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*-glycolylneuraminic 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, the loss of a cell-surface antigen can occur due to drift in isolated populations 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

All 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*-acetylneuraminic 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 *Alu* 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 to yet to be determined what form of selection could have driven this rapid fixation. *CMAH* (−/−) individuals lacking Neu5Gc might have experienced 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

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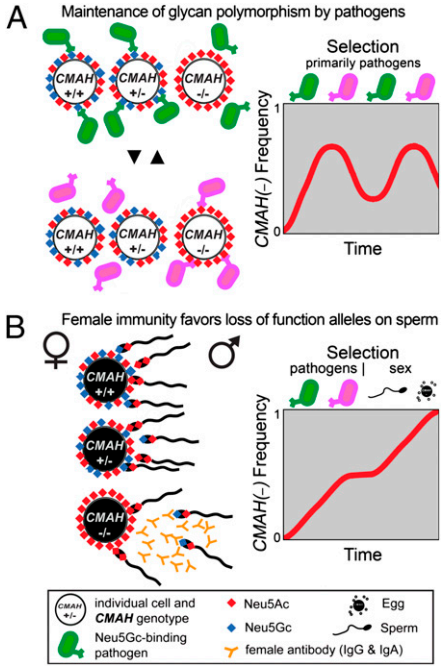

Fig. 1. Schematic of the interplay of natural and sexual selection acting on cell-surface Sias. (*A*) Natural selection by pathogens recognizing and exploiting Neu5Gc (blue diamond) as a receptor on host target cells can select for mutant *CMAH*(−) alleles that abolish expression of Neu5Gc in homozygote individuals. Such homozygous null individuals have only Neu5Ac on their cells (red diamonds) and, at higher frequencies, would be targeted by pathogens adapting to the host glycan change (magenta), resulting in maintenance of glycan polymorphism. (*B*) Anti-Neu5Gc antibody expressing *Cmah*(−/−) females favor loss-of-function alleles on sperm due to reproductive incompatibility with *Cmah*(+/−) or *Cmah*(+/+) males expressing Neu5Gc on their sperm. Once the frequency of the *Cmah*(−) allele reaches a critical level, this process can drive the fixation of the *Cmah*(−) allele in a population.

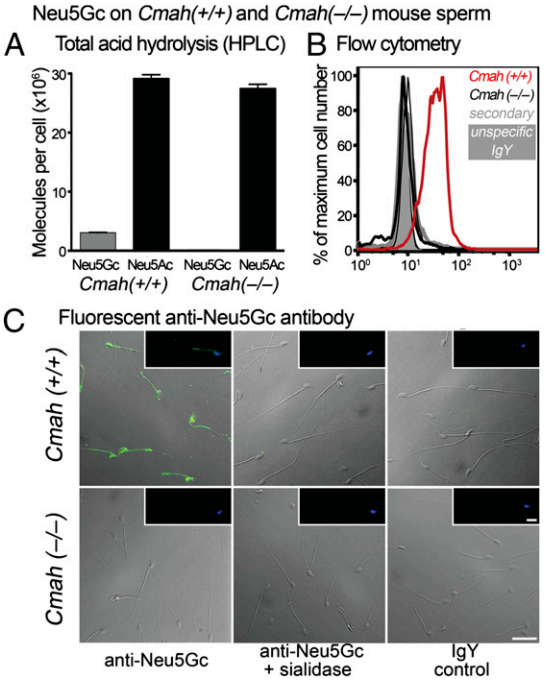

Fig. 2. Presence of Neu5Gc on sperm of wt but not *Cmah*(−/−) mice. (*A*) DMB HPLC measurement of Sias isolated by total acid hydrolysis for wt and *Cmah*(−/−) mouse sperm. No Neu5Gc was detected in the *Cmah*(−/−) sperm by this sensitive method. Sia content per sperm cell is shown for three different males of each genotype. (*B*) Flow cytometry detection of Neu5Gc in wt and *Cmah*(−/−) mouse sperm. The anti-Neu5Gc chicken IgY (20) stains Neu5Gc on wt mouse sperm but not on sperm from *Cmah*(−/−) mice. (*C*) Fluorescent detection of Neu5Gc in wt and *Cmah*(−/−) mouse sperm. An affinity-purified anti-Neu5Gc chicken IgY (20) stains Neu5Gc on head and midpiece of wt mouse sperm only (green). Signal is abolished upon treatment with sialidase. (Scale bars: 20 μm and *Inset*, 10 μm.)

