A Mouse Model for Dietary Xenosialitis

ANTIBODIES TO XENOGLYCAN CAN REDUCE FERTILITY*

Received for publication, May 20, 2016, and in revised form, June 28, 2016 Published, JBC Papers in Press, July 5, 2016, DOI 10.1074/jbc.M116.739169

Fang Ma1, Liwen Deng5, Patrick Secrest5, Linda Shi5, June Zhao5*, and Pascal Gagneux1*
From the 5Sichuan University – The Chinese University of Hong Kong Joint Laboratory for Reproductive Medicine, Key Laboratory of Obstetric, Gynecologic and Pediatric Diseases and Birth Defects of Ministry of Education, and 6Department of Obstetrics and Gynecology, West China Second University Hospital, Sichuan University, Chengdu 610041, China and the *Glycobiology Research and Training Center and Department of Pathology, 1Department of Bioengineering, and **Transgenic Mouse Core, University of California – San Diego, La Jolla, California 92039

Humans can incorporate the xenoglycan N-glycolylneuraminic acid (Neu5Gc) from the diet into reproductive tissues and secretions. Most humans also have circulating antibodies specific for this dietary xenoglycan. The potential for inflammation induced by incorporated Neu5Gc and circulating anti-Neu5Gc antibodies, termed xenosialitis, has been discussed as a factor influencing several human diseases. Potential effects of xenosialitis on human fertility remain unknown. Here, we investigate possible adverse effects of the presence of Neu5Gc on sperm or endometrium combined with anti-Neu5Gc antibodies in semen or uterine secretions in a mouse model. We use Cmah−/− mice, humanized for Neu5Gc deficiency. We find that the viability, migration, and capacitation of sperm with incorporated Neu5Gc are negatively affected when these are exposed to anti-Neu5Gc antibodies. In addition, we find that after copulation, activated uterine neutrophils and macrophages show increased phagocytosis of sperm in the presence of anti-Neu5Gc antibodies via the complement receptor 3 (C3R) and FcyγII/III (Fcγ receptor). Furthermore, Neu5Gc in endometrial explants combined with the presence of anti-Neu5Gc antibodies alters the receptivity and decidualization of endometrial explants. These studies provide mechanistic insights on how Neu5Gc on sperm and/or endometrium combined with anti-Neu5Gc antibodies in semen and uterine fluid might contribute to unexplained human infertility.

Infertility is a current global health issue affecting ~8–10% of couples in industrialized countries (1). It is a multifactorial problem, and a large fraction of cases (up to 30%) are classified as unexplained infertility (2, 3). Human anti-sperm antibodies (ASA)2 have been linked to human infertility, and have been detected in up to 12% of infertile couples as well as in a small fraction of fertile individuals (<2.5% of fertile men and <1.4% of fertile women) (4, 5). The mechanisms of the adverse effects of ASA on fertility include antibody binding to sperm, resulting in the inhibition of sperm movement, agglutination, impairment of cervical mucus penetration by sperm, suppression of recognition processes, and impairment of entry into the ovum (6). Furthermore, such adverse effects could also include complement-mediated effector function in the female reproductive tract (7–9). Additional immune factors could also be involved in idiopathic infertility. It has been proposed that circulating anti-sialic acid antibodies target incorporated non-human sialic acid and contribute to chronic inflammation in various tissues (10–12). Our objective is to determine whether xenoglycan-antibody interactions also affect human infertility (13) given tentative evidence from a clinical study (14). Sialic acids (Sias) are nine-carbon backbone, acidic, amino sugars that decorate the surfaces of all vertebrate cells in high abundance (tens to hundreds of millions of molecules per cell). Sias are typically found as terminal sugars on the branches of N-linked and O-linked glycans and glycolipids. These cell surface sugar molecules have many roles in immunity including their contribution as self-associated molecular patterns (15, 16). The two most common mammalian Sias are N-acetylglycolneuraminic acid (Neu5Ac) and Neu5Gc. Neu5Gc differs from Neu5Ac by a single oxygen atom added to the donor sugar nucleotide CMP Neu5Ac by the enzyme CMP Neu5Ac hydrolase (CMAH) (17). The single copy CMAH gene was inactivated in humans over 2 million years ago (18) by an Alu-mediated deletion of 487 bp including a 92-bp exon resulting in a frameshift mutation. As a consequence, humans are genetically incapable of producing Neu5Gc. Nevertheless, Neu5Gc has been detected in human tissues, both healthy and diseased. These include endothelia of blood vessels, certain epithelia (11, 19), fetal tissues (20), and carcinomas, especially of prostate, ovary, and breast (21–23). The absence of any alternative synthetic pathway for Neu5Gc was confirmed by characterizing the Sias of transgenic Cmah−/− mice, which bear the same Cmah gene mutation as found in humans (24). Furthermore, Neu5Gc can be metabolically incorporated into cultured human cells in vitro. In both normal and cancerous cells, exogenous Neu5Gc was shown

Sia, sialic acid; FeR, Fc receptor; LIF, leukemia inhibitory factor; PI, progesterone; Iodide; D4, gestation day 4.

*This work was supported by National Institutes of Health Grant GM10150GM095882. The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

1 To whom correspondence should be addressed: 9500 Gilman Dr., University of California, San Diego, La Jolla, CA 92093-0687. Tel.: 858-822-4030; Fax: 858-534-5611; E-mail: pgagneux@ucsd.edu.

