Sexual selection by female immunity against paternal antigens can fix loss of function alleles

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Humans lack the common mammalian cell surface molecule N-glycolyneuraminic acid (Neu5Gc) due to a CMAH gene inactivation, which occurred approximately three million years ago. Modern humans produce antibodies specific for Neu5Gc. We hypothesized that anti-Neu5Gc antibodies could enter the female reproductive tract and target Neu5Gc-positive sperm or fetal tissues, reducing reproductive compatibility. Indeed, female mice with a human-like Cmah(−/−) mutation and immunized to express anti-Neu5Gc antibodies show lower fertility with Neu5Gc-positive males, due to prezygotic incompatibilities. Human anti-Neu5Gc antibodies are also capable of targeting paternally derived antigens and mediate cytotoxicity against Neu5Gc-bearing chimpanzee sperm in vitro. Models of populations polymorphic for such antigens show that reproductive incompatibility by female immunity can drive loss-of-function alleles to fixation from moderate initial frequencies. Initially, if loss of a cell-surface antigen can occur in an isolated population or when natural selection favors the loss of a receptor exploited by pathogens, subsequently the same loss-of-function allele can come under sexual selection because it avoids being targeted by the female immune system. Thus, we provide evidence of a link between sexual selection and immune function: Antigenicity in females can select against foreign paternal antigens on sperm and rapidly fix loss-of-function alleles. Similar circumstances existed when the CMAH null allele was polymorphic in ancestral hominins, just before the divergence of Homo from australopithecines.

glycan antigen | sialic acid | xeno-antigens

A ll cell surfaces in nature are covered by a complex array of glycans that have numerous functions and are often subject to rapid evolution and diversification (1, 2). Sialic acids (Sias) are components of animal cell membranes that cap many of these cell surface glycans. Sias are involved in cellular recognition during infection, development, and immune regulation (3). In addition, Sias are exploited by pathogens as receptors to host cells (4). Sias are essential for vertebrate development and occur in a variety of modifications and glycosidic linkages to their underlying glycans (3). The two most abundant mammalian Sias are N-glycolyneuraminic acid (Neu5Gc) and N-acetyllactosaminic acid (Neu5Ac). Most mammalian cells have tens to hundreds of millions of molecules of each, in varying ratios on different cell types (5).

Most mammals have a conserved single copy gene coding for the enzyme cytidine monophosphate N-acetylneuraminic acid hydroxylase (CMAH), which modifies CMP-Neu5Ac into CMP-Neu5Gc (6). The human lineage lost Neu5Gc expression due to an inactivating mutation in the CMAH gene (7). Human cell surfaces thus differ from those of most mammals because they lack Neu5Gc molecules, and instead carry an excess of Neu5Ac molecules (8). Of the <70 known genes directly involved in Sia metabolism and recognition, over a dozen have undergone human-specific changes, possibly because the loss of Neu5Gc required subsequent reconfiguration of other aspects of human Sia biology (8). Pseudogenization of CMAH in hominins is estimated to have occurred about 3 million years ago (Mya). Molecular clock analyses of human and great ape genomic sequences and dating of the Ape element involved in the mutation yielded an age of >2.5 million years (9). Subsequent analyses of genomic DNA from 40 global populations estimated that the CMAH(−) mutation occurred 3.2 Mya and was fixed as far back as 2.9 Mya (10). The relatively short time span of <0.3 My between the origin of the CMAH(−) mutation and its fixation was interpreted as evidence of strong selection favoring the CMAH(−) allele. However, it is yet to be determined what form of selection could have driven this rapid fixation. CMAH(−) individuals lacking Neu5Gc might have had an initial selective advantage by avoiding pathogens targeting host Neu5Gc for invasion (11), but these pathogens would quickly compensate, and indeed many have (4). Given the importance of balancing selection in maintaining glycan polymorphisms in mammalian species (12–14), the question arises whether pathogen-mediated selection alone is a plausible explanation for the fixation of the human loss-of-function mutation.