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. 2*A*). 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. 2*C*) as well as by flow cytometry (Fig. 2*B*). 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. 3*A*). Control mice immunized with Sia-free bacteria and wt mice immunized with Neu5Gc containing 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. 3*B*). 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. 3*C*). 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. 3*D*). 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*A* 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

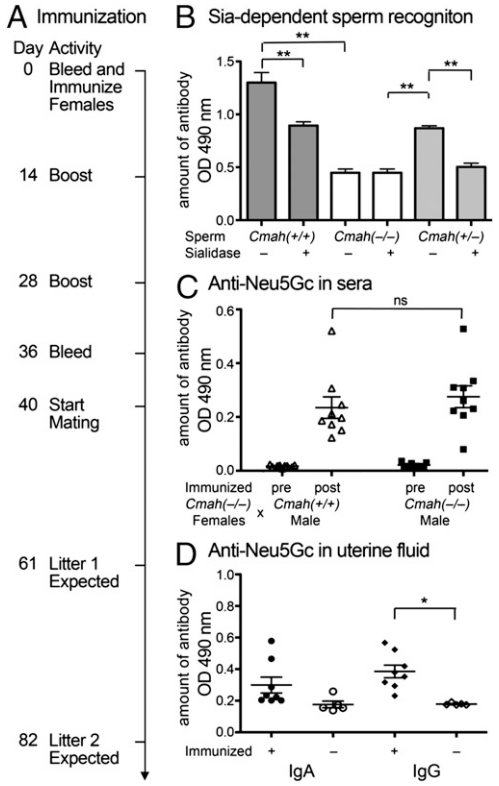

Fig. 3. Generation and characterization of qnti-Neu5Gc antibodies in *Cmah* (−/−) female mice. (*A*) Generation of anti-Neu5Gc antibodies in *Cmah*(−/−) mouse females. Female *Cmah*(−/−) mice were immunized by injection of heat-killed *H. influenzae* that had incorporated Neu5Gc (17). These female mice have been shown to generate antibodies against Neu5Gc, by ELISA and whole target cell assays. Control immunizations are done using the same *H. influenzae* glycolipid lacking Neu5Gc, due to culture in absence of Neu5Gc. (*B*) Antibodies from immunized *Cmah*(−/−) females recognize wt mouse sperm in a Sia-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 Sia-dependent nature of the interaction. All measures were carried out in triplicate. There was low background binding of anti-Neu5Gc mouse serum to all mouse sperm samples but no effect of sialidase treatment in *Cmah* (−/−) sperm. Heterozygote sperm were bound less than wt sperm but did show Sia-dependent antibody binding. Mock treatment included all reagents except the sialidase. (*C*) Anti-Neu5Gc IgG antibody titers of mouse sera from *Cmah*(−/−) mice before and two weeks after immunization with Neu5Gc containing bacterial glycolipid from *H. influenzae*. Data for 18 mouse sera analyzed by ELISA using plated Neu5Gc PAA targets are presented. Each group had a similar composition of low- and high-titer individuals and no significant differences in mean IgG levels (*t* test, two tailed, $P = 0.48$). (*D*) Anti-Neu5Gc antibodies measured in uterine fluid from naïve ($n = 5$) and immunized ($n = 8$) *Cmah*(−/−) mice. IgG was significantly higher (unpaired Student *t* test, $P = 0.022$).

born to each female (Fig. 4*A*). The reduced fertility of this cross-persisted over three consecutive litters. The effect could be due either to prezygotic effects (sperm killing or inactivation by female antibodies) or postzygotic incompatibility (failed implantation or embryo rejection). Fertility was also reduced by 30% in the progeny of crosses between heterozygote *Cmah*(+/−) males and immunized *Cmah*(−/−) females. Although all sperm from *Cmah*(+/−) males bear Neu5Gc, only half of their offspring with *Cmah*(−/−) females are expected to express Neu5Gc. There was no significant deviation from the expected 50% heterozygote offspring, indicating that most of the effect is prezygotic (Fig. 4*B*).

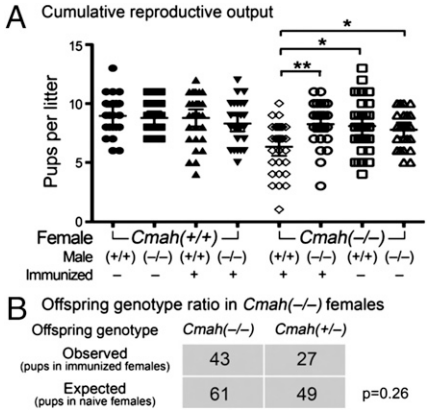

Fig. 4. Reduced reproductive success of *Cmah*(−/−) mice immunized against Neu5Gc. (*A*) The cumulative reproductive output of each cohort of nine females for three consecutive litters is shown. Bars show 95% confidence intervals. **, $P < 0.005$; *, $P < 0.05$ in unpaired Student *t* test. The scatter 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*(+/−) heterozygote 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. 5*A*), 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. 5*A*). 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. 5*B*). 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. 5*B*). 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 overcomes selection against *CMAH*(−) in females, and sexual selection switches from opposing to favoring the *CMAH*(−) allele, which can then drive it to fixation (Fig. 5*B*).