2 The abbreviations used are: ASA, anti-sperm antibodies; BWG, Biggers, Whitten, and Whittingham medium; CASA, computer-assisted sperm analysis; SCSA, sperm chromatin structural assay; CMAH, cytidine monophosphate N-acetylglycolneuraminic acid hydrolase; PSM, porcine submaxillary mucin; Neu5Ac, N-acetylglycolneuraminic acid; Neu5Gc, N-glycolylneuraminic acid; NTHI, nontypeable Haemophilus influenzae; SVF, seminal vesicle fluid;
to reach lysosomes via pinocytic/endocytic pathways, get imported in free form into the cytosol, and subsequently become available for activation and transfer to glycoconjugates (25). Dietary incorporation of Neu5Gc explains the presence of Neu5Gc in some human tissues. An experiment with human volunteers confirmed that ingested Neu5Gc can be taken up and incorporated into human sialo-glycoconjugates such as mucins (19). Human diets in most industrialized countries are typically rich in red meats (pork, beef, and lamb), which contain high levels of Neu5Gc and are thus likely to account for the accumulation of Neu5Gc in human tissues (26–29). Furthermore, the last decades have seen a strong increase in consumption of Neu5Gc-rich red meat in the United States (30). The Cmah−/− mouse (human-like model) has been used in dietary studies to prove that Neu5Gc can be metabolically incorporated following ingestion of Neu5Gc-rich glycoprotein (31). These experiments have shown that Neu5Gc is incorporated into the small intestine and subsequently appears in circulation at a steady-state level for several hours, followed by metabolic incorporation into multiple peripheral tissues (31).

The human immune system regards Neu5Gc-containing glycans structures as “foreign,” resulting in a polyclonal humoral response, with variable titers of circulating anti (α)-Neu5Gc antibodies among most humans (32, 33). The incorporation and accumulation of dietary Neu5Gc in the presence of an ongoing immune response against this non-human epitope make Neu5Gc the first known example of a “xeno-autoantigen” contributing to cancer and chronic inflammation (11, 34).

Human α-Neu5Gc antibodies appear during late infancy, correlating with weaning, exposure to dietary Neu5Gc (in formula based on bovine milk), and exposure to common bacteria, such as non-typeable Haemophilus influenzae (NTHi), which incorporate Neu5Gc into their cell surface lipooligosaccharides (35). Proposed as a human-specific mechanism, xenosialitis could exacerbate vascular pathologies such as atherosclerosis, and was also found to stimulate tumor growth in a human-like mouse model (36). A recent study demonstrated that Neu5Gc from diet increased the incidence of cancer in a mouse model (12).

We have previously shown that α-Neu5Gc antibodies can enter the female reproductive tract in mice and target Neu5Gc-positive sperm (37). Female mice genetically engineered with a human-like Cmah−/− mutation and immunized to express α-Neu5Gc antibodies show reduced fertility when mated with Neu5Gc-positive wild type males (13, 24). Furthermore, human α-Neu5Gc antibodies are also capable of targeting paternally derived antigens and can mediate cytotoxicity against Neu5Gc-bearing chimpanzee sperm in vitro (13), (37). Here, we investigate the possible consequences of the presence of Neu5Gc and α-Neu5Gc antibodies in the reproductive system in a mouse model humanized for its sialic acid and α-xenoglycan immune response.

Results

Mouse α-Neu5Gc Antibodies Bind to Neu5Gc on Motile Mouse Sperm—Cmah−/− Mice immunized against Neu5Gc produce antibodies in their sera (Fig. 1A) as well as in their uterine secretions (Fig. 1C). The binding of the antibody to motile sperm represents a crucial first step for its potential effect on sperm. We co-incubated WT C57/BL6 mouse sperm with Neu5Gc-containing WT seminal fluid. Neu5Gc on seminal fluid CD52 sialoglycoprotein after incubation of Cmah−/− mouse sperm with Neu5Gc-containing WT seminal fluid. Neu5Gc on CD52 was targeted by mouse α-Neu5Gc antibodies during preincubation as revealed by reduction in subsequent staining by commercial α-Neu5Gc antibody.

FIGURE 1. Serum α-Neu5Gc IgG and IgM binding of Neu5Gc on WT motile sperm. A, serum from immunized mice was incubated with WT motile sperm. Antibody binding to the sperm was characterized by staining with fluorescently labeled polyclonal goat-anti-mouse α-IgG and α-IgM antibodies (Santa Cruz Biotechnology) using flow cytometry, 10,000 events measured per sample, % of Max, percentage of maximum. B, binding by antibodies from sera of immunized Cmah−/− mice visualized by fluorescent microscopy was found mainly on the sperm head; red, IgG; green, IgM. Scale bar = 15 μm. C, production of α-Neu5Gc IgA and IgG antibodies targeting 2,3/2,6-linked Neu5Gc in Cmah−/− mouse uterine fluid immunized with NTHi 6 months earlier and detected by ELISA. D, mouse α-Neu5Gc antibodies targeting Neu5Gc on seminal fluid CD52 sialoglycoprotein after incubation of Cmah−/− mouse sperm with Neu5Gc-containing WT seminal fluid. Neu5Gc on CD52 was targeted by mouse α-Neu5Gc antibody during preincubation as revealed by reduction in subsequent staining by commercial α-Neu5Gc antibody.
Mouse Model for Dietary Reproductive Xenosialitis