We considered and tested an alternate hypothesis for explaining the rapid fixation of the CMAH(−) allele: that the loss of Neu5Gc may have affected reproductive compatibility in ancestral populations, and that sexual selection due to female immunity against sperm or embryos bearing antigenic Neu5Gc drove rapid fixation of the CMAH(−) allele after this allele had reached intermediate frequencies, due to either natural selection (perhaps pathogen-mediated) or genetic drift. The complete loss of a Sia variant is equivalent to the gain of a novel non-self Sia, which could become a novel immune target on sperm or fetal tissue (Fig. 1). Human antibody profiles against Neu5Gc are varied in level and composition, but all humans have circulating antibodies against Neu5Gc, which appear early in life (15–17). The antibody repertoire of females also extends to their reproductive tract, through which the sperm must pass and in which embryos must implant (18). Such a mechanism would be specific to humans; other old world primates tolerate this ubiquitous self-molecule, and their immune systems do not make antibodies targeting Neu5Gc.

Here, we address three key questions. First, can Neu5Gc on sperm or fetal tissues become functionally antigenic to CMAH(−) females? Second, does this reduce the reproductive compatibility of males and females with different Sia phenotypes? Third, could reproductive incompatibility by adaptive immunity accelerate the fixation of mutations that cause glycan antigens to be lost from cell surfaces? We tested the effect of Neu5Gc Sia mismatch and anti-Neu5Gc immunity on reproduction in vivo using wild-type (wt) and transgenic mice homozygous for the same inactivating mutation of the mouse Cmah as seen in humans (Cmah is the mouse ortholog of the human CMAH gene) (19). We...
also modeled the effect of this incompatibility to determine the circumstances under which a loss-of-function allele will have a selective advantage due to selection imposed by female immunity. Finally, we examined the impact of human anti-Neu5Gc antibodies and complement on chimpanzee sperm in vitro to ask whether human antibodies are also capable of targeting paternal antigens and whether this mechanism could have acted while CMAH was polymorphic in ancestral hominin populations.

**Results**

**Neu5Gc Is Absent from CMAH(−/−) Mice.** We found that each sperm cell contains ~30 million Sia molecules, with ~10% as Neu5Gc (Fig. 2A). The sperm of Cmah(−/−) mice had no detectable Neu5Gc but a similar amount of total Sia (~28 million molecules/cell). The surface localization of Neu5Gc on wt mouse sperm and the absence of Neu5Gc on Cmah(−/−) mouse sperm was confirmed by fluorescent staining, using a previously described anti-Neu5Gc antibody (20) (Fig. 2C) as well as by flow cytometry (Fig. 2B). Unlike adult humans, Cmah(−/−) mice do not have circulating antibodies against Neu5Gc (19). However, they can be immunized against Neu5Gc using a mechanism similar to that which induces human anti-Neu5Gc antibodies: incorporation of Neu5Gc into the lipo-oligosaccharides of the human-specific commensal microbe *Haemophilus influenzae* (17). As with humans, immunization with this Neu5Gc-expressing bacterium generates an anti-Neu5Gc response that varies among individual Cmah(−/−) mice. Consistent antibody levels could be achieved with a standard immunization schedule involving two boosts (Fig. 3C). Control mice immunized with Sia-free bacteria and wt mice immunized with Neu5Gc-expressing bacteria did not generate this response. We next confirmed that wt mouse sperm could be targeted by induced anti-Neu5Gc antibodies. This targeting was demonstrated by ELISAs using plated wt Cmah(+/+) control Cmah(−/−) sperm, and heterozygote Cmah(+/−) sperm. As predicted, antibody binding was only found when immunized female mouse serum was applied to wt or heterozygote sperm (Fig. 3B). Such antibody deposition would mark sperm for killing in vivo, via complement deposition and via antibody-dependent cellular cytotoxicity (ADCC). Two groups of immunized Cmah(−/−) female mice as well as control Cmah(+/+) females that do not produce antibodies even after immunization were bred with wt Cmah(+/+) or Cmah(−/−) males. Each group of 9 females had comparable levels of anti-Neu5Gc antibodies (Fig. 3C). Females immunized against Neu5Gc also had circulating anti-Neu5Gc IgG (produced by B-cell-derived plasma cells and filtering into uterine secretions) and to a lesser extent IgA (locally secreted by immune cells in the reproductive tract) in their uterine fluid compared with nonimmunized controls (Fig. 3D). These two classes of antibodies are found in the female reproductive tract and can target Neu5Gc on sperm and/or fetal tissues.