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. 5*B*), and increasing relative compatibility decreases this cost (Fig. 5*C*). 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.

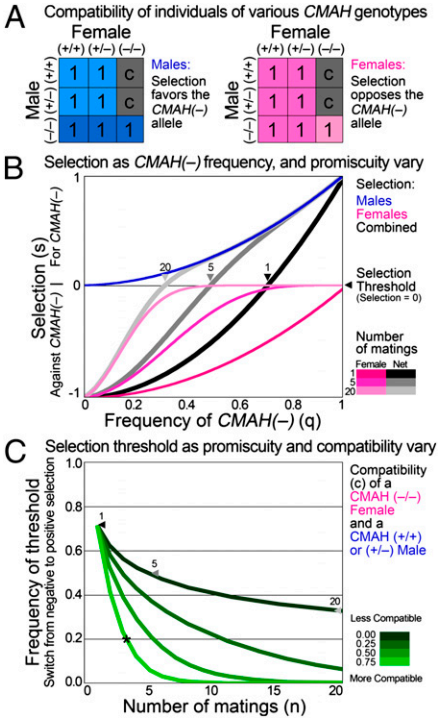

Fig. 5. Model of selection on the *CMAH*(–) allele. (*A*) Compatibility of various *CMAH* genotypes: c represents an antigenic cross with reduced compatibility. Selection favors *CMAH*(–/–) males, because they are compatible with females of all three *CMAH* genotypes. However, selection opposes *CMAH*(–/–) females, because they are only compatible with *CMAH*(–/–) males. (*B*) Sexual selection on the *CMAH*(–) allele in males (blue line) and females (pink lines). The black line represents the combined effect of selection in females and males. Selection on *CMAH*(–) goes from negative to positive at the selection threshold. Sexual selection cannot initiate positive selection on *CMAH*(–), but if pathogen pressure or genetic drift cause the *CMAH*(–) allele frequency to exceed the selection threshold, then sexual selection can drive *CMAH*(–) to fixation. The *CMAH*(–) allele exceeds the selection threshold at lower frequencies when females are more promiscuous (gray arrows). (*C*) Selection threshold as promiscuity and compatibility vary. Lines show the frequency at which *CMAH*(–) becomes favored by sexual selection for different antigenic cross compatibilities. Weak incompatibility imposes a lower reproductive cost on *CMAH*(–/–) females and lowers the selection threshold. The arrows show the relationship with Fig. 4*B*, increasing promiscuity lowers the selection threshold. The asterisk illustrates the following hypothetical case: a fertility loss of 25% (0.75 compatible) and a mating system in which individual females mate with three different males in their lifetime. In this case, the selection threshold frequency is 0.2. This translates in a switch from negative selection to positive selection on *CMAH*(–) when just 1 in 25 females ($0.2^2 = 0.04$) is homozygous *CMAH*(–/–).