α-Neu5Gc antibodies from NTHi-immunized Cmah−/− mice (35). We detected serum IgG and IgM α-Neu5Gc Ab bound to motile sperm by flow cytometry analysis (Fig. 1A). Immunohistochemistry revealed that most of the binding occurred to the sperm head (Fig. 1B). The target of mouse uterine α-Neu5Gc antibodies was measured by ELISA using glycan probes carrying the two most common linkage types of Neu5Gc: α2,3 and α2,6 (Fig. 1C). To verify the Neu5Gc specificity of the mouse antibodies, we also incubated Cmah−/− mouse sperm with seminal vesical fluid (SVF) from WT mice to decorate the Neu5Gc negative sperm with seminal sialoglycoproteins such as CD52 from Neu5Gc-positive WT mice. CD52 is a highly sialylated glyco-phosphoinositol-anchored glycopeptide abundantly transferred from SVF to the membrane of mature sperm during ejaculation (38). These SVF-preincubated sperm were then exposed to immune or control sera and subsequently stained in parallel with α-CD52 and commercial affinity-purified α-Neu5Gc IgY antibodies. The immune serum strongly reduced the staining for CD52, indicating that the Neu5Gc on CD52 targeted by the mouse α-Neu5Gc antibodies partially blocked CD52 staining (Fig. 1D).

Cmah−/− Mice Fed PSM-rich Chow Incorporate Neu5Gc in Their Sperm—Mice fed porcine submaxillary mucin (PSM)-containing chow for 6 months produce sperm that carry intermediate levels of Neu5Gc when compared with regular or soy chow-based controls that lack Neu5Gc, and wild type C57BL/6 mice that have about 20% of all their sperm sialic acids as Neu5Gc (Fig. 2, A and B). The specificity of the antibodies for sialic acid was confirmed by mild periodate treatment, which cleaves the side chain of sialic acid.

Effects of α-Neu5Gc Antibodies on WT Mouse Sperm prior to Fertilization—We explored the effects of α-Neu5Gc antibody binding to motile sperm containing Neu5Gc. Wild type epididymal sperm showed reduced viability after exposure to mouse sera with high titers of α-Neu5Gc antibody. SYBR-14 (live) and propidium iodide (PI, dead) staining followed by fluorescence measurements using FACS analysis revealed a strong increase in the fraction of dead and dying epididymal sperm after exposure to immune serum (Fig. 3A). Motility measured as curvilinear velocity and velocity (straight line velocity) measured by computer-assisted sperm analysis (CASA) were not affected even after exposure to immune sera with variant titers (Fig. 3B). There was no evidence for any effects of the antibodies on levels of sperm DNA damage as assayed by sperm chromatin structure assay (SCSA) (Fig. 3C) nor on the ability of sperm to bind eggs using a homologous sperm-egg binding assay (Fig. 3D).

On the other hand, a sperm migration assay through a filter showed reduced migration by epididymal sperm treated with high titer mouse α-Neu5Gc antibody from both sera and uterine fluid (Fig. 3E). This might reflect the ability of sperm to penetrate cervical mucus and/or to navigate morphologically complex passages in the female reproductive tract.

Calcium influx into the sperm cell is associated with the capacitation process, a key event for fertile sperm (39). We also stained sperm exposed to immune or control sera for intracellular calcium using the calcium specific fluorescent dye fluo3 and analyzed the sperm populations by flow cytometry. Calcium influx was reduced by an order of magnitude in sperm exposed to immune serum (Fig. 3F).

The local immune system is stimulated as sperm enter the female genital tract, and by 12–14 h after copulation, female cells outnumber sperm, especially in the uterus, where many sperm are phagocytosed and killed in neutrophil extracellular traps (40–42). To explore how the phagocytosis of sperm might be affected by α-Neu5Gc antibodies, we used WT-GFP mouse sperm (expressing green fluorescent protein driven by CAG promoter in their midpieces) as targets, exposing these to immune and control sera before in vitro co-incubation with activated uterine leukocytes from Cmah−/− females mated with a wild type male 14 h after mating. We then measured phagocytosis of GFP sperm using fluorescent cytometry to measure green fluorescence and staining by several fluorescent antibodies. We stained for neutrophils with anti-Ly6G antibody (marker: 6y-Gr1) and for macrophages using anti-F4/80...
Our experiments indicate that CD11b (C3R) might play a bigger role than FcR II/III, I (CD16/32, CD64) on neutrophils and macrophages (Fig. 4C).

**Effects of α-Neu5Gc Antibodies on Receptivity and Decidualization of Endometrium**—Results from our clinical study with in vitro fertilization patients pointed to failure of implantation as the main reason for low clinical pregnancy rates in women with xenoglycan and α-Neu5Gc antibodies in their uterus (14). No Neu5Gc was found in Cmah−/− mouse endometrium even after long time feeding with PSM in our present study, and the failure of this model might be due to differences in mice and human endometrial metabolism and regeneration. Thus, we exposed mouse estrus endometrium to free Neu5Gc in vitro to load the tissues with the xenoglycan and test the possibility of Neu5Gc accumulating in these tissues. Exogenous Neu5Gc accumulated in the luminal and glandular epithelia after exposure of endometrial explants to 5 mM Neu5Gc for 2 days (Fig. 5A). We then tested the effect of co-occurrence of Neu5Gc and α-Neu5Gc antibodies on the receptivity and decidualization of endometrium. Wild type endometrium explants of D4 (gestation day 4) were co-cultured with high serum levels of α-Neu5Gc antibodies for 2 days. We detected a strong reduction in both leukemia inhibitory factor (LIF), used as a marker of receptivity, and metalloproteinase inhibitor 3 (TIMP3), used as a marker of decidualization, indicating a possible effect of the xenoglycan and α-Neu5Gc antibodies on the endometrium’s receptivity and decidualization (Fig. 5, B and C).

