Gc in humans is due to a human-specific mutation in the *CMAH* gene that occurred approximately 2-3 million years ago and is fixed in the human population (15, 16). Like humans, *Cmah* null mice also lack the ability to synthesize Neu5Gc, ruling out an alternative pathway for Neu5Gc synthesis (17).

Incorporation of the Dietary Xeno-glycan, Neu5Gc, into Human Tissues

Despite the inability of human cells to synthesize Neu5Gc, humans can accumulate small amounts of dietary Neu5Gc in their tissues (18). Dietary sources that are rich in Neu5Gc include red meats such as beef, pork and lamb, and to a lesser extent, cow's milk products. Poultry and fish contain low/variable amounts of Neu5Gc while plants contain no Neu5Gc (18). Definitive proof of human metabolic incorporation of this dietary xeno-glycan comes from a human study demonstrating Neu5Gc incorporation into salivary mucins following ingestion of porcine-derived Neu5Gc (18).

Neu5Gc incorporation into human cells is mediated by macropinocytosis (19). Following macropinocytosis, exogenous Neu5Gc is transported via endosomes to the lysosome. Here, bound Neu5Gc can be released by lysosomal sialidases and free Neu5Gc can be transported into the cytosol through a specific lysosomal sialic acid transporter. Once in the cytosol, free Neu5Gc is apparently treated just as if it were Neu5Ac: It can enter the nucleus to be activated with CMP, transported into the golgi through a CMP-sialic acid transporter, and incorporated into glycoconjugates. Once on the cell surface, Neu5Gc-containing glycoconjugates can be recycled via macropinocytosis, repeating this process over again.

All Human Adults Have Circulating Antibodies Against Neu5Gc

In addition to incorporating small amounts of Neu5Gc from the diet, all human adults also have circulating antibodies against Neu5Gc (18, 20-23).

These IgA, IgG, and IgM anti-Neu5Gc antibodies have polyclonal binding specificity and recognize a wide range of different Neu5Gc-containing glycans. Human serum contains distinct populations of anti-Neu5Gc antibodies that not only recognize Neu5Gc, and modifications to the structure of Neu5Gc, but also recognize the specific linkages (ie. α2–3 or α2–6) and several underlying glycan residues. While these anti-Neu5Gc antibodies are found in all human adults, their binding specificity and titers are highly variable between individuals. Human anti-Neu5Gc IgG antibodies, which are present at levels as high as ~0.2% of total IgGs, can be dominated by either IgG2 or IgG3 subclasses. This IgG subclass variation between individuals suggests that anti-Neu5Gc antibodies can play diverse roles in complement and cell-mediated antibody effector functions.

Disease Implications of Human Anti-Neu5Gc Antibodies as "Xeno-Autoantibodies"

Because Neu5Gc incorporation is especially prominent along endothelial linings of blood vessels as well as on epithelial layers (18), we hypothesize that these potentially autoreactive anti-Neu5Gc "xeno-autoantibodies" likely contribute to heart disease, cancer, and autoimmunity which are all associated with chronic inflammation. Indeed, these antibodies have recently been shown to interact *in vitro* and *in vivo* with metabolically incorporated Neu5Gc to promote chronic inflammation and enhance carcinoma progression (24) and potentially vascular inflammation (25).

Sialic Acid Expression by Bacterial Commensals/Pathogens: An Evolutionary Perspective

Because sialic acid expression is largely restricted to higher animals of the *Deuterostome* lineage, including vertebrates, and a few bacterial pathogens that infect these vertebrate animals, it had been postulated that sialic acids represent a relatively new evolutionary invention that began in *Deuterostomes* and then later appeared in prokaryotes through convergent evolution or horizontal gene transfer (4). However, recent phylogenomic studies have demonstrated that sialic acids are related to a larger family of 9-carbon (nonulosonic) acidic sugars that include legionaminic, and pseudaminic acids and are related to the 8-carbon alpha keto acid, KDO (26). These studies conclude that nonulosonic acids originated in archaea and prokaryotes, were subsequently lost in various lineages such as plants, but flourished in the form of sialic acids in vertebrates. Later, by convergent evolution, prokaryotes independently adapted existing nonulosonic acid synthesis pathways to synthesize sialic acids mimicking vertebrate host glycans, a process referred to as molecular mimicry.

Sialylation Pathways and Functions in Bacterial Commensals/Pathogens

Bacterial commensals/pathogens can obtain sialic acid for cell surface decoration by *de novo* synthesis or by scavenging it from the host (Fig. 1-1). Bacteria known to synthesize sialic acid *de novo* include *Campylobacter jejuni*, *Escherichia coli* K1, most meningococcal strains, and *Streptococcus agalactiae*

(7). Sialic acid biosynthesis by these bacteria is achieved by the conversion of the common nucleotide sugar precursor, UDP-GlcNAc, to ManNAc, which is condensed with pyruvate to form Neu5Ac (27).

Bacterial sialic acids can be expressed on membrane bound lipopolysaccharides (LPS), capsular polysaccharides, or both. Like those found in mammals, bacterial sialic acids can be linked α2–3, α2–6, α2–8 or α2–9 to underlying Galβ1–4Glc or Galβ1–4GlcNAc moieties, and sometimes *O*-acetylated (28). However, the parent sialic acid structure synthesized by bacterial commensals/ pathogens is always Neu5Ac, as no microbe has ever been shown to synthesize Neu5Gc. In fact, the synthesis of Neu5Gc is entirely restricted to the *Deuterostome* lineage of animals (29).

In contrast to bacteria that can synthesize their own sialic acid, *Corynebacterium diphtheria* (*C. diphtheria*), *Neisseria gonorrhoeae* (*N. gonorrhoeae*), Non-typeable *Haemophilus influenzae* (NTHi) and *Haemophilus ducreyi* (*H. ducreyi*) lack the machinery necessary to synthesize sialic acid but have evolved multiple different pathways to scavenge host sialic acid: *C. diphtheria* uses a trans-sialidase to cleave bound sialic acid from host glycans and transfer it directly onto it's own glycans (30); *N. gonorrhoeae* uses an extracellular sialyltransferase to catalyze the transfer of host-derived CMP-sialic acid onto it's surface glycans (31); while NTHi and *H. ducreyi* transport free sialic acid into the cytoplasm, activate it with CMP, and transfer it onto cell surface glycans (32, 33). Interestingly, while NTHi and *H. ducreyi* are related bacterial species belonging to the same genus, NTHi captures and transports

sialic acid through a Tripartite ATP-independent periplasmic (TRAP) transporter (34, 35) while *H. ducreyi* uses an unrelated ATP-binding cassette (ABC) transporter (36).

This use of multiple independent pathways for sialic acid scavenging underscores the apparent evolutionary importance of sialic acid acquisition and expression in these bacterial commensals/pathogens. Indeed, bacterial sialylation has been shown to be a virulence factor, *in vivo* (32), and to protect from alternative complement pathway (37) or classical complement pathway killing (38) in human serum *in vitro*. Sialic acids expressed on bacteria can also dampen cell mediated immune defenses by engaging siglecs on host innate immune cells (39).

NTHi is a Human-Specific Commensal and Pathogen in Infants

Of the bacteria known to rely on host-derived sialic acid for cell surface decoration, only NTHi exists in humans as both a pathogen and a commensal. While NTHi asymptomatically colonizes most infants and adults along the nasopharynx (40, 41) it also represents a major cause of acute otitis (AOM) in infants. Interestingly, while *Streptococcus pneumoniae* once accounted for the majority of AOM cases, soon after the introduction of the heptavalent pneumococcal vaccine (PCV7) in 2000, NTHi became the most-common bacterium isolated from the middle ears of infants (42, 43). Although recent data suggest that non-PCV7 strains of *S. pneumoniae* are rapidly re-emerging, NTHi remains a dominant pathogen in AOM whose relative contribution to the disease will likely further increase following implementation of the recently

licensed 13-valent pneumococcal conjugate vaccine (PCV13) (44). There is currently no licensed vaccine against NTHi.

Colonization by NTHi during the first year of life is a highly dynamic process. Typically, an initial colonizing strain is carried for up to several months, lost, and then a new strain is acquired. Colonization patterns between infants in a given population can also be highly variable with some infants acquiring only one strain during the first 2 years of life and others sequentially acquiring up to 7 strains during the same time period (45). The duration of initial colonization also varies from less than one month to ten months, with the majority of strains lost within 2 months (45). Due to the transient nature of NTHi colonization during infancy, inadequate sampling frequency can lead to underestimates in infant NTHi colonization rates (46). For example, one study found that when infants (n=339) were evaluated for NTHi colonization by nasopharyngeal culture every 3 months for 12 months total, only 25.7% were found to have been colonized. In contrast, when a subcohort of infants (n=101) from the same population were evaluated weekly, the colonization rate jumped to 88%.

The frequency and duration of NTHi colonization positively correlate with rates of AOM in infants. In fact, infants who are prone to recurrent AOM are more than twice as likely to have a positive NTHi culture, even during healthy periods (47). While the exact mechanisms governing different NTHi colonization patterns between infants have yet to be elucidated, three important factors appear to be the speed, robustness, and specificity of the infant antibody

response directed against NTHi. While all infants generate strain-specific bactericidal antibodies during NTHi colonization and rarely acquire the same strain twice, AOM prone infants often fail to develop more broadly protective anti-NTHi antibodies (47).

Expression of Host-Derived Sialic Acid by NTHi

NTHi is a small, Gram-negative, non-encapsulated coccobacillus. Glycolipid molecules called lipooligosaccharides (LOS) are a major component of the outer membrane of NTHi. These LOS molecules are similar to the LPS molecules expressed by many other Gram-negative bacteria except that they are shorter and lack O-antigen repeating units. The non-repeating oligosaccharides found at the terminal ends of LOS on NTHi include sialylated Galβ1–4Glc and Galβ1–4GlcNAc moieties which resemble those found on human cell surface glycans. This molecular mimicry of host sialylated glycans confers protection against host immune defenses (7, 48, 49). Infact, NTHi is dependant on sialic acid expression for human serum resistance (34, 50), and for virulence in otitis media models using chinchillas (32, 51) and gerbils (52).

NTHi is incapable of synthesizing it's own sialic acid and therefore must scavange exogenous sialic acid from the host (32). Because NTHi also lacks a sialidase (7), sialic acid scavenged by NTHI must be in it's free form (non-glycosidically bound). This host-derived free sialic acid enters the bacterium through an ATP-independent periplasmic (TRAP) transporter. Unlike the sialic acid transporter of *Haemophilus ducreyi*, which is ATP dependent (36), the TRAP transporter of NTHi uses an electrochemical sodium gradient to drive the

import of sialic acid (53). The TRAP transporter is composed of three subunits, a periplasmic sialic acid binding protein, encoded by *siaP*, and two fused trans-membrane subunits encoded by a single gene, *siaT*. Disruption of either *siaP* or *siaT* results in complete loss of sialic acid uptake in NTHi, demonstrating the essential role of these genes for sialylation (34, 35, 54, 55). Before sialic acid is transferred onto LOS it must be activated by a CMP-sialic acid synthetase, SiaB (50), to form the nucleotide sugar donor, CMP-sialic acid. This CMP-activated sialic acid is used as a substrate by up to four different sialyltransferases Lic3A (56, 57), Lic3B (58), LsgB (57), and SiaA (57) that can transfer sialic acid onto Galβ1–4Glc (Lic3A and Lic3B) or Galβ1–4GlcNAc (LsgB and SiaA) moieties. The Lic3B sialyltransferase has been shown to be a bifunctional enzyme that can add sialic acid in a α2–3 linkage to Galβ1–4Glc or in a α2–8 linkage to another sialic acid, mimicking human disialylated gangliosides (57). Indeed, one NTHi strain expressing Lic3B was found to be associated with Miller-Fisher syndrome, a variant of Guillain-Barre syndrome mediated by autoantibodies against the disialylated ganglioside GQ1b (59).

In addition to sialylating cell surface LOS molecules, NTHi also sialylates biofilm exopolysaccharides *in vivo* (51) and *in vitro* (60). In fact, proper biofilm production is dependent on the addition of sialic acid to these secreted polysaccharide matrices (51, 52, 60) The SiaA sialytransferase has been shown to be the sole sialyltransferase responsible for sialylating NTHi biofilms. Interestingly, while Lic3A, Lic3B and LsgB are each α2–3 sialytransferases,

lectin analysis of NTHi biofilms suggests that SiaA is α2–6 sialyltransferase (60).

Unanswered Questions Regarding Origins and Implications of Human Xeno-Autoantibodies Against Neu5Gc

While previous studies have demonstrated that all human adults have circulating anti-Neu5Gc antibodies, the antigenic stimulus for these antibodies has not been identified. Given recent evidence linking anti-Neu5Gc antibodies to chronic inflammation and pathogenesis of dietary red meat-associated diseases (24, 25), it is important to understand when and how anti-Neu5Gc antibodies emerge in humans. Chapter 2 of this dissertation discusses studies elucidating the origins of anti-Neu5Gc antibodies and development of a human-like mouse model with anti-Neu5Gc antibodies. Chapter 3 and 4 of this dissertation describe the use of this human-like mouse model to further investigate the significance of anti-Neu5Gc antibodies.