**Female Mice with Anti-Neu5Gc Antibodies Show Reduced Fertility with Neu5Gc-Positive WT Males.** Having established conditions for testing possible reproductive incompatibility mediated by anti-Neu5Gc antibodies, we performed breeding experiments with cohorts of mice representing all possible pair-wise combinations of Neu5Gc- and control-immunized females with Cmah(+/+) and Cmah(−/−) males (all in C57Bl6 congenic background). Fig. 4.4 shows the eight combinations of breeding pairs used for the breeding experiment. Immunized Cmah(−/−) females breeding with wt Cmah(+/+) males had fewer pups per litter.
Reduced reproductive success of Cmah(−/−) mice immunized against Neu5Gc. (A) The cumulative reproductive output of each cohort of nine females for three consecutive litters is shown. Bars show 95% confidence intervals. **, P < 0.005; *, P < 0.05 in unpaired Student t test. The scatter plot shows the mating and immunization scheme, immunization negative female were control immunized with Sia-free bacteria. (B) Distribution of offspring in litters from Cmah(+/−) heterozygous males mated with immunized Cmah(−/−) females, neither significantly deviated from 50:50 (Fisher’s exact test, one-tailed).

Modeling Reproductive Incompatibility Caused by Female Immunity in Ancestral Hominins. Reproductive incompatibility between females and males due to the antigenicity of Neu5Gc could influence how selection operates on the Cmah(−) allele and perhaps explain its rapid fixation. We modeled the effect of sexual selection by female immunity against antigenic sperm or embryos. We calculated selection coefficients for the Cmah(−) allele by determining the strength and direction of selection in males and in females. Reproductive success was calculated using a pay-off matrix for all pair-wise genotype combinations (Fig. 5A), and by varying the level of female promiscuity and degree of reproductive incompatibility imposed by female immunity (Fig. 5). Selection favors Cmah(−/−) males because they are compatible with all females in the population, but males with one or more Cmah(+) allele are only compatible with a subset of females (Fig. 5A). As the frequency of the Cmah(−) allele increases, so does the proportion of females that are exclusively compatible with Cmah(−/−) males, and selection favoring the Cmah(−) allele in males increases correspondingly (Fig. 5B). Selection initially acts against Cmah(+/−) females, because they attack the Neu5Gc antigen and are thus only compatible with Cmah(−/−) males. However, as Cmah(−) increases in frequency, the probability of encountering a homozygous Cmah(−/) male increases and selection against Cmah(+/−) females decreases (Fig. 5B). The net effect is that the Cmah(−) allele faces negative selection at low frequencies, and positive selection at high frequencies. There is thus a frequency threshold, at which selection favoring Cmah(−) in males overrides selection against Cmah(−/) in females, and sexual selection switches from opposing to favoring the Cmah(−) allele, which can then drive it to fixation (Fig. 5B).

The selection threshold is influenced both by overall reproductive compatibility and by female promiscuity. Females that mate with multiple males increase their chances of encountering a compatible mate (Fig. 5 B and C). The model also predicts that the selection threshold occurs at lower frequency when the female immune response results in partial incompatibility (e.g., a 25% reduction as opposed to total loss of fertility). At low frequency, selection against Cmah(−) is dominated by the allele’s negative effects on females (Fig. 5B), and increasing relative compatibility decreases this cost (Fig. 5C). Allowing for partial incompatibility and scenarios where females mate with more than one male substantially lowers the threshold frequency at which sexual selection favors the Cmah(−) allele.
Chimpanzee species have an intact CMAH gene, but the loss of Neu5Gc expression in humans, caused directly by female immunity. We demonstrate in vivo that female immune response against sperm can cause reproductive incompatibility between individuals (and perhaps species) with mismatched cell-surface glycans. An analytical model of reproductive compatibility demonstrates that sexual selection can drive the fixation of loss-of-function alleles when their positive effect on male reproductive success exceeds their negative effect on females (Fig. 5). This form of sexual selection could thus explain the rapid fixation of CMAH(−) allele and loss of the Neu5Gc gene.