**Discussion**

This study demonstrates in a mouse model that exogenous Neu5Gc and α-Neu5Gc antibodies can contribute to immune-related reduction of fertility in males and females. Their co-occurrence in a mouse model was associated with several adverse effects. In humans, α-Neu5Gc antibodies appear after weaning, possibly promoted by the combination of dietary Neu5Gc and its presentation on antigens of NTHi (35). Together with the finding of reduced clinical pregnancy rates in the presence of Neu5Gc antigen and directed antibodies in human fertility patients (14), our results suggest that the uterine mucosal immune system can produce α-Neu5Gc antibodies independently in Cmah−/− mouse model immunized by NTHi (intraperitoneal injection). We detected dietary Neu5Gc on epididymal sperm from Cmah−/− mice fed with PSM without blood-testis barrier damage, suggesting that there might be another underlying mechanism for Neu5Gc accumulation on human sperm where the majority of men do not show incorporated Neu5Gc despite life-long red meat diets. In our previous study, we documented in vivo fertility reduction in female Cmah−/− mice with high titer α-Neu5Gc antibody and mated with WT C57B/L (13), suggesting that in humans, the presence of Neu5Gc on sperm and high titer α-Neu5Gc antibodies in female genital tract or in male semen could contribute to unexplained infertility.

Based on our analyses of the distribution of Neu5Gc and α-Neu5Gc antibodies in the human genital system and the physiology of the fertilization process, we propose that dietary xenosialitis could negatively affect human fertility in a variety of ways (Fig. 5). Binding of α-Neu5Gc antibodies to motile sperm

antibodies and found that phagocytosis was enhanced by 10.10 ± 2.3 and 5.6 ± 1.8%, respectively (n = 4, representative experiment shown in Fig. 4A). This indicates that α-Neu5Gc antibodies binding to sperm can recruit and activate leukocytes. To test for involvement of complement, we stained for C3R (complement receptor 3, marker CD11b) and found that FcR II/III (CD16/32) and FcR I (CD64) on neutrophils and macrophages were significantly occupied in the group treated with immune serum as quantified by FACS analysis. This suggests that these three complement receptors might play roles during phagocytosis mediated by α-Neu5Gc antibodies and indicates that the process is in part mediated by complement (Fig. 4B).
in vivo might critically contribute to the failure of sperm prior to fertilization, in line with reported mechanisms suggested from research on effects of human ASA (43, 44). Our results seem concordant with reports that ASA affect sperm fertilization capability after antibodies bind to antigen (Neu5Gc)-containing sperm through multiple key mechanisms. These include decreased viability, migration, calcium influx, and the increase of phagocytosis via the receptors of complement 3R (CD11b) and Fc IgG I/II/III (CD16/32/64) on mouse activated (15 h) neutrophils and macrophages entering the mated uterus as part of the leukocytic reaction. In contrast, we found no apparent effects of α-Neu5Gc antibody exposure on sperm motility, DNA damage, and the ability of sperm binding to the egg in vitro. With regard to the female reproductive tract, we found that free Neu5Gc was incorporated into the glandular and lumenal epithelia of mouse endometrium in vitro by means of three-dimensional culture system (45) and resulted in detrimental effects on decidualization and receptivity. This was apparent from staining with two markers for receptivity and decidualization, respectively, leukemia inhibitory factor (LIF) and tissue inhibitors of metalloprotease (TIMP3) (46, 47).

Fig. 6 depicts scenarios for potential interference in males or females. Anti-Neu5Gc antibodies of the male and/or female can bind to motile sperm and negatively affect a number of events prior to fertilization, including sperm viability, motility, migration, chromatin structure, ability to capacitate, phagocytosis by

**FIGURE 4. Effect of α-Neu5Gc antibody on interactions between Neu5Gc-containing sperm and uterine immune cells.** A, phagocytosis of GFP sperm by activated uterine neutrophils and macrophages analyzed by flow cytometry (left panel) and microscopy (right panel). Arrows indicate phagocytosed sperm (cells were examined in a DeltaVision real-time fluorescence microscope, Applied Precision). Scale bar = 15 um. B, representative results from flow cytometry analysis of Fc receptors on neutrophils and macrophages. C, quantification of Fc receptors on neutrophils and macrophages in three independent experiments. * indicates >0.5, and ** indicates >0.05. Error bars represent S.D.
leukocytes, adhesion of sperm to the oviductal epithelium, penetration of the cumulus layer, and sperm-egg binding. In addition, co-occurrence of Neu5Gc and α/β2,3-Neu5Gc antibodies in the female tract could affect the receptivity and decidualization of endometrium and thus compromise implantation.