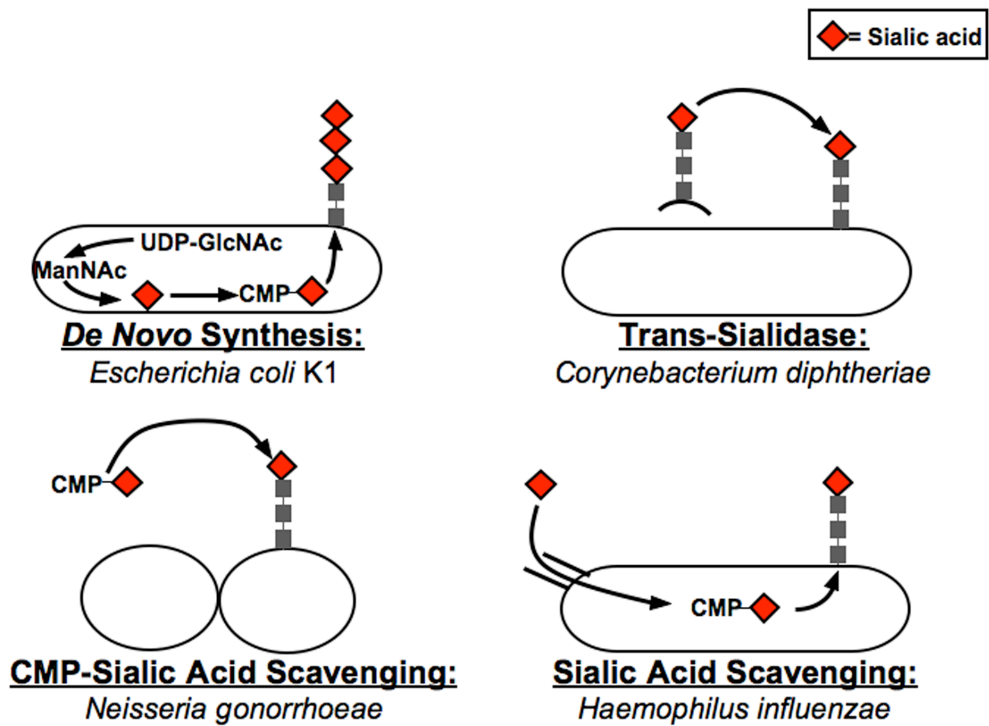

Figure 1-1. Pathways for bacterial sialylation.
This cartoon depicts four different mechanisms by which bacteria scavenge host sialic acid for cell surface sialylation, as described in the text of Chapter 1.

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CHAPTER 2

Novel Mechanism for the Generation of Human Xeno-Autoantibodies Against the Non-Human Sialic Acid N-glycolylneuraminic Acid

ABSTRACT

The non-human 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 human-like 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 non-typeable *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.

INTRODUCTION

Sialic acids (Sias) are monosaccharides with a shared 9-carbon backbone, typically found at the terminal ends of vertebrate cell surface and secreted glycoconjugates (1-5). Most mammals express two common Sias, *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc). However, humans are deficient in Neu5Gc synthesis, due to a human-specific mutation inactivating the *CMAH* gene responsible for converting CMP-Neu5Ac to CMP-Neu5Gc (6, 7). All human adults have varying levels of circulating IgM, IgG and IgA antibodies against Neu5Gc (8-12). 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 (9, 13, 14), through a mechanism involving macropinocytosis and delivery of free Neu5Gc to the cytosol via a lysosomal transporter (15, 16). Evidently, while 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 (14) and vascular inflammation (17).

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

post-natally in humans during the first year of life. Other post-natally acquired human antibodies against foreign glycans, e.g., blood group antibodies and anti-alpha-Gal antibodies, are thought to be induced by commensal bacteria expressing these epitopes (18, 19). However, while many bacteria can synthesize and express Neu5Ac (20, 21), 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 months (cord, 3 month, 6 month, and 12 month) 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 (11). All sera were from infants who had been exclusively breastfed for the first 3 months of life, and then switched to cow's milk based formula. Solid foods were also introduced starting at 3 months 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 months, appeared at

6 months and achieved almost adult levels at 12 months (Fig. 2-1 A). As expected due to 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 months, followed by increasing levels at 6 and 12 months (Fig 2-1 B). There was no difference between male and female anti-Neu5Gc IgM or IgG titers (data not shown). The reactivity of 12 month sera against Gcα2–6Lac was significantly reduced following truncation of the target epitope's Neu5Gc side chain by mild periodate oxidation (9), further demonstrating the specificity of the IgG antibodies for Neu5Gc-containing glycans (Fig. 2-1 C). The absence of anti-Neu5Gc IgM antibodies in cord sera suggests that anti-Neu5Gc antibodies are not germ-line encoded “natural antibodies” (22), but instead require a post-natal 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 months is also consistent with the half-life of maternally-derived IgG (23) and suggests that these infants lack the production of endogenous anti-Neu5Gc IgG antibodies at 3 months, 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 synthesis (13). However, despite consumption of ~1 mg/kg/day of Neu5Gc in their regular chow (data not shown, this represents ~5 times the amount present in the typical human Western diet (9)), *Cmah* null mice did not spontaneously generate anti-Neu5Gc IgM or IgG antibodies after 6–9 months (Fig. 2-2 A and B). In fact, as summarized in Table 2-1, 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 weeks; cross-fostering *Cmah* null pups with wild-type 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 (data not shown, see Table 2-1 for details). These negative results are also interesting because *Cmah* null mice actually have relatively hyper-reactive B cells, apparently due to loss of Neu5Gc-containing Siglec ligands (24, 25).

In contrast, deliberate immunization with an artificial immunogen rich in Neu5Gc, such as chimpanzee red blood cells (RBC), and complete Freund's adjuvant did elicit anti-Neu5Gc IgM and IgG antibodies in *Cmah* null, but not wild-type mice (14). 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. 2-2 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 (12).

Trace amounts of Neu5Gc are taken up and expressed on LOS of NTHi, a human-specific commensal/pathogen. Some other post-natally appearing anti-carbohydrate antibodies in humans such as anti-alpha-Gal and anti-blood group antibodies have been shown to be elicited in response to resident gut bacteria expressing these epitopes (18, 19, 26). Interestingly, we found that the appearance of anti-Neu5Gc antibodies in human infant sera coincides with the appearance of both anti-alpha-Gal (Fig 2-1D) and anti-blood group antibodies (data not shown) 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 (20, 21, 27). This molecular mimicry of host glycans can help pathogens evade, dampen, and/or inhibit host immune defenses (28-30). Several bacterial species including *Escherichia coli* K1, *Campylobacter jejuni*, most meningococcal strains and *Streptococcus agalactiae* are able to synthesize Sia *de novo* (21). 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 (1).

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 (31-34). 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 (35, 36), and then use an endogenous CMP-Sia synthetase (37) and sialyltransferases (38) to decorate its LOS. These sialylated LOS molecules are critical for NTHi human serum resistance (35, 37) and virulence in a chinchilla otitis media model (32, 39).

Of the bacteria mentioned above, only *Haemophilus 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 (40) in a Sia-free defined medium and analyzed the bacteria by flow cytometry using a Neu5Gc-specific chicken IgY antibody (41). When NTHi strain 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. 2-3 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 to no binding by the control IgY antibody (Fig. 2-3 A). Similar results were found (data not shown) using two other NTHi strains, Int1 (42) and

DH1 (43), as well as one encapsulated type b strain, Eagan (44). Thus, uptake and surface expression of Neu5Gc is a common feature among different strains of *Haemophilus influenzae*.

To further confirm Neu5Gc incorporation by NTHi strain 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 to treatment with heat-inactivated sialidase, demonstrating release of Neu5Gc from NTHi surface glycans by active sialidase (Fig. 2-3 B). Finally, no Neu5Gc expression was seen in NTHi strain 2019*siaT* (35), an isogenic mutant that lacks a critical Sia transporter (Fig. 6 A, top left panel). Taken together, 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 (45). 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 in order to ensure absence of Neu5Gc on the LOS of the bacteria. In all infants analyzed, we could detect IgM antibodies against NTHi that increased significantly between

birth, 3, 6 and 12 months (Fig. 2-4). Since it is unlikely that all infants in our cohort have had NHTi otitis media by 3 months 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 (46), and that infants generate antibodies against outer membrane proteins on NTHi during the course of asymptomatic colonization (47). 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 (see Table 2-1). 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). In order 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. 2-5 A and B). In contrast, *Cmah* null mice injected with Sia-free NTHi did not generate anti-Neu5Gc antibodies (Fig. 2-5 A and B). Multiple attempts to elicit anti-Neu5Gc antibodies in *Cmah* null mice via intranasal administration of Neu5Gc-expressing NTHi have been unsuccessful (data not shown). This is not surprising, given that mice show very rapid mucosal clearance of this human-specific microorganism and limited nasopharyngeal colonization (48). 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. 2-5 C) and adults (11).

NTHi can scavenge and express Neu5Gc from Neu5Gc-Containing Foods. NTHi that are colonizing the infant's oropharynyx 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 (49). 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 Figure 2-6 A, wild-type 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; data not shown), 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 Neu5G by NTHi. Furthermore, an even greater shift in anti-Neu5Gc staining (~4 fold increase in MFI compared to WT) was seen with a NTHi sialic acid lyase-deficient mutant (2019*nanA*; data not shown) which develops a hypersialylated phenotype in the presence of exogenous Sia (35). 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, while poultry contains only Neu5Ac and plants contain no Sia (9). NTHi do not produce a sialidase (21), and therefore require free (non-glycosidically bound) Sia for LOS sialylation. Indeed, when Sia levels were quantified in the baby food, considerable levels of free Neu5Gc (~2 μg/g; Table 2-2) were detected in red meat containing baby food but not in those containing poultry or plants (Table 2-2). Of course, many other oral and nasopharyngeal commensal bacteria express sialidases (50), 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 (11) 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 non-sialylated NTHi (Sia-Free NTHi) (Figure 2-6 B).

DISCUSSION

Here, we propose a model for how NTHi and dietary Neu5Gc cooperate to generate anti-Neu5Gc antibodies in humans. Taken together, 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 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 (51). Furthermore, LPS-induced secretion of BAFF and APRIL by dendritic cells and monocytes, could contribute to T-independent induction of class switch recombination (52, 53), especially in the setting of inflammation (54) which 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 vs. 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 over-reactivity of human B cells to stimulation (55).

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. While 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 (11) may reflect multiple routes of xeno-autoimmunization.

MATERIALS AND METHODS

Mice. *Cmah−/−* mice (13) were backcrossed onto a congenic C57BL/6 background. Wild-type C57BL/6 mice were purchased from Harlan Laboratories (Indianapolis, IN). Mice were fed standard chow (LabDiet, PicoLab Rodent Diet 20, #5053) and water ad libitum and maintained on a 12-hour 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 & Use Committee of the University of California, San Diego.

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 fifteen Chilean infants at birth (from cord blood), 3, 6 and 12 months of age. Serum from nine of the fifteen mothers in the study was obtained during to third trimester of pregnancy, with consent.

Bacteria, growth conditions, and baby food. NTHi strains 2019 (40) and 2019*siaT* (35) 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) supplemented with 1 μg/ml protoporphyrin IX (Sigma), 1 μg/ml L-histidine (Sigma), 10 μg/ml β- Nicotinamide adenine dinucleotide (Sigma), 0.1 mg/ml hypoxanthine (Sigma), 0.1 mg/ml uracil (Sigma), and 0.8 mM sodium pyruvate (Gibco)(35, 56). 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 (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) (41), control chicken IgY (1:2,000; Jackson ImmunoResearch), or PBS alone. After washing

once with PBS, bacteria were resuspended in Fluorescein isothiocyanate (FITC) donkey anti-chicken antibody (1:200; Jackson ImmunoResearch) for 1 h at RT and analyzed by flow cytometry (FACSCalibur, BD Biosciences). 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. Following filter sterilization, the filtrate was added to an equal volume of mid-log bacteria in sialic acid-free media, 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 following 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 mU active or heat-inactivated (10 minutes at 100°C) *Vibrio cholerae* sialidase (Sigma) for 3 hours 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 (Costar), 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 RT, 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 (1:5,000; Jackson ImmunoResearch) for 1 h at RT, washed as before and then developed with *p*-nitrophenyl phosphate, with product measured at 405 nm wavelength on a SpectraMax 250 (Molecular Devices).

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 OD_{600} 0.4) intraperitoneally into *Cmah* null and wild-type (C57BL/6) mice (age 5−8 weeks, female). All mice were injected a total of three times at two-week intervals. No adjuvant was used with any of the bacterial injections. Erythrocyte immunizations were performed as described previously (14). Briefly, *Cmah* null mice (age 6−9 weeks, male and female) were injected intraperitoneally with 200 μg chimpanzee (Neu5Gc-rich; Yerkes National Primate Research Center, Emory University, GA) or human (Neu5Gc-free) erythrocyte membrane ghosts in 100 μl PBS with equal volume Freund's complete adjuvant (Difco), and boosted twice (two weeks and eight weeks 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 Neu5Gcα-PAA (Glycotech) by ELISA as previously described (14). Briefly, 96-well plates (Costar 9018) were coated overnight at 4°C with 250 ng/well Neu5Gcα-PAA 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 RT. Sera were added to the wells for 2 h at RT 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), 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 (Molecular

Devices). Alternatively, mouse anti-Neu5Gc antibodies against Neu5Gc α2–6 or α2–3 linked to Galβ1–4Glcβ-HSA, or Galβ1–4GlcNAcβ-HSA (synthesized as described previously (57)) 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 Neu5Gcα2–6 or α2–3 linked to Galβ1–4Glcβ-HSA (57) and Galα1–3Gal-Polyacrylamide (alpha-Gal-PAA; Glycotech) by ELISA, as previously described (11). Briefly, 96-well microtiter plates (Costar 9018, Corning) were coated overnight at 4°C, in triplicate, with saturating concentrations of Neu5Gc containing glycoconjugates α2–6 or α2–3 linked to Galβ1–4Glcβ-HSA, HSA (Sigma) alone or with saturating concentrations of alpha-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). Wells were blocked for 2 h at RT with 1% ovalbumin (Grade V, Sigma, free of Neu5Gc) in PBS, followed by incubation with serum samples diluted 1:100 in the same blocking solution for 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) 1:6,000–1:7,000 diluted in PBS. After washing three

times with PBST, wells were developed with *O*-phenylenediamine in a citrate-PO_4 buffer, pH 5.5, and absorbance was measured at 490 nm on a SpectraMax 250 (Molecular Devices). Anti-glycan Ig values were defined by subtracting readings obtained from HSA or PAA alone from those obtained from the Neu5Gc-glycoconjugate-HSA or alpha-Gal PAA antigens respectively, and were quantified into ng/μl 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 Neu5Gcα2–6Galβ1–4Glcβ-HSA glycoconjugate, which selectively truncates the side chain of sialic acids while maintaining their negatively charged carboxyl group (58). Neu5Gcα2–6Galβ1–4Glcβ-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 Neu5Gcα2–6Galβ1–4Glcβ-HSA or HSA alone, wells were washed twice with 60 μl PBS, pH 6.5. Wells were periodate treated with 80 μl fresh 2 mM sodium metaperiodate ($NaIO_4$; Fisher Scientific) in PBS, pH 6.5, at 4°C in the dark for 20 minutes and then quenched with 20 μl 100 mM sodium borohydride ($NaBH_4$; EMD Chemicals; 20 mM final concentration) in PBS, pH 6.5 for 10 minutes at RT in the dark. Mock treatment was performed in parallel by premixing $NaIO_4$ with $NaBH_4$, which renders the $NaIO_4$ inactive. Borates formed during the borohydride treatment were removed by washing wells three times with 60 μl of

50 mM NaOAc/100 mM NaCl pH 5.5. Wells were then washed twice with 60 μl 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 (11). 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 non-glycosidically 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 x g for 10 minutes at 4°C. The supernantant was then filtered through a 10,000 molecular weight cut off filter and sialic acids in the filtrate were derivatized with 1,2-diamino-4,5-methylenedioxybenzene (DMB; Sigma-Aldrich) as described previously (59) 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; San Diego, CA).