Closest Living Relatives of Humans Have Abundant Neu5Gc on Their Sperm, Which Can Be Attacked by Human Anti-Neu5Gc Antibodies and Complement. Chimpanzee species have an intact CMAH gene, and their sperm Sias should thus be similar to the ancestral condition of Australopithecine hominins before the loss of Neu5Gc expression. In fact, levels of Neu5Gc on chimpanzee sperm were much higher than on wt mouse sperm (Fig. 6 A and B). In keeping with this, and with the much higher levels of complement in human compared with mouse sera (21), we could demonstrate direct killing of chimpanzee sperm by human serum and complement deposition (Fig. 6 C and D). The Neu5Gc-specificity of these interactions was confirmed by showing that it could be inhibited by Neu5Gc-rich chimpanzee serum or by heat inactivation of the human serum (Fig. 6 D).

Discussion
It has been repeatedly suggested that evolution will forge links between sexual reproduction and immune function via the choice and display of traits that indicate immune competence (22–24). Here we provide evidence of another relationship between immune function and sexual selection: reproductive incompatibility caused directly by female immunity. We demonstrate in vivo that female immune response against sperm can cause reproductive incompatibility between individuals (and perhaps species) with mismatched cell-surface glycans. An analytical model of reproductive compatibility demonstrates that sexual selection can drive the fixation of loss-of-function alleles when their positive effect on male reproductive success exceeds their negative effect on females (Fig. 5). This form of sexual selection could thus explain the rapid fixation of CMAH(−) allele and loss of the Neu5Gc gene.
Several conditions are required for female immunity to cause reproductive incompatibility by selecting against males that express foreign cell-surface antigens; namely: immunization, contact, and response. All three of these conditions were met when ancestral hominin populations were still naturally polymorphic at the CMAH locus. First, females must be immunized against foreign antigens. Immunization to the lost Neu5Gc in ancestral hominin populations could have occurred one of two ways. Hominin ancestors increased eating of Neu5Gc-rich red meat via scavenging and hunting about 3 Mya (25). This would have exposed ancestral populations to Neu5Gc through diet and direct contact with the bodily fluids of their prey (26). Additionally, microbial presentation of dietary Neu5Gc could have provided consistent immunization of most individuals (17). Circulating anti-Neu5Gc antibodies have been detected in all humans tested so far (16). Second, females must come into close contact with antigens expressed on sperm or embryonic tissues. Our experiments show that mouse and chimpanzee sperm express Neu5Gc and that female antibodies against Neu5Gc exist in the reproductive tract. Third, female immune responses must reduce the cycle of pairing with males that bear foreign antigens by directly targeting their sperm or the resulting embryos.

Our observations still raise some questions about the precise nature of the Neu5Gc based incompatibility; most importantly, we do not yet know the mechanisms or relative contributions of prezygotic versus postzygotic incompatibilities in the hominin ancestors. However, our experiments do show that CMAH(−/−) females suffer reduced compatibility with CMAH(+/+) and CMAH(+/-) males as a result of their immune response to the foreign Neu5Gc antigen, and our models function regardless of whether this is a result of pre- or postzygotic effects. Sexual selection by female immunity could have influenced the maintenance or elimination of other glycan polymorphisms. Humans and all their Old World primate relatives have also lost the α-Gal epitope. The enzyme responsible for synthesizing this common mammalian glycan, α1-3-galactosyltransferase or Gal-T, has been inactivated in the Old World monkey and hominoid lineage (27, 28). There are also resident glycan polymorphisms in humans (blood groups for example) and other mammals (including some species which maintain polymorphism in Neu5Gc itself) (29). We predict that glycan antigens, which remain polyvalent, will have features allowing them to avoid being targeted by the female immune system by interrupting one or more of the above-mentioned three operational requirements of incompatibility by immunity. For example, anti-A and anti-B blood group antibodies tend to be large IgM molecules, which diffuse much less freely into the female reproductive tract and across the placenta (30).