A clear answer to the age-old question of why hundreds of millions of sperm are required for the fertilization of a single ovum by a single sperm remains elusive. The factors favoring the precarious survival of a small minority of sperm that reach the fallopian tube remain poorly understood but may well involve differences in the sperm glycocalyx of individual sperm (48), differences in protein folding including haploid gene products on the sperm membrane, sperm chromatin structure and/or epigenetic modifications, and hormonal effects mediated by the female (49, 50). Unexplained infertility remains a troubling problem, and new approaches for understanding this multifactorial condition are needed. Exogenous, dietary Neu5Gc and α-Neu5Gc antibodies (xenosialitis) in humans could be novel factors contributing to unexplained human infertility. This phenomenon shares mechanisms with ASA, a well accepted cause for human infertility as an immunologic factor targeting certain substances on sperm that have been known to be antigenic for more than a century (51), but also includes autoimmunity in the female. Evidence from our findings in a humanized mouse model together with our published clinical findings (14) provide justification to study xenosialitis as a factor underlying human infertility by means of large-scale, comprehensive, and multicenter research. In light of the many profound differences between human and mouse reproductive biology, ranging from sperm count and mating behavior to female reproductive tract anatomy, diet, and immunology, our findings can only hint at potential mechanisms in humans.

**FIGURE 5.** Incorporation of Neu5Gc, effect of α-Neu5Gc antibody on the receptivity and decidualization of the endometrium. A, incorporation of Neu5Gc in Cmah−/− estrus endometrium fragment (three-dimensional culture) in vitro for 48 h. Exogenous Neu5Gc was incorporated on epithelia of lumen and gland in vitro (black triangles). Scale bar: 200 μm. B and C, the effect of α-Neu5Gc antibody on the receptivity and decidualization of WT D4 endometrium (three-dimensional culture) in vitro for 48 h. Red, LIF; green, TIMP3; blue, DAPI; gray arrow, no expression; white arrow, positive expression. Tissues were examined in a DeltaVision real-time fluorescence microscope, Applied Precision. Scale bar = 200 μm. HE, hematoxylin/Eosin; EF, endometrium fragment.
Future investigation of xenosialitis in human infertility promises to lead to important insights into causes of human idiopathic infertility and open avenues for intervention.

**Experimental Procedures**

**Mouse Sperm Collection**—WT C57BL/6 mice were purchased from Harlan Laboratories, and Cmah<sup>-/-</sup> (24), C57BL/6 and CAG GFP mice (52) were provided by the Laboratory of Dr. Ajit Varki at University of California, San Diego. All mice were kept and utilized under University of California, San Diego Institutional Animal Care and Use Committee (IACUC) Protocol S01227. Sperm were harvested from the cauda epididymis of sacrificed 12–20-week-old males. The cauda epididymis was squashed in a drop of 50 µl of Biggers, Whitten, and Whittingham medium (BWW) or human tubal fluid (Irvine Scientific), and sperm were subjected to a swim-up procedure in 100 µl of BWW at 37 °C under 5% CO<sub>2</sub> for 20 min following published procedures (53).

**In Vitro SVF Incubation with Sperm**—Seminal vesicles were isolated from blood vessels and accessory glands by careful dissection. The gland fluid contents were manually expressed into microcentrifuge tubes. 20 µl of SVF were then mixed with 100 µl of physiological BWW and placed in a humidity chamber at 37 °C for 10 min, and then centrifuged at 1000 × g for 5 min, before using the supernatant for sperm incubation. Epididymal sperm were added to the diluted SVF. 10<sup>6</sup> sperm in 50 µl of diluted SVF were incubated at 37 °C under 5% CO<sub>2</sub> for 15 min (54, 55).

**Mouse Model for Human Exogenous Neu5Gc Incorporation**—Cmah<sup>-/-</sup> mice were fed with specially formulated chow containing Neu5Gc (added in the form of PSM (1.28 g of mucin/kg)) (56), and soy chow was fed to a control group for 6 months. Epididymal sperm and male and female genital organs were collected for measurement of incorporated Neu5Gc.

**Mouse Model for Human Exogenous α-Neu5Gc Antibody by NTHi-immunized Mice**—As described by Taylor et al. (35), NTHi strain 2019 or 2019siaT was grown to mid-log (A<sub>600</sub> 0.3–0.4) in Neu5Gc medium (Inalco Pharmaceuticals, San Luis Obispo, CA), washed, heat-killed, and injected (200 µl of A<sub>600</sub> 0.4) intraperitoneally into Cmah<sup>-/-</sup> mice (age 5–8 weeks, female). All mice were injected a total of three times for boosting at 2-week intervals. No adjuvant was used with any of the
bacterial injections. Serum for antibody analysis was collected after the second boost, and uterine flush was collected 6 months after initial immunization.

**Determination of Neu5Gc Antigen Targeted by α-Neu5Gc Antibodies**—Motile epididymal sperm from WT and Cmah<sup>−/−</sup> mice were preincubated with wild type mouse SVF (method as described above) and subsequently co-incubated with mouse sera and uterine fluid containing high levels of α-Neu5Gc Ab (detected as above) at 37 °C under 5% CO<sub>2</sub> for 1 h in BWW. Sperm were subsequently washed and stained with fluorescently labeled anti-mouse IgA/G/M (1:200 BioLegend) in 1% human serum albumin and PBS for 30 min at 4 °C. Changes in CD52 and Neu5Gc content on and Cmah<sup>−/−</sup> epididymal sperm after preincubation with wild type SVF were evaluated using commercial polyclonal α-Neu5Gc IgY (1:1000, Sialix) and polyclonal α-CD52 (1:500, Santa Cruz Biotechnology). NaIO<sub>4</sub> treatment truncates the side chain of Neu5Gc and confirms the specificity of the antibody (57).