ACKNOWLEDGEMENTS

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Chapter 2, in full, is a reprint of the material as it appears in *The Journal of Experimental Medicine,* 207(8):1637-46, 2010. The dissertation author was the primary author and Dr. Ajit Varki directed and supervised the research that forms the basis of this chapter.

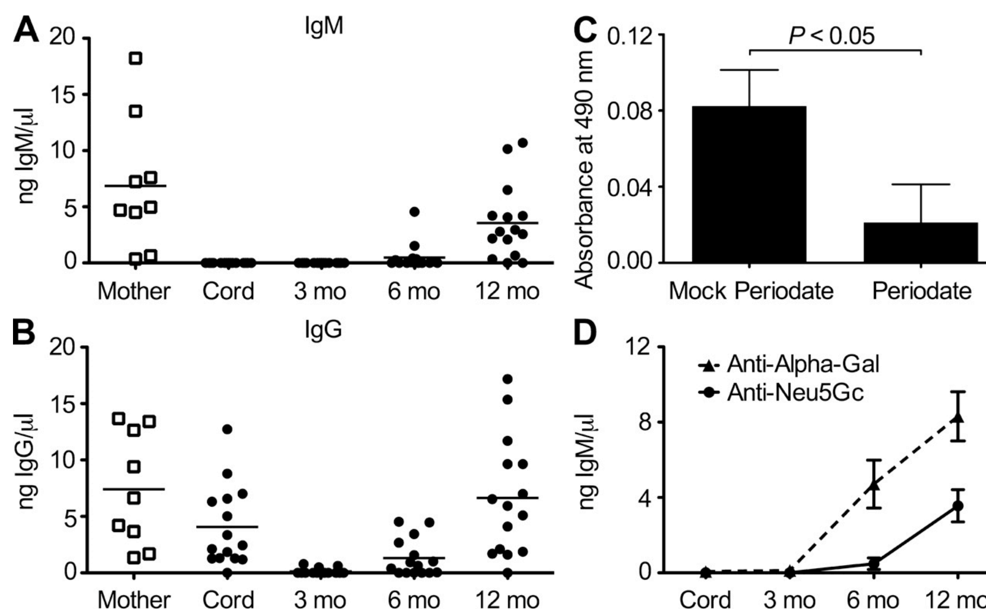

Figure 2-1. Anti-Neu5Gc antibodies in human infants.
(A and B) Levels of anti-Neu5Gc IgM (A) and IgG (B) antibodies in infant sera (*n*=15, filled circles) from birth (cord), 3, 6, and 12 months (for each infant) and adult sera from the pregnant mothers (*n*=9, open squares) were measured by ELISA against Neu5Gcα2–6Lacβ-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) Neu5Gcα2–6Lacβ-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 months of age (*n*=15). Bars represent mean absorbance values at OD_{490} ± SEM. Statistical analysis was performed using an unpaired two-tailed Student's *t*-test. (D) Infant sera (same infants as in Fig. 2-1 A and B) were analyzed by ELISA for IgM antibodies against alpha-Gal-PAA (dashed line). For comparison, anti-alpha-Gal IgM levels were plotted with anti-Neu5Gc IgM levels (solid line, same data as in Fig. 2-1 A). Values represent mean IgM levels ± SEM.

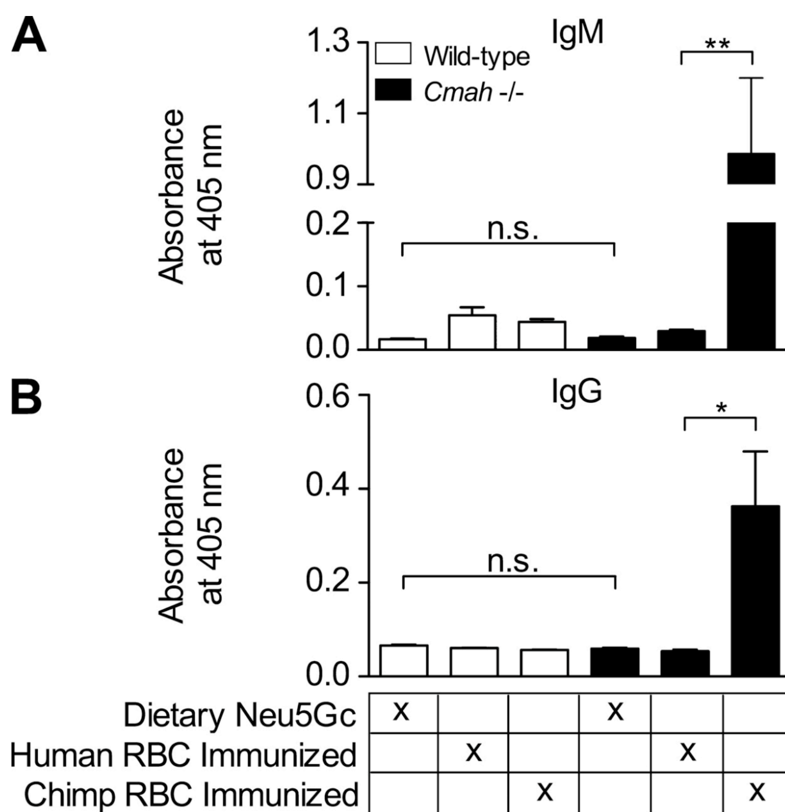

Figure 2-2. Dietary Neu5Gc does not elicit anti-Neu5Gc antibodies.
(A and B) Wild-type (open bars, *n*=8) and *Cmah* –/– (filled bars, *n*=8) were fed ~1 mg/kg/day Neu5Gc (from normal chow) after weaning (4–6 weeks total), immunized with human RBC ghosts (Wild-type *n*=4, *Cmah* –/–*n*=4), or immunized with chimpanzee RBC ghosts (Wild-type *n*=4, *Cmah* –/– *n*=4). Sera were analyzed by ELISA for IgM (A, note the broken y-axis) and IgG (B) antibodies against Neu5Gcα-PAA and shown as mean absorbance values at OD_{405} ± SEM. Statistical analysis was performed using an unpaired two-tailed Student's *t*-test. n.s, not significant, *, $P < 0.05$ and **, $P < 0.01$. Data are representative of > three independent experiments.

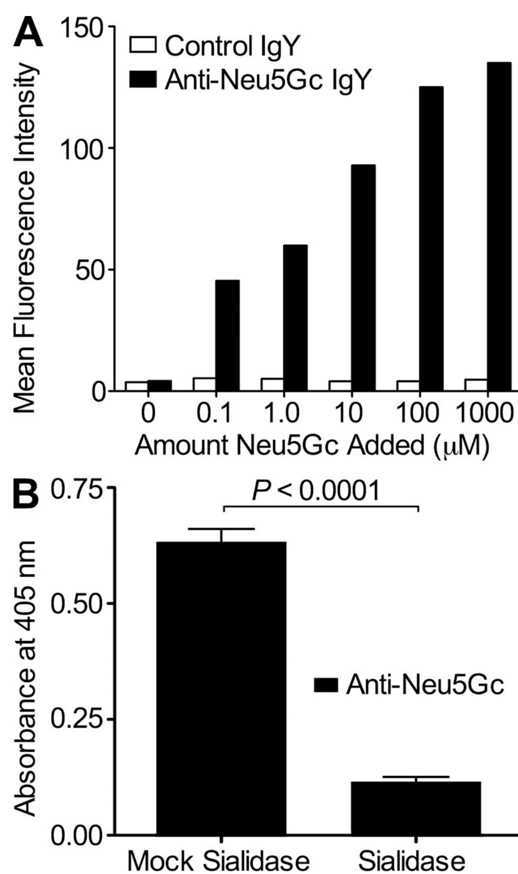

Figure 2-3. NTHi can efficiently take up and incorporate Neu5Gc.
(A) Dose dependent uptake and expression of NTHi (2019) grown in a Sia-free defined media with 0.1 μM–1 mM Neu5Gc. Neu5Gc was detected by flow cytometry analysis using a chicken anti-Neu5Gc IgY antibody. (B) 2019 grown in 1 mM Neu5Gc were 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 > three independent experiments and show the mean of triplicate absorbance values at OD_{405}. Error bars represent SD.

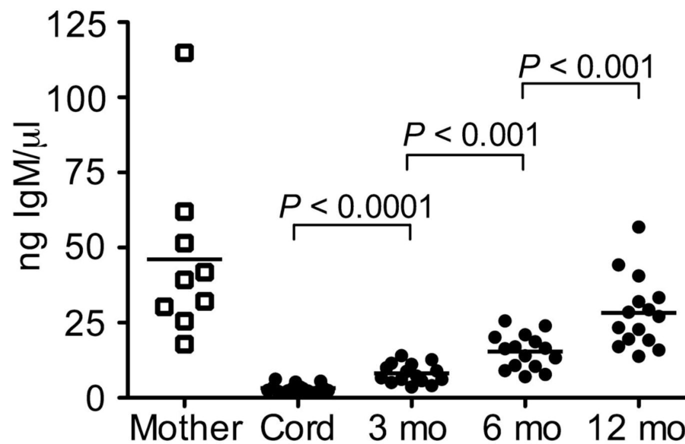

Figure 2-4. Anti-NTHi antibodies in human infants.
Infant sera (same infants analyzed in Fig. 2-1, *n*=15) were analyzed by whole-cell ELISA for IgM antibodies against NTHi strain 2019 grown in Sia-free media (filled circles). Adult sera (*n*=9) obtained from the pregnant mothers of the infants in this 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.

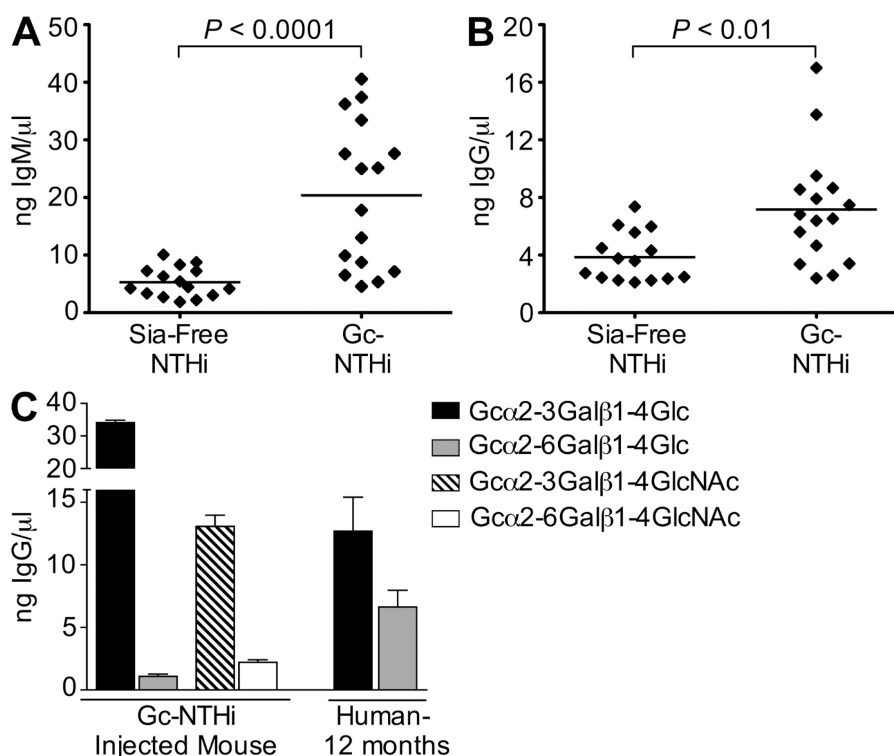

Figure 2-5. Neu5Gc expressed on NTHi induces anti-Neu5Gc antibodies in *Cmah* -/- mice.

(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 two week intervals. Sera collected following 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 prior to 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 Neu5Gcα2–3Galβ1–4Glcβ-HSA (black bar), Neu5Gcα2–6Galβ1–4Glcβ-HSA (gray bar), Neu5Gcα2–3Galβ1–4GlcNAcβ-HSA (white hashed bar), and Neu5Gcα2-6Galβ1-4GlcNAc-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 month old infants (*n*=15) was analyzed for levels of anti-Neu5Gc antibodies against Neu5Gcα2–3Galβ1–4Glcβ-HSA (black bar), Neu5Gcα2–6Galβ1–4Glcβ-HSA (gray bar) (same data as shown in Figure 2-1 B). Values represent mean IgG levels, quantified according to a human IgG standard curve. Error bars represent SEM. Data are representative of > three independent experiments.

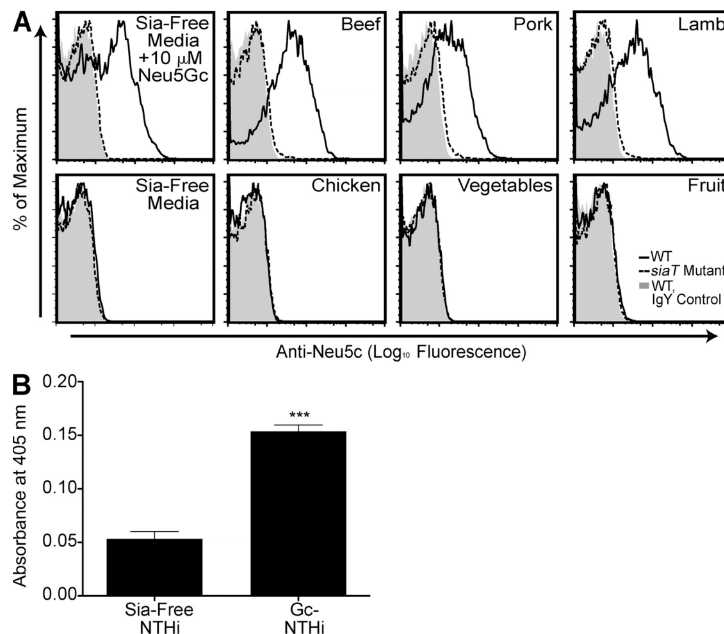

Figure 2-6. NTHi uptake and expression of Neu5Gc from baby food and its recognition by purified human anti-Neu5Gc antibodies.
(A) Wild-type NTHi strain 2019 (WT, solid line) and Sia transporter mutant strain of 2019 (*siaT* Mutant, dashed line) were grown in Sia-free media with or without the addition 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 shown), and IgY control for *siaT* Mutant (not shown) all showed similar shifts compared to 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 as the mean of triplicate values at OD_{405}. 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.