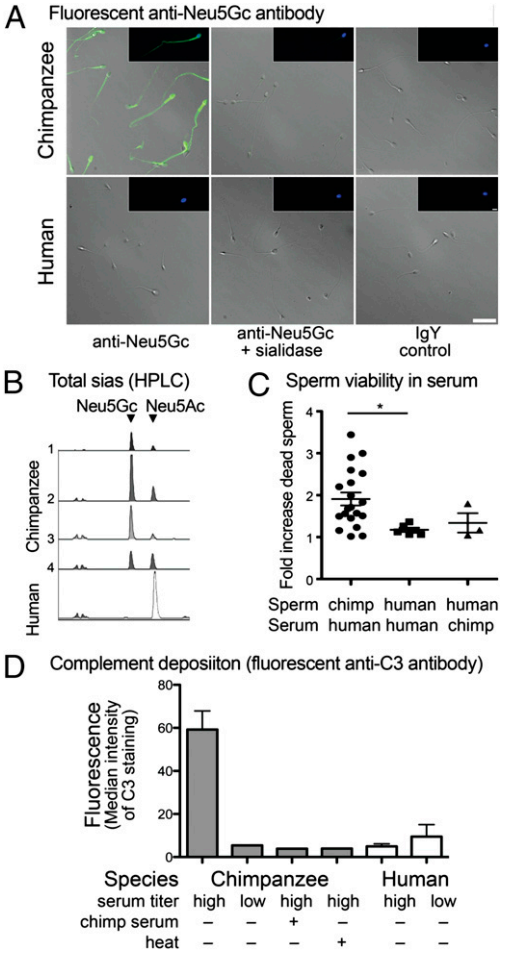

Fig. 6. Negative effect of human anti-Neu5Gc antibodies on live chimpanzee sperm. (*A*) Fluorescent detection of Neu5Gc on chimpanzee and human sperm. Anti-Neu5Gc chicken IgY(20) stains Neu5Gc on head and midpiece of chimpanzee sperm only (green). Signal is abolished upon treatment with sialidase. (Scale bars: 20 μm and *Inset*, 10 μm.) (*B*) High levels of Neu5Gc on sperm from four different chimpanzees. HPLC profile of total Sias released from chimpanzee and human sperm and base treated, as determined by DMB derivatization and separation via HPLC, as described in *Materials and Methods*. (*C*) Negative effect of exposure to human serum with high levels of anti-Neu5Gc antibodies on chimpanzee sperm viability ($n = 6$, three different experiments). Chimpanzee sperm exposed to high anti-Neu5Gc human serum show increased mortality. There was no difference in effect of human serum or chimpanzee serum on human sperm cell mortality. Values represent fold increase in mortality over background mortality of sperm not exposed to serum. (*D*) Increased complement deposition on chimpanzee sperm ($n = 2$) incubated in human serum containing high levels of anti-Neu5Gc antibodies compared with low-level anti-Neu5Gc human serum. For complement binding, median fluorescence intensity of staining by an anti-C3 antibody in high anti-Ne5Gc antibody titer human serum is inhibited by heat treatment or addition of chimpanzee serum. Bars show SEM.

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 *Australopithecene* 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

antigen in the common hominin ancestor after the *CMAH*(−) allele had reached intermediate frequencies due to pathogen mediated natural selection or genetic drift in local populations (Fig. 1).

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 hominid 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 success of pairings 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 hominid 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 polymorphic, 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 case 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 ancestral australopithecine 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. 5*A* shows a compatibility matrix that describes the reproductive output of all possible *CMAH* genotype combinations. In males, selection favors the *CMAH*(−) allele; in females, selection acts against the *CMAH*(−) allele (Fig. 5*A*). 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 $1 - q^2$, where q is the frequency of the *CMAH*(−) allele. For completely incompatible crosses the disadvantage to *CMAH*(+/+) and *CMAH*(+/−) males, $s_m = q^2$. c 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: $s_m = (1 - c) \times q^2$. The expected frequency the *CMAH*(−) allele after a single generation of selection in males is given by the standard equation describing selection against a dominant allele: $q_1 = q - s_m q + s_m q^2/1 - s_m(1 - q^2)$.

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 q^2. Partial compatibility raises the probability of encountering a compatible *CMAH*(−/−) male (ε) by a fraction proportional to the compatibility of a *CMAH*(−/−) female with *CMAH*(+/+) and *CMAH*(+/−) males: $\varepsilon = q^2 + c(1 - q^2)$.

The strength of selection against homozygous *CMAH*(−/−) females (s_f) is the proportion of *CMAH*(−/−) females that did not successfully reproduce relative to *CMAH*(+/+) and *CMAH*(+/−) females. s_f 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 (ε). 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: $q_1 = q - s_f q/1 - s_f q^2$.

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 by equally weighting 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 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 (35)]. Sperm were further subjected to a swim up procedure in 500 μL of SSB at 37 °C, 5% CO_2 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-polyacrylamide beads as plated targets (16). Sia dependence of antibody binding was confirmed by mild periodate 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 postsacrifice flushing of the uteri with PBS.

EVOLUTION

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 3× with 200 μL of PBS (0.1%) per well. For sialidase treatment, 5 mU of *A. ureafaciens* sialidase (AUS) in buffer (50 mM $NaPO_4$, 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 1:100 in TBS ovalbumin (100 μL 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.

Neu5Gc Antigens for ELISA. Antigen was prepared from homogenate of *H. influenzae* that was precultured with or without Neu5Gc, as described (17). The latter served as the control antigen.

Hominid Sperm. Chimpanzee sperm were collected by a noninvasive method (36). The samples from a total of six different chimpanzees were shipped at RT and analyzed side-by-side with human samples that were collected the same day as the chimpanzee samples and kept at RT until arrival of the chimpanzee samples (UCSD protocol 040613).

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 antibodes). 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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