**Viability, Migration, and Calcium Assay of Sperm**—For sperm viability analysis, sperm density was adjusted in BWW, and staining was performed according to the manufacturer’s instructions using the LIVE/DEAD<sup>®</sup> Sperm Viability Kit (SYBR-14/PI, Invitrogen) (58), prior to evaluation of the sample by flow cytometry (BD Biosciences FACSCalibur). For the sperm migration assay, 300 μl of BWW were added to each well of a 24-well plate (BD Biosciences), and then inserts (BD Biosciences, pore size 3 um) were set, and 10<sup>5</sup> sperm were added. Sperm that migrated into the lower chamber were counted (this simple system was designed with the help of Noritaka Hirohashi (Ochanomizu University, Tokyo, Japan). We determined sperm capacitation status according to the instructions in Markoe and Suarez (59). 10<sup>5</sup> sperm were co-incubated with the loading concentration of the fluorescent calcium dye FLO3 (1 mM in DMSO, Invitrogen) and then analyzed by flow cytometry and imaged by microscopy.

**Phagocytosis of Sperm by Uterine Activated Leukocytes and Determination of Potential Receptors on Neutrophils**—A single (C57BL/6) was caged with two females in estrus stage, and then females were examined the next morning for the presence of vaginal plugs as an indication of successful mating. Mated males (C57BL/6) were caged with two females in estrus stage, and sperm attached to the zona pellucida were counted (63).

**Mouse Model for Dietary Reproductive Xenosialitis**

Mouse Endometrial Fragment Culture in Vitro—Endometrium from Cmah<sup>−/−</sup> estrus mice mated at day 4 (D4) to WT mice (as above) was squashed in PBS (Gibco) at 4 °C following the methods described in Colette et al. (45). Tissues were gently dissected into small fragments (1 mm<sup>2</sup>) using a scalpel in fresh PBS at 4 °C. Ten pieces per dish were placed on 12-mm-diameter tissue culture inserts (Teflon membrane, pore size: 0.4 μm, BD Biosciences) in 24-well culture plates. Organ cultures were maintained in serum-free conditions in Dulbecco’s modified Eagle’s medium/Ham F12 1:1 (DMEM/F-12, Gibco) supplemented with 1% insulin-transferrin-selenium (ITS+, Sigma, Steinheim, Germany), 0.1% EX-CYTE (Millipore). Endometrial explants were cultured for 24–48 h at 37 °C in a humidified atmosphere under 5% CO<sub>2</sub> in air. For the endometrium of D4 mice, 63.5 nmol/liter progesterone, 7.14 nmol/liter estradiol were added in the no-sera medium (60). Endometrial explants were cultured with and without exposure to 5 mM Neu5Gc (Inalco Pharmaceuticals) for 2 days.

**Analysis of Sperm Motility and DNA Fragment**—Sperm motility was measured by CASA, and both straight line velocity and curvilinear velocity data were collected and then analyzed. Sperm DNA fragmentation was determined by SCSA, measuring DNA fragmentation index and high DNA stainability, and sperm were stained by adding 1.2 ml of acridine orange (chromatographically purified, Invitrogen) for 5 min prior to analysis by flow cytometry (61).

**In Vitro Capacitation of Mouse Sperm**—Mouse epididymal sperm were incubated in 5 mg/ml BSA (Sigma), 20 mM NaHCO<sub>3</sub>, BWW, at 37 °C under 5% CO<sub>2</sub> for 2 h following published methods (62).

**Sperm-Egg Binding**—Three-week-old female mice were superovulated by sequential administration of pregnant mare’s serum gonadotropin (Sigma) and human chorionic gonadotropin (Sigma), and then the oocytes were collected by flushing the oviduct and were stripped of the cumulus cell using hyaluronidase (Invitrogen). Fresh epididymal cauda sperm were incubated in capacitation medium and then added to the oocytes for co-incubation for 30 min at 37 °C. The eggs were individually aspirated into a new drop of medium and placed on glass, kept on ice for 5 min, stained with DAPI (Sigma) at a concentration of 1:50,000 at room temperature for 2 min, and coverslipped, and sperm attached to the zona pellucida were counted (63).

**Statistics**—Data were graphed and analyzed using GraphPad Prism 5. Two-tailed Student’s t tests were used to evaluate significance and calculate p values, with threshold values as described under “Results” or in the figure legends. Error bars represent standard deviation of mean values. A p value of less than 0.05 was considered significant., * indicates >0.5, ** indicates >0.05, and *** indicates >0.005.

**Author Contributions**—P. G. and F. M. conceived the experiments, F. M. and L. D. carried out the experiments, P. S. helped with mouse husbandry, L. S. helped with sperm motility experiments, and J. Z. helped with sperm zona binding experiments. P. G. and F. M. wrote the manuscript, all authors proof read the manuscript.

**Acknowledgments**—We thank Ajit Varki for Cmah<sup>−/−</sup> mice, Nissi Varki for precious advice on histology, and Noritaka for help setting up the sperm migration assay. We gratefully acknowledge the UCSF Neuroscience Microscopy Facility (National Institutes of Health Grant P30 NS047101).
Mouse Model for Dietary Reproductive Xenosialitis