Table 2-1. 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) (9).
Adding Free Neu5Gc to Drinking Water	Maximum exposure 1 mg/ml in drinking water for 12 weeks.
Feeding Cow's Milk	Cow's milk contains ~ 8 μg/g total Neu5Gc (9). Gavaged 250 μl 5 d/week for 4 weeks.
Feeding Goat Cheese	Rich in Neu5Gc-containing glycolipids, 39.9 μg/g total Neu5Gc (9)
Feeding Mucin	Very rich in Neu5Gc. Mixed in with regular mouse chow.
Breast Feeding/Fostering by WT females	Wild-type 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.

Table 2-2. 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[a] μg/g	10.19	5.40	5.13	<0.07	<0.07
Free Neu5Gc[a] μg/g	2.68	1.56	0.98	<0.03	<0.03

[a]Total and free (non-glycosidically bound) Neu5Gc in commercial baby foods was determined by DMB-HPLC as described in *Materials and Methods.* Data represent mean values from two independent experiments. Previous studies have shown that plants are devoid of sialic acids (60)

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CHAPTER 3

Reduced Efficacy of a Neu5Gc-Glycosylated Biotherapeutic in the Presence of Anti-Neu5Gc Antibodies Generated by NTHi

INTRODUCTION

Chapter 2 of this dissertation describes the generation of a novel human-like mouse model using *Cmah* null mice immunized with Neu5Gc-expressing NTHi, to generate anti-Neu5Gc antibodies. The anti-Neu5Gc antibody titer and specificity elicited in these mice reflect what is seen in humans and therefore can be used to investigate the significance of these xeno-antibodies *in vivo*. Here we describe the use of this human-like mouse model to study the effect of anti-Neu5Gc antibodies on clearance of Neu5Gc-containing biotherapeutics.

Therapeutic glycoproteins, including antibodies, growth factors, cytokines, hormones and clotting factors, generate sales with annual double-digit growth rates (1). They must often be produced in mammalian expression systems because of the crucial influence of the location, number and structure of N-glycans on their yields, bioactivity, solubility, stability against proteolysis, immunogenicity and rate of clearance from the bloodstream (2-4).

Two differences between the protein glycosylation apparatus of humans and rodents account for major potential differences between the N-glycans on glycoproteins made in cultured human cells and those made using rodent cell lines. First, humans cannot synthesize a terminal Galα1-3Gal motif (known as alpha-Gal) on N-glycans. As a consequence, they express antibodies against this structure (5). Second, unlike other mammals, humans cannot biosynthesize the sialic acid Neu5Gc because the human gene *CMAH*, encoding CMP-*N*-acetylneuraminic acid hydroxylase, the enzyme responsible for producing CMP-Neu5Gc from CMP-*N*-acetylneuraminic acid (CMP-Neu5Ac), is irreversibly

mutated (6). The use of cultured human cells to address this issue is not a solution, as Neu5Gc can be taken up from animal products present in the culture medium and then metabolically incorporated into secreted glycoproteins (7).

Owing largely to limitations of the assays originally used to detect anti-Neu5Gc antibodies, including the fact that only a small number of possible Neu5Gc-containing epitopes were tested, healthy humans were long believed to show no immune reaction to Neu5Gc (8). Subsequent reports that all humans possess anti-Neu5Gc antibodies (9), sometimes at high levels, approaching 0.1–0.2% of circulating IgG (9, 10) have led to re-evaluation of the potential significance of Neu5Gc contamination (3, 4). Especially in light of trends toward administering increasingly higher amounts of certain biotherapeutics over longer periods of time, some biopharmaceutical companies are exploring steps to reduce levels of Neu5Gc in their products (11).

Given that they are produced using non-human cell lines, animal serum or serum-derived factors, or a combination of these, it is likely that most recombinant therapeutic glycoproteins carry some Neu5Gc. However, given the diversity of products and production protocols, it is difficult to make generalizations. Thus, we chose to compare two US Food and Drug Administration (FDA)-approved monoclonal antibodies with the same therapeutic target, the EGF receptor. The first, Erbitux (cetuximab, obtained from the University of California, San Diego Pharmacy), is a chimeric antibody

produced in mouse myeloma cells (12, 13). The second, Vectibix (panitumumab, obtained from Amgen), is a fully human antibody produced in Chinese hamster ovary (CHO) cells (14). The samples studied were preparations that would normally be administered to patients.

RESULTS

We first performed enzyme-linked immunosorbent assays (ELISAs) using an affinity-purified polyclonal chicken Neu5Gc-specific antibody preparation that is highly monospecific for Neu5Gc (15), alongside a nonreactive control IgY. Bound Neu5Gc was easily detectable on cetuximab but not on panitumumab (data not shown). Sialidase pretreatment abolished binding, confirming specificity. Western blot analysis also showed sialidase-sensitive anti-Neu5Gc IgY reactivity on the heavy chains of cetuximab but not those of panitumumab (data not shown). The specificity of anti-Neu5Gc IgY binding was reaffirmed by pretreatment with mild sodium periodate under conditions that selectively cleave sialic acid side chains (data not shown) and abolish reactivity of such antibodies (9, 15). Finally, we quantified sialic acids on the therapeutic antibodies. Panitumumab carries 0.22 mol of sialic acids per mole of protein, with <0.1% Neu5Gc. In contrast, cetuximab carries 1.84 mol of sialic acids per mole of protein, mostly as Neu5Gc (data not shown). The differences probably reflect different cell-expression systems. For example, in contrast to CHO cells, murine myeloma cell lines express a greater proportion of sialic acids as Neu5Gc (data not shown). Pull-down assays of cetuximab with SNA-agarose (modified with the lectin *Sambucus nigra* agglutinin, which

recognizes α2-6-linked sialic acids), followed by ELISAs of unbound proteins, showed that only about half of cetuximab molecules actually carry bound sialic acids and Neu5Gc (data not shown). Such heterogeneity is typical for glycoproteins.

We next evaluated whether Neu5Gc affects clearance rate when circulating anti-Neu5Gc antibodies are present. To mimic the situation in humans, we used the mice mentioned earlier with a human-like defect in the *Cmah* gene (16). Such mice were previously used and make anti-Neu5Gc antibodies upon immunization with glycosidically bound, but not free, Neu5Gc (17-19). However, these previous studies used whole rodent or chimpanzee cells for immunization (17, 18), an artificial approach. In contrast, feeding of Neu5Gc (which is present in mouse chow) does not induce a human-like immune response in the mutant mice (19). We could not immunize the mice with cetuximab itself, as other antibodies directed against the partly human IgG protein backbone would confound any results. To most closely mimic the situation in humans, we therefore immunized with Neu5Gc-loaded *Haemophilus influenzae* (see Materials and methods); this is very similar to the mechanism by which human Neu5Gc-specific antibodies appear to be generated naturally (Chapter 2 of this dissertation (19)). Given the great variability in isotypes and affinities of the naturally occurring human anti-Neu5Gc antibodies, as well as their different relative reactivities against various Neu5Gc-containing antigens (10), it is impractical to model all possible human conditions. We therefore chose to mimic a situation in a human with relatively high levels of the IgG

antibodies against the kind of Neu5Gc epitope (Neu5Gcα2-6Galβ1-4Glc-) found in cetuximab (20). It also happens that this epitope is commonly recognized by human anti-Neu5Gc antibodies (10).

Each of the therapeutic antibodies, cetuximab and panitumumab, was injected intravenously at levels estimated to ensure a concentration of 1 μg ml^{-1} in the extracellular fluid volume according to mouse body weight (21). Next, sera pooled from naïve, control-immunized or Neu5Gc-immunized syngeneic mice were passively transferred via intraperitoneal injection, ensuring equal starting concentrations of circulating Neu5Gc-specific antibodies. Anti-Neu5Gc IgG levels in the pooled sera from Neu5Gc-immunized mice were quantified using ELISA with a Neu5Gcα2-6Galβ1-4Glc-conjugate as a target, as previously describe (10) (97.5 μg ml^{-1}, data not shown). The amount of pooled antibody injected was then calculated to achieve an approximate starting concentration of 4 μg ml^{-1} IgG in the extracellular fluid volumes of the mice, which is about a four fold excess of anti-Neu5Gc antibodies compared to the injected drug in the mice, and similar to levels found in some humans (10).

Clearance was monitored by a sandwich ELISA specific for human IgG-Fc. Although both drugs had a similar clearance rate in mice pre-injected with serum from naïve or control-immunized mice, circulating levels of cetuximab decreased significantly ($P < 0.001$) when Neu5Gc-specific antibodies were pre-injected (Fig. 3-1). Assuming that a similar interaction between cetuximab and circulating anti-Neu5Gc antibodies occurs in patients, there could be relevant

effects on clearance rate and efficacy. This might help to explain the wide range of half-life values reported for such antibodies in clinical studies (13, 14).

DISCUSSION

Despite their successful use for a variety of indications, infusion-related reactions, immunogenicity and accelerated clearance remain important concerns for many therapeutic glycoproteins (3, 22). The incidence and severity of an immune reaction depends on the interplay of infused agents with the immune system and can vary greatly from patient to patient. Understanding the underlying nature of these events will help to identify patients at risk with the use of specific markers. Humanized and fully human antibodies have been developed to reduce immunogenicity due to peptide epitopes (1). However, the potential immunogenicity of the glycans they carry has not been as well considered. It is known that immune reactions can be mediated by binding of pre-existing IgEs against the non-human alpha-Gal epitope carried by some agents, such as cetuximab (12). However, in our studies alpha-Gal residues are not an issue, as *Cmah*-null mice already express this sequence and do not have antibodies against it.

A further concern arises here because pre-existing antibodies against a glycan on a glycoprotein can secondarily enhance antibody reactivity against the underlying protein backbone (23), perhaps because immune complexes are cleared efficiently by Fc receptors into dendritic cells and other antigen-presenting cells (24, 25). Such a mechanism might help explain why patients' immunogenicity to some glycoprotein therapeutics sometimes increases over

time (23, 26, 27). If this were true, it would likely have a further impact in long-term replacement therapy with recombinant therapeutic glycoproteins.

Additional studies done by others in the lab showed that affinity-purified human anti-Neu5Gc antibodies also reacted with cetuximab and not to panitumumab, and that human sera with high titer anti-Neu5Gc antibodies formed immune complexes with cetuximab (28). Taken together, our findings suggest that the potential significance of the presence of Neu5Gc on glycoprotein biotherapeutics should be revisited. Despite a natural tendency to downplay potential new problems involving currently useful drugs, it is worthwhile to consider lessons from other fields, where initial enthusiasm was not balanced by full appreciation of immunological implications (29). With this in mind, we have also suggested that Neu5Gc contamination of stem cells and other cell types intended for human therapy could pose risks (30, 31). In addition, others have recently reported that *Cmah*-null mice can reject Neu5Gc-positive wild-type organ transplants via complement-fixing Neu5Gc-specific antibodies (18).

MATERIALS AND METHODS

Mice. The *Cmah*-null mice used for this study have been described previously (16) and were backcrossed to C57Bl/6 mice for over ten generations. All experiments were approved by the University of California, San Diego Institutional Review Board committee responsible for approving animal experiments.

Generation of murine Neu5Gc-specific antibodies. *Haemophilus influenzae* strain 2019 (32) was grown to mid log phase in sialic acid–free media (33) with or without addition of 1 mM Neu5Gc (19), heat-killed and injected intraperitoneally (200 µl of culture at an absorbance of 600 nm of 0.4) into *Cmah*-null mice.

Effects of anti-Neu5Gc antibodies on *in vivo* kinetics of therapeutic antibodies. Cetuximab or panitumumab in PBS (0.24 µg per gram mouse body weight) were injected intravenously, and 14 h later, mouse serum pooled from syngeneic *Cmah*-null mice containing anti-Neu5Gc antibodies (or pooled serum from syngeneic naïve or control-immunized mice) was passively transferred via intraperitoneal injection into syngeneic *Cmah*-null mice that were prescreened for the absence of pre-existing antibodies against human IgG. Mice were bled 0, 2, 8, 32, 56 and 80 h after the passive transfer of mouse serum. For quantification of therapeutic antibody concentrations in the sera, wells of ELISA plates were coated with 1 µg of anti-human IgG (Biorad), then blocked with TBST for 2 h and incubated with 1:500 dilutions of the sera in each well. Captured therapeutic antibodies were detected by HRP-conjugated anti-human Fc (Jackson; 1:10,000), with development by *O*-phenylenediamine in citrate-phosphate buffer, pH 5.5, and absorbance measured at 495 nm (n = 5 for injections of both control sera groups; n = 10 for injections of anti-Neu5Gc serum groups).

Quantification of Neu5Gc-specific IgG antibodies in Neu5Gc-immunized mice. A Neu5Gcα2-6Galβ1-4Glc-conjugate (10) (1 µg per well) and serial

dilutions of mouse IgG as standards (0.625–20 ng per well) were used for coating overnight, then blocked with PBST for 2 h and incubated with pooled serum from Neu5Gc-immunized mice (1:250 dilution) for 2 h at 25 °C. Binding of mouse IgG was detected using HRP-conjugated goat anti-mouse IgG-Fc (Jackson; 1:10,000 in PBST) and developed with *O*-phenylenediamine in citrate-phosphate buffer, pH 5.5, with absorbance measured at 490 nm. ELISA samples were studied in triplicate.