Sexual selection by female immunity may be a powerful determinant of antigen polymorphism, particularly in mammals. Indeed, reproductive incompatibilities due to antigen mismatch may affect the evolution of the female immune response itself. Mammals with more invasive placentas (where fetal trophoblast is in more intimate contact with maternal tissue) are also more prone to hybridization as a result of a down-regulation of the maternal immune response due to the mother’s greater exposure to foreign fetal antigens (31). Our models of sexual selection show that selection due to female immunity against such foreign antigens can fix loss-of-function alleles from moderate initial frequencies, and our in vivo experiments show that the conditions for the operation of this form of sexual selection are met in the success of human CMAH(−/−). Thus, sexual selection by female immunity could explain the rapid fixation of the CMAH(−/−) allele and complete loss of Neu5Gc early in the divergence of the hominin lineage. Given the timing estimates and effectiveness of the mechanisms described here, it is also possible that the CMAH mutation resulted in a reproductive barrier among central australopitheneic populations, eventually fixing in the Homo lineage that gave rise to modern humans (32) and Neanderthals. In keeping with this result, analysis of fossil Sia revealed that Neanderthal bones also only contained Neu5Ac and no Neu5Gc (9), a factor that could help explain why they could hybridize with modern humans (33).

Materials and Methods

Model of Selection on the CMAH(−/−) Allele. Homozygous CMAH(−/−) females have a nonself immune reaction to the Neu5Gc Sia on the sperm or embryos of CMAH(+/+) and CMAH(+/-) males. Fig. 5A shows a compatibility matrix that describes the reproductive output of all possible CMAH genotype combinations, with selection favoring the CMAH(−/−) allele over CMAH(+/+) males against the CMAH(−/−) allele (Fig. 5A). The effect of selection and expected frequency change of the CMAH(−/−) allele over a single generation was calculated for each 0.01 frequency increment between 0 and 1 by substituting selection inferred from the compatibility matrix into standard equations for calculating frequency change by selection on dominant alleles (34).

Males. The proportion of females compatible with a CMAH(−/−) male = 1. The proportion compatible with a CMAH(+/+) or CMAH(+/-) male was calculated as 1−q2, where q is the frequency of the CMAH(−/−) allele. For completely incompatible crosses the disadvantage to CMAH(+/+) and CMAH(+/-) males, sm = q2, is the compatibility, the fraction of the cross which is restored in cases of partial incompatibility. The strength of selection against CMAH(+/+) and CMAH(+/-) males is thus described by: sm = (1−q)2. The expected frequency of the CMAH(−/−) allele over a single generation of selection in males is given by the standard equation describing selection against a dominant allele: qf = q−smq1 − smq2.

Females. The strength of selection in females depends on the probability of encountering a compatible mate. For completely incompatible crosses, homozygous CMAH(−/−) females can only be fertilized by CMAH(−/−) males, which are encountered in each mating attempt with a probability of q2. Partial compatibility raises the probability of encountering a compatible CMAH(−/−) male (q) by a fraction proportional to the compatibility of a CMAH(−/−) female with CMAH(+/+) and CMAH(+/-) males: e=q+(1−q2). The strength of selection against homozygous CMAH(−/−) females (sF) is the proportion of CMAH(−/−) females that did not successfully reproduce relative to CMAH(+/+) and CMAH(+/-) females. sF is calculated as the binomial probability that a CMAH(−/−) female will encounter zero (k = 0) compatible mates in a given number of mating attempts (n) with a given probability of encountering a compatible mate (e). The expected frequency of the CMAH(−/−) allele after a single generation of selection in females is given by the standard equation describing selection favoring a dominant allele: qf = q−snq1 − snq2.

The combined effect of selection in males and females and the expected change in frequency of the CMAH(−/−) allele over a single generation was calculated equally weighted (16). We next calculated the negative effect in females and the positive effect in males (assuming a 50:50 population sex ratio). Scripts were written in Perl by S. Springer (SI Appendix).

Mice. The transgenic Cmah(−/−) mice have been described (19) and were used under protocol S01227. Age-matched female mice were immunized, and cohorts of females with similar spectra of anti-Neu5Gc titers were formed. Females were bred with a single male. Litters were removed at weaning.