References

1. Smith, C., Grimm, M., and Schwegel, M. (2012) Treatment of infertility in women. J. Am. Pharm. Assoc. 52, e27–e42
2. Krausz, C. (2011) Male infertility: pathogenesis and clinical diagnosis. Best Pract. Res. Clin. Endocrinol. Metab. 25, 271–285
3. Ray, A., Shah, A., Gudi, A., and Homburg, R. (2012) Unexplained infertility: an update and review of practice. Reprod. Biomed. Online 24, 591–602
4. Hendry, W. F. (1989) Detection and treatment of antisympatotaxial antibodies in men. Reprod. Fertil. Dev. 1, 205–220; discussion 220–222
5. Kremer, J., and Jager, S. (1992) The significance of antisympar antibodies for sperm-cervical mucus interaction. Hum. Reprod. 7, 781–784
6. Diekman, A. B., Norton, E. J., Westbrook, V. A., Klotz, K. L., Naaby-Hansen, S., and Herr, J. C. (2000) Anti-sperm antibodies from infertile patients and their cognate sperm antigens: a review. Identity between SAGA-1, the H6-3C4 antigen, and CD52. Ann. N.Y. Acad. Sci. 999, 1637–1646
7. Samraj, A. N., Pearce, O. M., Läubli, H., Crittenden, A. N., Bergfeld, A. K., Pham, T., Gregg, C. J., Karp, F., Chow, R., Padler-Karavani, V., Cao, H., and Varki, A. (2011) Since there are PAMPs and DAMPs, there must be SAMPs? Glycan “self-associated molecular patterns” dampen innate immunity. But pathogens can mimic them. Mol. Cell Biol. 31, 4320–4346
8. Varki, A., and Schauer, R. (2009) Sialic Acids. In Essentials of Glycobiology, 2nd edn., M. D. Paquette and J. L. Valentine (eds), Oxford University Press, Oxford, pp. 15–33
9. Varki, A. (2011) Since there are PAMPs and DAMPs, there must be SAMPs? Glycan “self-associated molecular patterns” dampen innate immunity. But pathogens can mimic them. Mol. Cell Biol. 31, 4320–4346
10. Varki, A. (2001) Loss of CMP-N-acetylneuraminic acid hydroxylase occurred prior to brain expansion during human evolution. Proc. Natl. Acad. Sci. U.S.A. 99, 11736–11741
11. Varki, A. (2011) Since there are PAMPs and DAMPs, there must be SAMPs? Glycan “self-associated molecular patterns” dampen innate immunity. But pathogens can mimic them. Mol. Cell Biol. 31, 4320–4346
12. Varki, A., and Schauer, R. (2009) Sialic Acids. In Essentials of Glycobiology, pp. 199–218, Cold Spring Harbor Press, Plainview, NY
13. Chou, H. H., Hayakawa, T., Diaz, S., Knings, M., Indriati, E., Leakey, M., Paabo, S., Satta, Y., Takahata, N., and Varki, A. (2002) Inactivation of CMP-N-acetylneuraminic acid hydroxylase occurred prior to brain expansion during human evolution. Proc. Natl. Acad. Sci. U.S.A. 99, 11736–11741
14. Miyoshi, I., Higashi, H., Hirabayashi, Y., Kato, S., and Naiki, M. (1986) Detection of 4-O-acetyl-N-glycolylneuraminyl lactosylceramide as one of tumor-associated antigens in human colon cancer tissues by specific antibody. Mol. Immunol. 23, 631–638
15. Devine, P. L., Clark, B. A., Birrell, G. W., Layton, G. T., Ward, B. G., Alewoud, P. F., and McKenzie, I. F. (1991) The breast tumor-associated epitope defined by monoclonal antibody 3E12 is an O-linked mucin carbohydrate containing N-glycolylneuraminic acid. Cancer Res. 51, 5826–5836
16. Miyoshi, I., Higashi, H., Hirabayashi, Y., Kato, S., and Naiki, M. (1986) Detection of 4-O-acetyl-N-glycolylneuraminyl lactosylceramide as one of tumor-associated antigens in human colon cancer tissues by specific antibody. Mol. Immunol. 23, 631–638
17. Devine, P. L., Clark, B. A., Birrell, G. W., Layton, G. T., Ward, B. G., Alewoud, P. F., and McKenzie, I. F. (1991) The breast tumor-associated epitope defined by monoclonal antibody 3E12 is an O-linked mucin carbohydrate containing N-glycolylneuraminic acid. Cancer Res. 51, 5826–5836
18. Miyoshi, I., Higashi, H., Hirabayashi, Y., Kato, S., and Naiki, M. (1986) Detection of 4-O-acetyl-N-glycolylneuraminyl lactosylceramide as one of tumor-associated antigens in human colon cancer tissues by specific antibody. Mol. Immunol. 23, 631–638
19. Devine, P. L., Clark, B. A., Birrell, G. W., Layton, G. T., Ward, B. G., Alewoud, P. F., and McKenzie, I. F. (1991) The breast tumor-associated epitope defined by monoclonal antibody 3E12 is an O-linked mucin carbohydrate containing N-glycolylneuraminic acid. Cancer Res. 51, 5826–5836
20. Miyoshi, I., Higashi, H., Hirabayashi, Y., Kato, S., and Naiki, M. (1986) Detection of 4-O-acetyl-N-glycolylneuraminyl lactosylceramide as one of tumor-associated antigens in human colon cancer tissues by specific antibody. Mol. Immunol. 23, 631–638
21. Miyoshi, I., Higashi, H., Hirabayashi, Y., Kato, S., and Naiki, M. (1986) Detection of 4-O-acetyl-N-glycolylneuraminyl lactosylceramide as one of tumor-associated antigens in human colon cancer tissues by specific antibody. Mol. Immunol. 23, 631–638
22. Miyoshi, I., Higashi, H., Hirabayashi, Y., Kato, S., and Naiki, M. (1986) Detection of 4-O-acetyl-N-glycolylneuraminyl lactosylceramide as one of tumor-associated antigens in human colon cancer tissues by specific antibody. Mol. Immunol. 23, 631–638
41. Thompson, L. A., Barratt, C. L., Bolton, A. E., and Cooke, I. D. (1992) The leukocytic reaction of the human uterine cervix. *Am. J. Reprod. Immunol.* 28, 85–89