Levels of anti-Neu5Gc IgG after injections of the antibodies. *Cmah*-null mice were injected intravenously with 4 μg antibody per gram of mouse body weight in PBS weekly for 3 weeks. Mice were bled initially, and again 1 week after the third intravenous injection. Wells of ELISA plates were coated with 1:1,000 dilutions of human (Neu5Gc-deficient) or chimpanzee (Neu5Gc-positive) serum glycoproteins (note that the only major difference between human and chimp serum glycosylation is the absence or presence of Neu5Gc; (34)). Alternatively, wells were coated with human or bovine fibrinogen, which carry Neu5Ac or Neu5Gc on otherwise identical N-glycans (35). Wells were then blocked with TBST for 2 h followed by incubation with 1:100 dilutions of the mouse sera. Binding of the mouse antibodies was detected using HRP-conjugated goat anti-mouse IgG Fc fragment (1:10,000 in TBST). Neu5Gc-specific binding (change in absorbance at 495 nm) was determined by subtracting the background signal of the wells coated with human serum or human fibrinogen (no Neu5Gc) from the signal of chimpanzee serum–coated or bovine fibrinogen–coated wells (containing Neu5Gc). Data were obtained in

triplicate (*n* = 5 for injection of mouse IgG; *n* = 4 for injection of panitumumab; *n* = 6 for injection of cetuximab).

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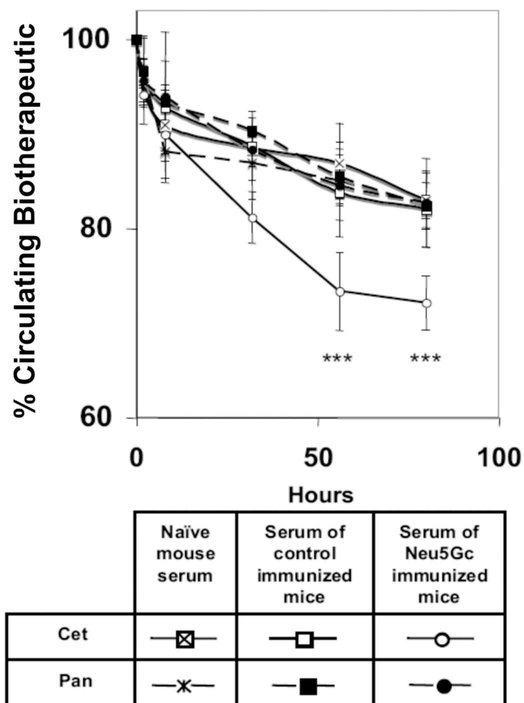

Figure 3-1. Effects of Neu5Gc-specific antibodies on the kinetics of therapeutic antibodies in mice with a human-like Neu5Gc deficiency.
Cmah-null mice were first injected intravenously with either of the therapeutic antibodies, cetuximab (Cet) or panitumumab (Pan). Serum from *Cmah*-null mice containing anti-Neu5Gc antibodies (or serum from naïve mice or control-immunized mice) was then passively transferred by intraperitoneal injection. Mice were bled periodically after the passive transfer of serum. Concentrations of Cet or Pan in the isolated sera were determined by sandwich ELISA. Absorbance was measured at 495 nm. The *y* axis starts at 60% to better display the difference in kinetics. Error bars, s.d.; ***$P < 0.001$, unpaired two-tailed *t*-test.

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CHAPTER 4

Potential Role of Anti-Neu5Gc Antibodies Generated by NTHi in Infertility and Reproductive Incompatibility During Human Evolution

INTRODUCTION

The *Alu* mediated inactivating mutation in the human *CMAH* gene was originally estimated to have occurred approximately 2.7 million years ago (1, 2). More recent analysis has estimated that this mutation occurred 3.2 million years ago and its subsequent fixation (estimated time of most recent common ancestor) reaching as far back as 2.9 million years ago. (3). The short time span of 0.3 million years between estimated mutation and fixation has been interpreted as evidence for strong selection by pathogens, possibly in combination with demographic events, i.e. small populations favoring rapid fixation.

Here we propose an alternative or complementary hypothesis for explaining such a rapid fixation. We propose that the loss of Neu5Gc may have affected reproductive compatibility within ancestral populations and might have provided a mechanism for rapid fixation based on female immunity to male sperm bearing Neu5Gc-containing glycans.

This hypothesis is based upon the fact that female reproductive fluid can contain high levels of antibodies and complement derived from the bloodstream (4). Meanwhile sperm cells are covered by an especially thick sialic acid-rich glycocalyx (5, 6). Thus, there was a time approximately 3 million years ago (after the initial mutation in the *CMAH* gene but before it's fixation in the population) when human ancestors were polymorphic for Neu5Gc expression, and fetus or sperm expressing Neu5Gc could be at risk for immune attack by maternal anti-Neu5Gc antibodies. Here we test the hypothesis that anti-

Neu5Gc antibodies in females homozygous for the inactive *CMAH* allele could have contributed to glycan-mediated reproductive incompatibility with heterozygous or wild-type (WT) males, contributing to accelerated fixation of the inactive allele in the human lineage. We test this hypothesis using the human-like mouse model described in Chapter 2 of this dissertation.

RESULTS

Mouse anti-Neu5Gc antibodies can recognize Neu5Gc on WT mouse sperm. Unlike adult humans, *Cmah* null mice do not spontaneously generate antibodies against Neu5Gc (7). However, as described in Chapter 2 of this thesis, these mice can be immunized against Neu5Gc using a mechanism likely similar to that by which humans are naturally induced to generate anti-Neu5Gc antibodies. This involves uptake and incorporation of Neu5Gc into cell surface LOS molecules of nontypeable *Haemophilus influenzae*. This immunization method has been shown to induce anti-Neu5Gc IgM and IgG antibodies with similar titer and specificity to those found in humans, generating a human-like mouse model. Conversely, control mice can be immunized with sialic acid-free bacteria and do not generate anti-Neu5Gc antibodies (Chapter 2 of this dissertation).

The expression of Neu5Gc on WT mouse sperm was first confirmed by HPLC analysis as well as staining with a Neu5Gc-specific polyclonal chicken IgY antibody (8) (data not shown). Next, we asked whether anti-Neu5Gc antibodies induced in our human-like mouse model could recognize Neu5Gc-expressing sperm. We investigated this in an ELISA assay using immobilized

WT, *Cmah* heterozygote, and control *Cmah* null mouse sperm. As predicted, sialidase sensitive anti-Neu5Gc antibody binding was only noted when immunized female mouse serum was applied to WT or *Cmah* heterozygote sperm but not to *Cmah* null sperm (Fig. 4-1).

Female *Cmah* null mice with anti-Neu5Gc antibodies show reduced fertility with Neu5Gc-positive WT males. Having established that anti-Neu5Gc antibodies generated in female *Cmah* null mice specifically recognize Neu5Gc-containing sperm, we performed breeding experiments with cohorts of mice representing all possible pair-wise combinations of Neu5Gc- and control-immunized females with WT and *Cmah* null males (all in a C57BL/6 congenic background). Of the eight combinations of breeding pairs used, only the immunized *Cmah* null females bred with WT males showed significantly reduced fertility as determined by a 25% reduction in litter size (Fig. 4-2). While we assume that this reduced fertility is mostly due to pre-zygotic effects, i.e., sperm killing or inactivation by female antibodies, there is also the possibility of post-zygotic incompatibility. To investigate this possibility, we performed crosses between heterozygote males and immunized *Cmah* null females and studied the genotypes of the progeny. While fertility was again significantly reduced in immunized females (by 30%) and a lower than expected fraction of heterozygote pups was observed, the latter effect was only marginally significant in a Chi square test (Fig. 4-3). Thus post-zygotic antibody-mediated incompatibility is likely a lesser contributor to the overall reproductive incompatibility.

DISCUSSION

In this study we show an anti-Neu5Gc antibody dependent mechanism of reproductive incompatibility between *Cmah* null female and WT male mice. The apparently short time to fixation of the *CMAH* mutation in humans suggests that strong selection by pathogens was likely a major driving force for the complete loss of this functional enzyme in humans. While it is impossible to know the exact selective pressures that were involved, it is conceivable that the loss of Neu5Gc could have provided a selective advantage against a Neu5Gc-binding pathogen. Indeed, one possible candidate for such a pathogen would be *P. falciparum*, a Neu5Ac-binding protozoan parasite responsible for the most serious form of malaria in humans, which has recently been shown to have evolved from *P. reichenowi* which preferentially binds Neu5Gc and infects chimpanzees (9, 10). While such a Neu5Gc-binding pathogen likely represents the initial selective force for the *CMAH* mutation, our current study suggests that reproductive isolation subsequent to the induction of anti-Neu5Gc antibodies in *Cmah* null female human ancestors could also have contributed to the fixation of the inactive *CMAH* allele. Furthermore, our *in vivo* data point to the possible existence of a sympatric speciation mechanism based on the loss of Neu5Gc and subsequent induction of anti-Neu5Gc antibodies. Interestingly, the estimated timing of the *CMAH* mutation roughly coincides with the estimated time of speciation of the genus *Homo*. Therefore, the possibility exists that this mechanism of cell surface sialic acid mismatch and immune reaction could

have contributed to both the fixation of the *CMAH* mutation and the speciation of the genus *Homo*.

MATERIALS AND METHODS

Mice. *Cmah*−/− mice (7) were backcrossed onto a congenic C57BL/6 background. Wild-type C57BL/6 mice were purchased from Harlan Laboratories (Indianapolis, IN). Mice were fed standard chow (LabDiet, PicoLab Rodent Diet 20, #5053) and water ad libitum and maintained on a 12-hour 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 & Use Committee of the University of California, San Diego.

Generation of anti-Neu5Gc antibodies in mice and breeding set-up. As described in Chapter 2 of this dissertation, 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 OD_{600} 0.4) intraperitoneally into *Cmah*-null and WT (C57BL/6) mice (age 8-12 weeks, female). All mice were injected a total of three times at 2 week intervals. No adjuvant was used with any of the bacterial injections. For the fertility study, all possible combinations of naïve WT and *Cmah* null male mice were paired with Neu5Gc-immunized and control immunized WT and *Cmah* null female mice. Breeding experiments were carried out by pairing each female with a single male. Litters were removed at weaning. Serum samples from female mice were collected before the immunization (pre-immune), after the three immunizations, and after each litter.

Litter size and date of birth were recorded and neonatal mice were weighted, sacrificed, and bled for the collection of serum. The study was terminated 5 months after the breeding pairs were set up.

Mouse Sperm. Mouse sperm were harvested from 12 to 20 week old males immediately after sacrificing the animals from the cauda epididymis. Cauda epididymis tissue was minced and kept on a shaker at room temperature for 10 minutes, followed by 30 sec centrifugation at 500 g and a swim up procedure in sperm storage buffer (SSB, 110 mM NaCl, 27.2 mM KCl, 0.36 mM NaH_2PO_4, 0.49 mM $MgCl_2$, 2.40 mM$CaCl_2$, 25.00 mM HEPES, 5.00 mM MES/ 2-(N-morpholino)ethanesulfonic acid, 25.00 mM lactic acid, pH 5.5 (11)). Sperm were further subjected to a swim up procedure in 500 μl SSB @ 37C & 5%CO_2 for 30min, before collection of the supernatant.

Antibody-Sperm binding assays. Antibody-Sperm binding assays by mouse immune sera were performed on plated and freshly fixed wild type and *Cmah*$^{-/-}$ and *Cmah*$^{-/+}$ epididymal sperm. Epididymal sperm were collected immediately after sacrifice of male mice. Sperm were diluted to a concentration of 8 million /ml in BWW HSA (21 mM HEPES, 21.5 mM Lactic acid, 91.06 mM NaCl, 4.78 mM KCl, 1.71 mM $CaCl_2$, 1.71 mM KH_2PHO_4, 2.44 mM $MgSO_4$, 5.55 mM Glucose, 4mM $NaHCO_3$, 0.25 mM Sodium pyruvate, 1% HSA, pH 7.5, filter sterilized and stored at 4ºC and warmed to room temperature prior to use). 100 μl of this suspension was added to each well of a COSTAR microplate. Plates were spun down at 250 *g* for 5 minutes at room temperature. Supernatant was discarded by gently flicking the plate on paper towel. Cell

densities were verified under microscope. Plate was allowed to air dry for 10 minutes. The plated sperm were fixed using freshly thawed formaldehyde adjusted to 1%. 200 μl of 1% paraformaldehyde in PBS was added to each well. After fixation plate was washed three times with 200 μl PBS + 0.1% Tween per well. *Arthrobacter ureafaciens* sialidase (AUS) treatment: 5 mU of AUS in AUS buffer (50 mM sodium acetate, pH 5.5) were added to each well and sample was incubated at 37°C for 2 hours. Control was a sham treatment with buffer and heat-treated AUS. Plate was blocked with TBS + 1% ovalbumin at room temperature for 1 hour. Serum was added at a concentration of 1:100 in TBS + 1% ovalbumin, 100 μl per well and incubated at room temperature for 2 hours. Plate was washed three times with 150 μl of TBS + 1% ovalbumin per well and then blotted. Secondary antibody (Donkey anti-mouse IgG-alkaline phosphatase) was added at a concentration of 1:500 in TBS and incubated for 30 min at room temperature. Plate was washed three times with TBS, and then developed with *p*-nitrophenyl phosphate, with product measured at 405 nm wavelength on a SpectraMax 250 (MDS Analytical Technologies) plate reader.

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Cohen, Patrick Secrest, Ajit Varki, and Pascal Gagneux. Dr. Pascal Gagneux directed and supervised the research that forms the basis for this chapter.