Mouse Sperm. Mouse sperm were harvested from the cauda epididymis of 12- to 20-wk-old males immediately after sacrificing the animals. Cauda epididymis tissue was minced and kept on a shaker at room temperature (RT) for 10 min, followed by 30 s of centrifugation at 300 × g and a swim up procedure in sperm storage buffer [SSB: 110 mM NaCl/27.2 mM KCl/0.36 mM Na2HPO4/0.49 mM MgCl2/2.40 mM CaCl2/25.00 mM Hepes/5.00 mM Mes/2-(N-morpholino)ethanesulfonic acid/25.00 mM lactic acid, pH 5.5 (35)]. Sperm were further subjected to a swim up procedure in 500 μL of SSB at 37 °C, 5% CO2 for 30 min before collection of the supernatant.

Mouse Immunization and Determination of Anti-Neu5Gc Immune Response. Antibody titers in mouse serum and mouse uterine fluids were determined by ELISA using Neu5Gc- and Neu5Ac-polycrylamide beads as plated targets (16). The dependence of antibody binding on the Neu5Gc antigen was used as a treatment to destroy the side chain of Sia and controlled for by mock treatment without the premixed periodate and sodium borohydride (16). Uterine fluid was collected by immediate post sacrifice flushing of the uteri with PBS.
Antibody–Sperm Binding Assays. Antibody–sperm binding by mouse immune sera was studied on plated and freshly fixed sperm of all three possible Cmah genotypes, which was collected immediately after sacrificing the male mice. Sperm were diluted to a concentration of 8 million/mL in Biggers Whitten Whittingham, 0.05% human serum albumin. A total of 100 μL of this suspension was added to each well of a COSTAR microplate. Plates were spun down at 250 × g for 5 min at RT. Supernatant was discarded. Cell densities were verified under microscope. The plate was allowed to air dry for 10 min. The plated sperm were fixed using freshly thawed formaldehyde adjusted to 1%. A total of 200 μL of 1% pfa in PBS was added to each well. After fixation, the plate was washed 3x with 200 μL of PBS (0.1%) per well. For sialidase treatment, 5 μL of A. ureafaciens sialidase (AUS) in buffer (50 mM NaPO₄, pH 6) were added to each well, and the sample was incubated at 37 °C for 2 h. Controls were treated with buffer or heat-treated AUS. The plate was blocked with 1% ovalbumin at RT for 1 h, serum was added at a concentration of 20 million sperm in 100 μL of BWW (1% ovalbumin per well) and incubated at RT for 2 h. The plate was washed three times with 150 μL of TBS (1%) ovalbumin per well and then blotted. Secondary antibody (donkey anti-mouse IgG) was added at concentration of 1:500 in TBS and incubated for 30 min at RT. The plate was washed three times with TBS before adding alkaline phosphatase substrate, developing in the dark, and reading at 490 nm.

Sample Preparation for Fluorescence Microscopy. For details see SI Materials and Methods.

Human Sera. Human sera were collected by venipuncture from human volunteers under University of California, San Diego, IRB-approved protocol 080677x.

Sialic Acid Analysis. For details see SI Materials and Methods.

Hominin Sperm Exposure to Anti-Neu5Gc Antibodies in Serum. Sperm were exposed to sera in a 1:2 dilution (20 million sperm in 50 μL of BWW medium plus 50 μL of neat serum) for 2 h at RT. Sera included human sera with high or low tiers of anti-Neu5Gc and chimpanzee sera (no anti-Neu5Gc antibodies). Anti-Neu5Gc content of human sera had been previously determined by ELISA using Neu5Gc target probes (16). Samples were then washed and stained for cell death with propidium iodine (PI, 15 min at RT) or for complement deposition with anti-C3 monoclonal antibody [1 h at RT, C3 (6C9), Santa Cruz Biotechnology]. Staining was quantified by flow cytometry. Sera were untreated or heat treated (30 min at 56 °C) to inactivate complement. Chimpanzee serum was added as an inhibitor due to its high content of Neu5Gc. The effect of serum exposure was measured by staining with PI followed by flow cytometry. Negative controls were incubated in BWW buffer.

Statistics. Statistical analyses were performed using Prism v5.0a (GraphPad Software).

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