42. Hahn, S., Giaglis, S., Hoesli, I., and Hasler, P. (2012) Neutrophil NETs in reproduction: from infertility to preeclampsia and the possibility of fetal loss. *Front. Immunol.* 3, 362

43. Peters, A. J., and Coulam, C. B. (1992) Sperm antibodies. *Am. J. Reprod. Immunol.* 27, 156–162

44. Kutteh, W. H., Kilian, M., Ermel, L. D., Byrd, E. W., and Mestecky, J. (1994) Antisperm antibodies (ASAs) in infertile males: subclass distribution of IgA antibodies and the effect of an IgA1 protease on sperm-bound antibodies. *Am. J. Reprod. Immunol.* 31, 77–83

45. Colette, S., Defrère, S., Lousse, J. C., Van Langendonckt, A., Gotteland, J. P., Loumaye, E., and Donnez, J. (2011) Inhibition of steroid sulfatase decreases endometriosis in an in vivo murine model. *Hum. Reprod.* 26, 1362–1370

46. Aghajanova, L. (2004) Leukemia inhibitory factor and human embryo implantation. *Ann. N.Y. Acad. Sci.* 1034, 176–183

47. Godbole, G., Suman, P., Gupta, S. K., and Modi, D. (2011) Decidualized endometrial stromal cell derived factors promote trophoblast invasion. *Mol. Reprod. Dev.* 82, 635–650

48. Sutovsky, P. (2012) Cell biology solves mysteries of reproduction. *Cell Tissue Res.* 349, 631–632

50. Foote, R. H. (2003) Fertility estimation: a review of past experience and future prospects. *Anim. Reprod. Sci.* 75, 119–139

51. Chamley, L. W., and Clarke, G. N. (2007) Antisperm antibodies and conception. *Semin. Immunopathol.* 29, 169–184

52. Deng, L., Song, J., Gao, X., Wang, J., Yu, H., Chen, X., Varki, N., Naito-Matsui, Y., Galán, J. E., and Varki, A. (2014) Host adaptation of a bacterial toxin from the human pathogen *Salmonella Typhi.* *Cell* 159, 1290–1299

53. Nixon, B., MacIntyre, D. A., Mitchell, L. A., Gibbs, G. M., O’Bryan, M., and Aitken, R. J. (2006) The identification of mouse sperm-surface-associated proteins and characterization of their ability to act as decapacitation factors. *Biol. Reprod.* 74, 275–287

54. Minelli, A., Liguori, L., Bellezza, I., Renieri, T., and Castellini, C. (2003) Effects of diadenosine polyphosphates and seminal fluid vesicles on rabbit sperm cells. *Reproduction* 125, 827–835

55. Buttke, D. E., Nelson, J. L., Schlegel, P. N., Hunnicutt, G. R., and Travis, A. J. (2006) Visualization of GM1 with cholera toxin B in live epididymal versus ejaculated bull, mouse, and human spermatozoa. *Biol. Reprod.* 74, 889–895

56. Banda, K., Gregg, C. J., Chow, R., Varki, N. M., and Varki, A. (2012) Metabolism of vertebrate amino sugars with N-glycolyl groups: mechanisms underlying gastrointestinal incorporation of the non-human sialic acid xeno-autoantigen N-glycolyneuraminic acid. *J. Biol. Chem.* 287, 28852–28864

57. Higashi, H., Hirabayashi, Y., Fukui, Y., Nakii, M., Matsumoto, M., Ueda, S., and Kato, S. (1985) Characterization of N-glycolyneuraminic acid-containing gangliosides as tumor-associated Hanganutziu-Deicher antigen in human colon cancer. *Cancer Res.* 45, 3796–3802

58. Mantovani, A. (2006) Risk assessment of endocrine disrupters: the role of toxicological studies. *Ann. N.Y. Acad. Sci.* 1076, 239–252

59. Marquez, B., and Suarez, S. S. (2007) Bovine sperm hyperactivation is promoted by alkaline-stimulated Ca2+ influx. *Biol. Reprod.* 76, 660–665

60. Kombe, A., Siros, J., and Goff, A. K. (2003) Prolonged progesterone treatment of endometrial epithelial cells modifies the effect of estradiol on their sensitivity to oxytocin. *Steroids* 68, 651–658

61. Balasuriya, A., Speyer, B., Serhal, P., Doshi, A., and Harper, J. C. (2011) Sperm chromatin dispersion test in the assessment of DNA fragmentation and aneuploidy in human spermatozoa. *Reprod. Biomed. Online* 22, 428–436

62. Iba, K. N., Salicioni, A. M., Arcelay, E., Chertihin, O., Kumari, S., Herr, J. C., and Visconti, P. E. (2006) Evidence for the involvement of proline-directed serine/threonine phosphorylation in sperm capacitation. *Mol. Hum. Reprod.* 12, 781–789

63. Mitchell, L. A., Nixon, B., and Aitken, R. J. (2007) Analysis of chaperone proteins associated with human spermatozoa during capacitation. *Mol. Hum. Reprod.* 13, 605–613