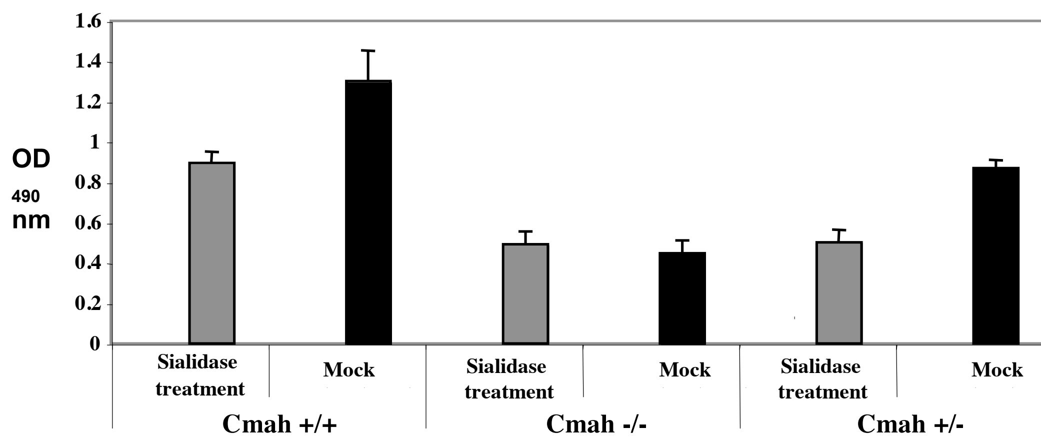
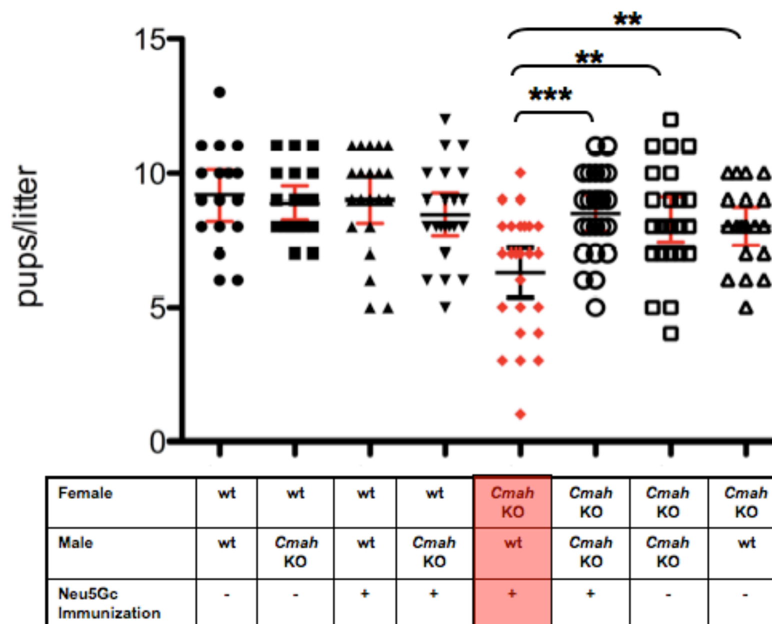
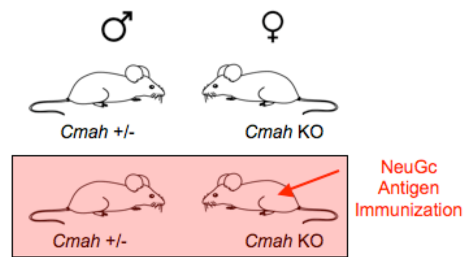


Figure 4-1. Antibodies from immunized *Cmah*$^{-/-}$ females recognize WT mouse sperm in a sialic acid dependent manner.
Results for an ELISA using immunized mouse sera on plated, fixed mouse sperm from WT (*n*=3), *Cmah*$^{-/-}$ (*n*=2) and *Cmah*$^{-/+}$ (*n*=1) males. The binding of high anti-Neu5Gc serum was reduced after treatment with bacterial sialidase (AUS) indicating the sialic acid-dependent nature of the interaction. Anti-Neu5Gc activity of the sera was determined by ELISA using polyacrylamide probes bearing Neu5Gc or Neu5Ac sialic acids respectively. Heterozygote sperm were bound less than WT sperm but did show sialic acid dependent antibody binding. Mock treatment included all reagents except the sialidase.



Female	wt	wt	wt	wt	*Cmah* KO	*Cmah* KO	*Cmah* KO	*Cmah* KO
Male	wt	*Cmah* KO	wt	*Cmah* KO	wt	*Cmah* KO	*Cmah* KO	wt
Neu5Gc Immunization	-	-	+	+	+	+	-	-

Figure 4-2. Reduced fertility in Neu5Gc immunized female *Cmah* null mice mated with WT males.
The cumulative reproductive output of each cohort of nine females for three consecutive litters is shown. Bars show 95% confidence intervals for unpaired Student t-test. *** indicates $P < 0.0005$ and ** $P < 0.05$. The table below the scatter plot shows the mating and immunization scheme.



Deviation from Mendelian 50:50 ratio of -/- and +/- pups?

	-/-	+/-		
immune female	43	27	Total=	70
expected	35	35	p value = 0.055	
naive female	61	49	Total=	110
expected	55	55	p value = 0.25	

Figure 4-3. Investigating postzygotic effects of anti-Neu5Gc dependent reproductive incompatibility.
Skewed distribution of offspring in litters from Neu5Gc immunized *Cmah*$^{-/+}$ males mated with immunized *Cmah*$^{-/-}$ females.

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CHAPTER 5

Differential Utilization of Human and Non-Human Sialic Acids by NTHi

INTRODUCTION

Nontypeable *Haemophilus influenzae* (NTHi) is an obligate human nasopharyngeal commensal and opportunistic pathogen. NTHi cannot endogenously synthesize sialic acids but instead sialylate their cell surface LOS molecules using host-derived sialic acid. This LOS sialylation is essential for human serum resistance *in vitro* (1, 2) and virulence in otitis media models *in vivo* (3-5).

NTHi can also catabolize the common human sialic acid, *N*-acetylneuraminic acid (Neu5Ac), to be used as a source of carbon, nitrogen and energy (6, 7). The degradation of Neu5Ac by NTHi is carried out by five enzymes encoded by a highly conserved gene cluster (8). A similar sialic acid degradation pathway with homologous genes has been well characterized in *Escherichia coli* (*E. coli*) (6). Sialic acid breakdown is initiated in the cytosol by the sialic acid lyase, NanA, which cleaves sialic acid to yield ManNAc and pyruvate. Next, NanK kinase phosphorylates ManNAc to ManNAc-6-P, which is subsequently converted to GlcNAc-6-P via the NanE epimerase. The NagA deacetylase converts GlcNAc-6-P to GlcN-6-P and the final enzyme, NagB, deaminates GlcN-6-P to form Fruc-6-P which can enter into the glycolysis pathway. UDP-GlcNAc production from intermediates of this degradation pathway, can also be used as a precursor to cell wall or LOS biosynthesis (9, 10)(Fig. 5-1).

Catabolism of Neu5Ac is not essential for survival or host infectivity of NTHi as deletion of the *nanA* gene results in increased fitness in an infant rat

model (10) and enhanced human serum resistance, presumably from the resulting hypersialylation of this mutant (1, 11). These results suggest that Neu5Ac is not a critical source of nutrition for NTHi. In fact, sialic acid catabolism is repressed by SiaR, which is a transcriptional regulator of the sialic acid catabolic and transport operons (8, 11). Instead, it is hypothesized that the major role of sialic acid catabolism is to prevent the toxic accumulation of sialic acid. In support of this hypothesis, a *siaRnanA* double mutant of NTHi is unable to grow in the presence of Neu5Ac (11).

Unlike most mammals which express two major sialic acids, Neu5Ac and *N*-glycolylneuraminic acid (Neu5Gc), humans are deficient in Neu5Gc synthesis and express an excess of Neu5Ac on their cell surfaces and secretions (12, 13). Additionally, all humans have circulating antibodies against Neu5Gc-containing glycans (14-18).

Chapter 2 of this dissertation includes the novel finding that NTHi can take up and express the non-human sialic acid Neu5Gc. Here we expand upon this finding and describe a constellation of findings related to the differential expression and catabolism of Neu5Ac and Neu5Gc by NTHi.

RESULTS

Neu5Gc stimulates growth of NTHi

During the course of studies investigating the uptake and expression of Neu5Gc by NTHi (Chapter 2 of this dissertation), it was observed that NTHi consistently grew faster in media containing 1mM Neu5Gc as compared to 1mM Neu5Ac (unpublished data). To investigate whether exogenous Neu5Gc

indeed stimulates growth of NTHi, WT and NanA deficient strains were grown in sialic acid-free media alone or with increasing amounts of Neu5Ac or Neu5Gc. Consistent with studies by others showing that Neu5Ac does not stimulate growth under normal glucose conditions (19), neither the WT nor the NanA deficient strain showed any growth enhancement in the presence of exogenous Neu5Ac (Fig. 5-2 A and B). In contrast, when Neu5Gc (0.01 mM-1 mM) was added to the media, there was a dose dependent increase in growth as compared to media alone (Fig. 5-2 C). There was no increase in growth of the *nanA* mutant strain grown in the presence of Neu5Gc (Fig. 5-2 D) demonstrating that growth stimulation by Neu5Gc is dependant on the sialic acid catabolic pathway.

The NTHi sialic acid lyase (NanA) preferentially catabolizes the human sialic acid, Neu5Ac

The Neu5Gc-induced growth stimulation of NTHi suggests that the NanA lyase, or another enzyme further downstream in the sialic acid catabolic pathway, may preferentially degrade Neu5Gc or one of it's breakdown products. In order to determine the sialic acid preference of the NanA lyase, a competition lyase assay was performed in which equal amounts of Neu5Ac and Neu5Gc (500 μM each) were added to cell lysates prepared from WT and NanA deficient NTHi strains. Aliquots of these lysates were collected at 1-hour intervals and the breakdown of each sialic acid was analyzed by quantifying the amount of Neu5Ac and Neu5Gc by HPLC. As shown in Fig. 5-3 A, both Neu5Ac and Neu5Gc levels in the lysates diminished over time in the WT strain, indicating

the NanA lyase of NTHi is capable degrading both of these sialic acids. However, the rate of Neu5Ac disappearance from the lysate was considerably faster than that of Neu5Gc, demonstrating preferential degradation of Neu5Ac over Neu5Gc (Fig. 5-3 A). No decrease in either Neu5Ac or Neu5Gc was seen at any time point in the lysate of the *nanA* mutant, confirming that the disappearance of Neu5Ac and Neu5Gc in the WT strain is due to the lyase activity of NanA (Fig. 5-3 B). Thus, at a single concentration in a competition study Neu5Ac is the preferred substrate for the lyase. In the future more detailed kinetic studies may help to better define the extent of this difference.

Preferential expression of Neu5Ac on LOS of NTHi

In Chapter 2 of this dissertation we show that NTHi can take up and express free Neu5Gc. We next asked whether NTHi has a preference for incorporating Neu5Ac or Neu5Gc onto cell surface LOS molecules. WT NTHi strains 2019 (20), DH1(21), and Int1 (22) were grown in media containing equal amounts (50 μM each) of Neu5Ac and Neu5Gc and cell surface sialic acid was analyzed by HPLC. As shown in Fig. 5-4, all three strains were found to express approximately 2-4-fold more Neu5Ac as compared to Neu5Gc. A preference for Neu5Ac LOS expression was also found for the encapsulated type b strain, Eagan (data not shown) (23), suggesting that this preference for incorporating Neu5Ac into LOS is common among *H. influenzae* strains.

Differential sialic acid expression preferences of two related bacteria with different mammalian hosts

Having shown that NTHi preferentially expresses Neu5Ac, we asked whether the preference for Neu5Ac expression was a general feature of all bacteria that scavange sialic acid. To investigate this, we compared the sialic acid expression of NTHi strain 2019, with that of a related bacterium in the same family, *Histophilus somni (H. somni*; Formally *Haemophilus somnus)* strain 2336. *H. somni* has previously been shown to incorporate exogenous Neu5Ac to sialylate it's LOS (24). Both strains were grown overnight on chocolate agar and cell surface sialic acids were analyzed by HPLC. Under these growth conditions, NTHi was found to express approximately 75% more Neu5Ac than Neu5Gc, while *H. somni* strain 2336 showed the opposite pattern, preferentially expressing Neu5Gc (Fig. 5-5). Interestingly, *H. somni* is a bovine commensal and pathogen suggesting that the sialic acid expression pattern of these two bacteria may not be random, but instead may mimic the sialic acid expression of their respective hosts (bovine tissues can be rich in Neu5Gc).

Analysis of sialic acid expression comparing three pathogenic *H. somni* strains (649, 8025, and 2336) and three commensal strains (1P, 127P, and 129Pt) revealed that all three pathogenic strains preferentially express Neu5Gc while one commensal strain (127P), preferentially expresses Neu5Ac, in a manner similar to NTHi. The other two commensal strains, 1P and 129Pt, did not express any sialic acid (Fig. 5-6). These results indicate that the preferential expression of Neu5Gc may be a virulence factor for *H. somni.*

Further studies are limited by the fact that we have been unable to find a defined media in which *H. somni* will grow (unpublished data), a requirement for controlling sialic acid intake.

Human anti-Neu5Gc antibodies can kill Neu5Gc-expressing NTHi

We have previously shown that purified human anti-Neu5Gc antibodies specifically recognize Neu5Gc-expressing NTHi (Chapter 2 of this dissertation). These results, together with those of the present study showing that NTHi preferentially expresses Neu5Ac on cell surface LOS molecules (Fig. 5-4) suggest that the expression of Neu5Gc could have a detrimental effect on NTHi. To test this hypothesis, we analyzed the effect of Neu5Ac or Neu5Gc expression by NTHi on human serum sensitivity. Others have previously shown that Neu5Ac expression on LOS is required to protect NTHi against complement mediated killing in human serum (1, 2). Consistent with these studies, we show that NTHi grown in the absence of sialic acid are serum-sensitive, while Neu5Ac-expressing NTHi are serum-resistant (Fig. 5-7). Interestingly, we found that Neu5Gc-expressing NTHi showed similar serum sensitivity compared to sialic acid-free NTHi (Fig. 5-7).

All humans have circulating antibodies against Neu5Gc, as this “xeno-glycan” is not expressed in humans. To investigate whether this serum sensitivity of Neu5Gc-expressing NTHi (Fig. 5-7) was due to anti-Neu5Gc antibodies, we performed the serum killing assay in the presence of glycosidically-bound Neu5Gc on a polyacrylamide scaffold (Neu5Gcα-PAA) to block anti-Neu5Gc antibodies (17) or as a negative control, 2 μg glycosidically-

bound Neu5Ac (Neu5Acα-PAA). Indeed, the presence of Neu5Gcα-PAA, but not Neu5Acα-PAA rendered Neu5Gc-expressing NTHi serum-resistant (Fig. 5-7). Furthermore, no killing of Neu5Gc-expressing NTHi was seen in the absence of calcium (unpublished data), demonstrating that the killing of Neu5Gc-expressing NTHi in human serum is most likely mediated by the classical complement pathway. Together, these data indicate that human anti-Neu5Gc antibodies in serum can efficiently and specifically kill Neu5Gc-expressing NTHi via classical complement pathway activation.

DISCUSSION

In this chapter, we present data investigating the differential utilization of Neu5Ac and Neu5Gc by NTHi. We show the surprising finding that this human-specific commensal/pathogen grows better on the non-human sialic acid Neu5Gc. The fact that Neu5Gc-dependent growth stimulation was not seen in the *nanA* mutant of NTHi suggests that this stimulation is depended on Neu5Gc catabolism. NanA is the first enzyme in the sialic acid degradation pathway of NTHi. Here we show that while the NanA lyase of NTHi strain 2019 is capable of degrading both Neu5Ac and Neu5Gc, it's preferred substrate appears to be Neu5Ac. Similarly, others have found that the *E.coli* NanA lyase Vmax for Neu5Ac is 5-fold higher than that of Neu5Gc (25). This result does not explain why NTHi grows better in Neu5Gc than Neu5Ac but suggests that one of the enzymes further down the catabolic pathway might preferentially recognize the glycolyl group of one of the breakdown products of Neu5Gc (ManNGc-6-P and GlcNGc-6-P). Alternatively, the presence of an acetyl group on the breakdown

products of Neu5Ac, may also cause these intermediates to be preferentially shunted into a pathway to generate UDP-GlcNAc, the precursor of peptidoglycan and LOS - while the breakdown products of Neu5Gc may primarily be used to generate Fruc-6-P to enter glycolysis. Interestingly, it has been proposed that a putative isomerase or epimerase from *H. influenzae*, YhcH, may be involved in Neu5Gc catabolism, although functional studies are lacking (26). Further studies are currently underway in our lab to investigate the catabolic fate of Neu5Gc.

We determined that NTHi preferentially expresses Neu5Ac on cell surface LOS molecules. Previous studies by others have shown that the siaP component of the sialic acid transporter has an approximately 2-fold higher affinity for Neu5Ac than Neu5Gc which may contribute to this finding (19). Future studies to determine the sialic acid preferences of the CMP-sialic acid sythetase and the 3 sialyltransferases of NTHi strain 2019 will help to characterize the differential utilization of Neu5Ac and Neu5Gc by NTHi.

We have found that *H. somni*, another Pasteurellaceae family bacterium that expresses host-derived sialic acid, preferentially incorporated Neu5Gc into its cell surface LOS. While the human host of NTHi is deficient in Neu5Gc synthesis, this sialic acid is abundant in the bovine host of *H. somni* suggesting that the sialylation patterns of these bacteria may have evolved to best mimic host sialic acid expression patterns. Interestingly, preferential Neu5Gc expression by *H. somni* strains appears to correlate positively with virulence. Future studies comparing Neu5Gc and Neu5Ac expression in resistance to host

immune responses should help define the roles of these sialic acids in *H. somni*.

Our results also suggest that preferential expression of Neu5Ac over Neu5Gc is conserved among *H. influenzae* strains. Given that significant levels of free Neu5Gc can be available for uptake by NTHi from diets containing red meat products (Chapter 2 of this dissertation), we propose that preferential Neu5Ac expression by NTHi likely protects against host recognition and killing mediated by human anti-Neu5Gc antibodies (Fig. 5-7).

The efficient killing of Neu5Gc-expressing NTHi mediated by pre-existing anti-Neu5Gc antibodies in human serum suggests that this could even represent a novel antimicrobial strategy. Thus Neu5Gc, delivered as a bolus injection or inhalant could be used as a “Trojan Horse” to target NTHi for anti-Neu5Gc mediated killing. The development of novel therapeutics against NTHi is important, as there is currently no vaccine against this organism. Indeed, others have also proposed the use of sialic acid analogs with high affinity for the sialic acid transporter to block sialic acid transport in NTHi (27).

Previous studies have shown that human serum killing of NTHi is dependent on the classical complement pathway (28) and that human serum survival is dependent on LOS sialylation with Neu5Ac (1, 2). However, the exact mechanism by which Neu5Ac confers protection in human serum is not known. Here, we show that Neu5Gc–expressing NTHi are killed in human serum but survive when anti-Neu5Gc antibodies are blocked. The fact that both Neu5Ac and Neu5Gc are capable of protecting NTHi in human serum (when anti-

Neu5Gc antibodies are blocked) suggests that these sialic acids are likely masking the underlying immunogenic LOS glycan structures. A similar mechanism has been demonstrated for the protection of sialylated meningococci in human serum (29).

MATERIALS AND METHODS

Bacteria and growth conditions. *H. influenzae* strains were generously provided by Michael Apicella, Department of Microbiology, University of Iowa (Strains 2019 (20) and 2019*nanA* (1)), Michel Gilbert, Institute for Biological Sciences, National Research Council, Ottawa, Ontario, K1A OR6, Canada (NTHi strain DH1 (21)) and Victor Nizet, Department of Pediatrics, University of California, San Diego (NTHi strain Int1 (22) and *H. influenzae* type b strain, Eagan (23)). *H. somni* strains (strains 649, 8025, 2336,1P, 127P, and 129Pt (30-32)) were a generous gift from Lynette Corbeil, Department of Pathology, University of California, San Diego.

Sialic acid-free NTHi stocks were prepared by passaging bacteria several times in sialic acid-free media: RPMI 1640 media (Sigma) supplemented with 1 μg/ml protoporphyrin IX (Sigma), 1 μg/ml L-histidine (Sigma), 10 μg/ml β-nicotinamide adenine dinucleotide (Sigma), 0.1 mg/ml hypoxanthine (Sigma), 0.1 mg/ml uracil (Sigma), and 0.8 mM sodium pyruvate (Gibco) (1, 33). The absence of sialic acid was confirmed by HPLC and mass spectrometry.

Human serum samples. Normal human sera was obtained from apparently healthy adult blood donors at University of California, San Diego School of

Medicine with approval from the Institutional Review Board. Written, informed consent was obtained in advance from the volunteers. To assure confidentiality, samples were de-identified and coded with an S number, then aliquoted and stored at −80°C. For this particular study, we specifically chose one serum (S34), which had previously been shown to contain high levels of anti-Neu5Gc antibodies. Sera used for the killing assay were freshly frozen and never thawed in order to preserve complement activity.

Sialic acid lyase assay. NTHi strains 2019 and 2019*nanA* were grown to mid-log (OD_{600}~0.3−0.4) in sialic acid-free media and equal amounts of bacteria (approximately 20 ml of each) were centrifuged at 20,817 *g* for 10 min at 4°C. The pellets were resuspended in 1 ml PBS and centrifuged at 20,817 x g for 10 minutes. Next the pellets were resuspended in 200 μl lysis buffer (50 mM Tris HCl, 2 mM EDTA, 100 μg/ml lysozyme, pH 8.0) and incubated for 15 min on ice. The pellets were then sonicated (3x20sec-30sec; level 4-5 on a Fisher Scientific 550 sonic dismembrator), and incubated on ice in between for at least 20 sec. Following centrifugation at 20,817 *g* for 10 min the supernatants (soluble lysate fraction) containing the lyase were transferred to new tubes. The sialic acid lyase activity assay was carried out in a 1 ml reaction volume containing 100 mM sodium phosphate buffer, pH 7.2, equal amounts (500 μM final) of Neu5Ac and Neu5Gc as well as 180 μl of the freshly prepared soluble lysate fraction of NTHi strains 2019 and 2019*nanA*, respectively. At 0 min, 1 hr, 2 hr, 3 hr time points 100 μl aliquots were removed and placed directly in 100 μl 0.2 M H_2SO_4 (to stop lyase activity (10)) and then frozen. The samples were

then passed through a Microcon-10 filter, derivatized with DMB, and analyzed by HPLC to measure the amount of remaining sialic acids as described below.

NTHi growth curves. NTHi strains 2019 and 2019*nanA* were grown to mid-log (OD_{600}~0.3−0.4) in sialic acid-free media and diluted to OD_{600}~0.25 in sialic acid-free media alone, or with various amounts of Neu5Gc (Inalco) or Neu5Ac (Inalco) added (0.01 mM, 0.1 mM or 1 mM). Bacterial growth was monitored by measuring the OD_{600} every hour for 5 hr total.

Comparison of Neu5Ac and Neu5Gc sialic acid expression. *H. influenzae* strains grown to early-log (OD_{600}~0.2−0.3) in sialic acid-free media, were diluted 1:1 in fresh sialic acid-free media with 50 μM (final concentration) each, Neu5Ac (Inalco) and Neu5Gc (Inalco) and grown for 3.5 hr at 37°C. Alternatively, NTHi strain 2019 and *H. somni* strains 649, 8025, 2336,1P, 127P, and 129Pt were grown overnight on Chocolate II Agar plates (BD) at 37°C in a candle jar. Bacterial pellets were resuspended in 20 mM Tris·HCl/10 mM $MgCl_2$ (pH 7.4), and subjected to four rounds of freeze-thawing. The pellet was then washed well and resuspended in 200 μl 2M acetic acid and heated to 80°C for 3 hr to release sialic acids. Following centrifugation at 10,000 *g* for 10 min at 4°C, the supernatant was filtered through a 10,000 molecular weight cut off filter and sialic acids in the filtrate were derivatized with 1,2-diamino-4,5-methylenedioxybenzene (DMB; Sigma-Aldrich) as described previously (34) 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.

Serum killing assay. NTHi strain 2019 was grown to mid-log (OD_{600}~0.3−0.4) in sialic acid-free media, diluted 1:1 in fresh sialic acid-free media alone or with 500 μM (final concentration) of either Neu5Ac (Inalco) and Neu5Gc (Inalco). After growing for an additional 3 hr at 37°C, bacteria were pelleted and resuspended to an OD_{600} of 0.2 in Gelatin Veronal Buffer with Calcium and Magnesium (GVB+/+; 3.2 mM diethyl barbituric acid, 1.8 mM sodium barbital, 145 mM NaCl, 0.02% NaN_3, 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, 0.1% gelatin). Serum killing assay was performed in a sterile 96-well round bottom plate in 100 μl total volume as follows: 10 μl freshly frozen human serum or heat inactivated human serum (30 min at 56°C) was added to 83 μl GVB+/+ and allowed to incubate for 5 min at room temperature with 2 μl (2 μg total) Neu5Gcα-PAA or Neu5Acα-PAA (Glycotech) or milli-Q water before adding 5 μl bacteria (OD_{600} of 0.2 in GVB+/+). The plate was incubated 30 min at 37°C, with gentle shaking. Serial 1/10 dilutions were plated on chocolate agar and incubated overnight at 37°C with 5% CO_2 and colonies were counted the following day. The percent survival was calculated by comparing the number of CFU incubated in normal serum to the CFU incubated in the heat-inactivated serum.

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generously providing *H. influenzae* strains. Many thanks to Jason Lehmann for help designing/performing the *H. somni* experiments and to Anne Bergfeld for help designing/performing the lyase assay and for helpful discussions.

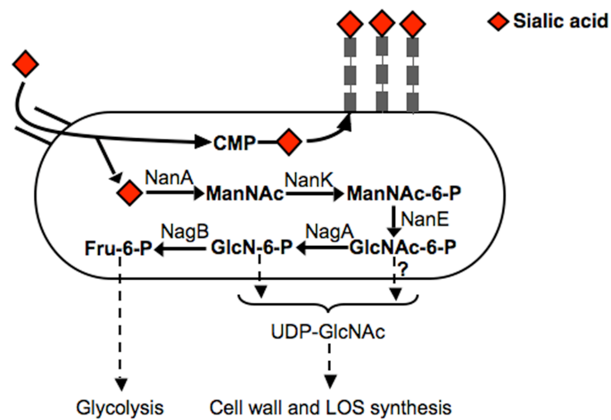

Figure 5-1. Sialic acid catabolism in NTHi.
This cartoon depicts the catabolic pathway of sialic acid (Neu5Ac) in NTHi as described in chapter 5. The catabolic fate of Neu5Gc in NTHi has not been previously studied.

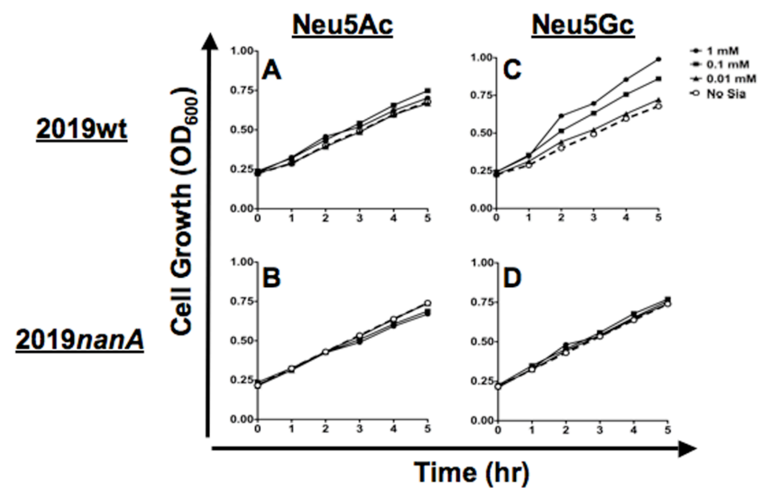

Figure 5-2. Neu5Gc-induced growth stimulation of NTHi.
WT NTHi strain 2019 (2019wt) and NanA sialic acid lyase deficient NTHi strain (2019*nanA*) were grown for 5 hours in 0 (open circles), 0.01mM (closed triangles), 0.1 mM (closed squares), or 1mM (closed circles) Neu5Ac or Neu5Gc. Bacterial growth was monitored hourly by measuring the OD_{600} of each culture. Data are representative of two independent experiments.

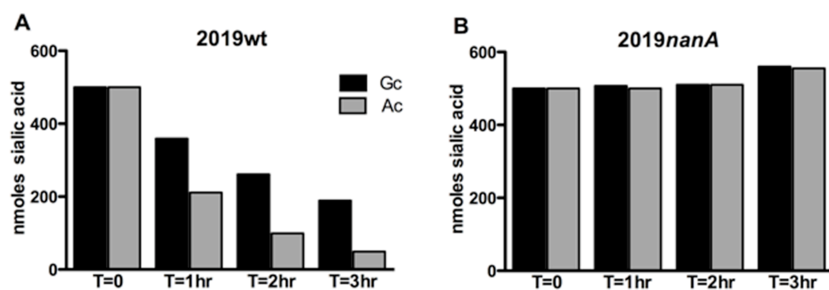

Figure 5-3. Comparison of Neu5Ac and Neu5Gc degradation in NTHi. Equal amounts of Neu5Ac and Neu5Gc (500 μM each) were added to cell lysates prepared from WT and NanA deficient NTHi strains. In this competition lyase assay, breakdown of each sialic acid was analyzed by quantifying the amount of Neu5Ac and Neu5Gc remaining in the lysates at 1-hour intervals by HPLC.

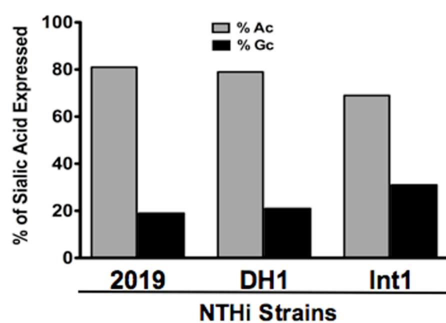

Figure 5-4. Preferential expression of Neu5Ac on LOS of NTHi.
NTHi strains 2019, DH1 and Int1 were grown for 3.5 hr in sialic acid-free media with equal amounts of Neu5Ac and Neu5Gc (50 μM each). Membrane bound sialic acid was analyzed by HPLC. Neu5Ac and Neu5Gc values for each bacterium are expressed as percent of total sialic acid. The data are representative of two independent experiments.

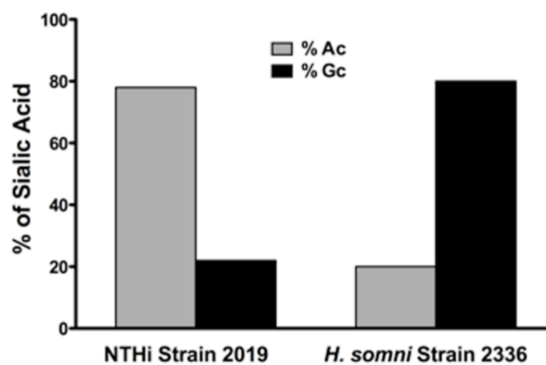

Figure 5-5. Comparison of cell surface sialic acid expression on NTHi and *H. somni*.
NTHi Strain 2019 and *H. somni* strain 2336 grown on chocolate agar were analyzed for sialic acid expression by HPLC. Neu5Ac and Neu5Gc values for each bacterium are expressed as percent of total sialic acid.

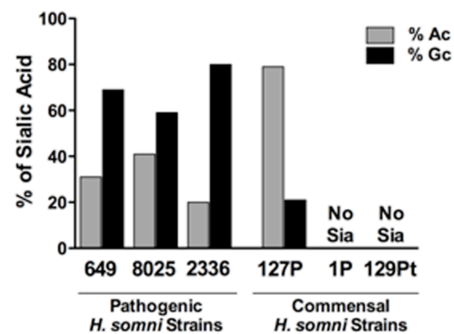

Figure 5-6. Comparison of cell surface sialic acid expression patterns on pathogenic and commensal *H. somni* strains.

HPLC analysis of membrane bound sialic acid expression comparing pathogenic (strains 649, 8025, and 2336) and commensal (strains 1P, 127P, and 129Pt) strains of *H. somni* grown on chocolate agar. Neu5Ac and Neu5Gc values for each bacterium are expressed as percent of total sialic acid.

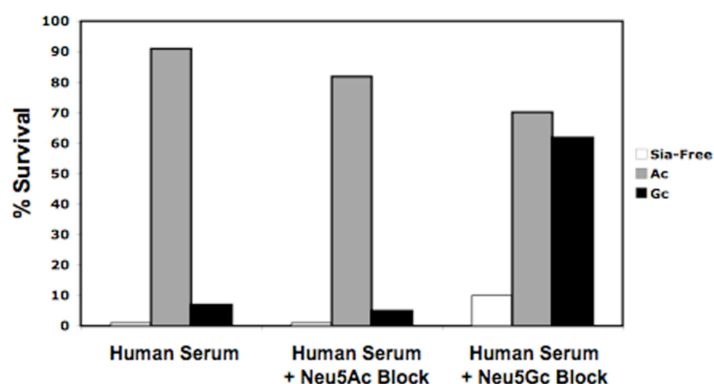

Figure 5-7. Killing of Neu5Gc-expressing NTHi in normal human serum. NTHi strain 2019 was grown in sialic acid-free media alone or with 500 μM (final concentration) of either Neu5Ac and Neu5Gc. Serum sensitivity was analyzed by incubating these bacteria in 10% normal human serum and counting colonies following serial dilution and growth on chocolate agar. In parallel serum killing assays, Neu5Gcα-PAA (Neu5Gc block) or Neu5Acα-PAA (Neu5Ac block; negative control) were used to try to block anti-Neu5Gc antibodies present in the serum. The percent survival was calculated by comparing the number of CFU incubated in normal serum to the CFU incubated in the heat-inactivated serum. The data are representative of three independent experiments.

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CHAPTER 6

Conclusions and Future Perspectives

The primary goal of the research presented in this dissertation was to investigate the origins and other implications of human antibodies against the non-human sialic acid Neu5Gc. In Chapter 2 of this dissertation, we show that anti-Neu5Gc IgM and IgG antibodies arise during the first year of life (1). Interestingly, anti-Neu5Gc IgM antibodies were not present in cord blood serum, indicating that these antibodies are not germ-line encoded "natural antibodies" but, are instead generated by a post-natal antigenic stimulus. Although the appearance of anti-Neu5Gc antibodies correlates with the introduction of Neu5Gc in the diet, we determined that dietary Neu5Gc alone cannot stimulate anti-Neu5Gc antibodies in human-like *Cmah* null (Neu5Gc-deficient) mice. Thus dietary Neu5Gc is unlikely to represent the sole antigenic stimulus required for inducing anti-Neu5Gc antibodies. Instead, we present evidence for a novel mechanism, in which a human-specific commensal bacterium, NTHi, can scavenge and express dietary Neu5Gc from within the human host and likely contributes to the generation of human anti-Neu5Gc antibodies (1).

These studies have raised many new questions. While NTHi is the first human commensal known to scavenge and express dietary Neu5Gc, it is possible that other respiratory, oral and/or gut bacteria can do the same. Indeed, the wide variation in anti-Neu5Gc titers and specificity (2) between adult individuals may reflect exposure to different Neu5Gc-expressing bacteria. One strategy for identifying other bacteria that can take up and express exogenous Neu5Gc would be to use sequence alignment to specifically look for bacteria

that lack a sialic acid synthase gene and yet possess a transporter gene with homology to the sialic acid TRAP transporter of NTHi. One such commensal bacterium meeting these criteria is the oral anaerobe *Fusobacterium nucleatum* subsp. *vincentii* (3). A more direct method would be to incubate bacteria from different segments of the human gastrointestinal tract with free Neu5Gc. Bacterial cells that stain positive for Neu5Gc expression could then be isolated by flow cytometric sorting or capillary tube capture and identified by single cell genomic sequencing (4).

Another factor that could contribute to the variability in the levels of anti-Neu5Gc responses between human individuals (2) and mice (Chapter 2 of this dissertation) could be the expression of Siglecs on B-cells (5). Siglecs are sialic acid-binding immunoglobulin-like lectins that are expressed mainly on leukocytes that can mediate activating or inhibitory signals (6). Others have recently shown evidence that ligation of Siglec-2/CD22 and/or Siglec G found on mouse B cells by high affinity sialoglycans can induce B-cell tolerance to T-independent antigens (5). Therefore, it is possible that the variable response seen in the mice is due to the fact that the antigen being used is capable of ligating not only the cognate B cell receptors, but also inhibitory Siglecs on the very same B cells. The corresponding Siglec orthologs on human B-cells (CD22 and Siglec 10) could likewise dampen B-cell responses to Neu5Gc-containing antigens.

Dietary ingestion of Neu5Gc by infants or mothers could be another factor influencing both the timing of the appearance of anti-Neu5Gc antibodies

and their levels. Along these lines, we are currently investigating whether ingestion of cow's milk formula (which contains Neu5Gc) vs. human mother's milk (which is Neu5Gc-free) tolerizes or sensitizes infants to Neu5Gc. To model formula feeding in mice, we are using *Cmah* null mice that are cross-fostered with WT mothers.

In our analysis of infant serum, we found that anti-Neu5Gc antibodies appeared starting at 6 months and correlated with the introduction of Neu5Gc in the diet. These results raise the question of whether the timing of the introduction of Neu5Gc in the diet influences the timing of the appearance of anti-Neu5Gc antibodies. Future studies comparing sera from infants who were exclusively formula-fed from birth and compared with those who had been exclusively breast-fed should help answer this question. We are also interested in investigating whether ongoing dietary Neu5Gc in adults correlates with anti-Neu5Gc levels and whether these antibody levels can be altered by removing Neu5Gc from the diet for a period of time.

As mentioned earlier, we have also shown that ingestion of dietary Neu5Gc alone by normal healthy *Cmah* null mice does not induce anti-Neu5Gc antibodies (Chapter 2 of this dissertation). However, studies are currently underway to determine whether dietary Neu5Gc can stimulate anti-Neu5Gc antibodies in the context of gut inflammation caused by infection (ie. rotavirus) or disease (ie. colitis). Indeed, a subset of intestinal B-cells was recently identified which rapidly expands in response to gut inflammation, even in the absence of antigen (7).

Previous characterizations of human anti-Neu5Gc antibodies have focused on IgM, IgG and IgA isotypes present in the serum (2, 8, 9). In future studies we will investigate whether IgE anti-Neu5Gc antibodies are also present in human serum and whether these antibodies correlate with reported cases of red meat allergy. Another research area of interest is the characterization of mucosal IgM and IgA anti-Neu5Gc responses.

In Chapter 2 we show that maternal IgG antibodies against Neu5Gc cross the placental barrier and can be found in serum from cord blood at comparable levels compared to serum. While humans cannot synthesize endogenous Neu5Gc, this foreign glycan is particularly enriched in fetal and placental tissues (8), presumably incorporated from the maternal diet. Thus, the potential exists for these maternally derived anti-Neu5Gc antibodies to act as anti-fetal antibodies. Indeed, we have shown that when *Cmah* null famale mice with anti-Neu5Gc antibodies are crossed with *Cmah* heterozygous males, they produce a lower than expected fraction of heterozygote pups (Chapter 4 of this dissertation). While these results did not quite reach statistical significance at the 95% confidence limits (P=0.055) they suggest that maternal anti-Neu5Gc antibodies could react against fetal incorporated Neu5Gc and contribute to post-zygotic infertility. Future studies will help to define the role of anti-Neu5Gc antibodies in maternal-fetal immune responses.

In additional work, we have recently demonstrated that the presence of Neu5Gc on glycoprotein biotherapeutics can induce anti-Neu5Gc antibodies in *Cmah* null mice (10). Thus, the possibility exists that other biotherapeutic or

pharmaceutical products that contain Neu5Gc could also induce or enhance anti-Neu5Gc antibodies in humans. In this regard, studies are currently underway to investigate the possibility that common childhood vaccines contain Neu5Gc.

A major unanswered question is whether anti-Neu5Gc antibodies arise in a T-independent or T-dependent manner. Interestingly, while most anti-carbohydrate antibodies are thought to arise by a T-cell independent mechanism, there is evidence that the generation of antibodies recognizing alpha-Gal carbohydrates is T-cell dependent (11). On the other hand, T-independent mechanisms of B-cell activation, Ig secretion and class switching are known to be facilitated by TLRs, (12) and TNF-family ligands APRIL and/or BAFF (13, 14). 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. To specifically address the question of T-cell dependence vs. independence, our lab has recently produced *Cmah* null nude (T-cell deficient) mice. Current studies are also underway to determine if antibodies against Neu5Gc arise from a restricted set of immunoglobulin V-region genes and whether these anti-Neu5Gc V-genes undergo somatic mutation. This requires the cloning of human anti-Neu5Gc antibodies from single memory B cells.

In Chapter 5 of this dissertation we show that pre-existing anti-Neu5Gc antibodies present in human serum can efficiently kill Neu5Gc-expressing NTHi. This result suggests that Neu5Gc could potentially be used during the course of

infection as a novel antimicrobial strategy to target NTHi for anti-Neu5Gc mediated killing. For example, this might be particularly useful in clearing chronic infections, Of course such an approach might almost be accompanied by boosting of pre-existing anti-Neu5Gc antibody titers. The individual so treated may thus have to minimize future Neu5Gc dietary intake to avoid the possibility of a xenoautoantibody reaction against normal tissues.

Finally, evidence that anti-Neu5Gc can contribute to carcinoma progression (15) and vascular inflammation (16), suggests a possible role for these antibodies as biomarkers for diagnosing and/or predicting risks of cancer and/or atherosclerosis. Studies are also currently underway to identify anti-Neu5Gc antibodies recognizing cancer-specific antigens that could be used therapeutically.

In conclusion, we have presented evidence of a novel mechanism for the generation of anti-Neu5Gc xeno-autoantibodies in humans. In Chapter 2 we show for the first time that anti-Neu5Gc antibodies arise during infancy, likely in response to expression of dietary Neu5Gc on the commensal bacterium, NTHi. Additionally, we recapitulated this human condition in a mouse model by using NTHi to induce anti-Neu5Gc antibodies *Cmah* null mice. This human-like mouse model has been instrumental in defining the implications of anti-Neu5Gc antibodies with regard to clearance of Neu5Gc-containing biotherapeutics and reproductive incompatibility (Chapters 3 and 4, respectively). Lastly, in Chapter 5 we have investigated the differential expression and catabolism of human and non-human sialic acid by NTHi and proposed a novel anti-Neu5Gc antibody

dependent antimicrobial approach against NTHi. This work has also raised many new questions that have opened several avenues for future research.

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