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# Loss of N-Glycolylneuraminic Acid in Humans: Mechanisms, Consequences, and Implications for Hominid Evolution

AJIT VARKI

*Glycobiology Research and Training Center and Departments of Medicine and Cellular and Molecular Medicine, University of California, San Diego, La Jolla, California 92093*

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*ABSTRACT* The surface of all mammalian cells is covered with a dense and complex array of sugar chains, which are frequently terminated by members of a family of molecules called sialic acids. One particular sialic acid called N-glycolylneuraminic acid (Neu5Gc) is widely expressed on most mammalian tissues, but is not easily detectable on human cells. In fact, it provokes an immune response in adult humans. The human deficiency of Neu5Gc is explained by an inactivating mutation in the gene encoding CMP-N-acetylneuraminic acid hydroxylase, the rate-limiting enzyme in generating Neu5Gc in cells of other mammals. This deficiency also results in an excess of the precursor sialic acid N-acetylneuraminic acid (Neu5Ac) in humans. This mutation appears universal to modern humans, occurred sometime after our last common ancestor with the great apes, and happens to be one of the first known human-great ape genetic differences with an obvious biochemical readout. While the original selection mechanisms and major biological consequences of this human-specific mutation remain uncertain, several interesting clues are currently being pursued. First, there is evidence that the human condition can explain differences in susceptibility or resistance to certain microbial pathogens. Second, the functions of some endogenous receptors for sialic acids in the immune system may be altered by this difference. Third, despite the lack of any obvious alternate pathway for synthesis, Neu5Gc has been reported in human tumors and possibly in human fetal tissues, and traces have even been detected in normal human tissues. One possible explanation is that this represents accumulation of Neu5Gc from dietary sources of animal origin. Finally, a markedly reduced expression of hydroxylase in the brains of other mammals raises the possibility that the human-specific mutation of this enzyme could have played a role in human brain evolution. Yrbk Phys Anthropol 44:54–69, 2001.



## GLOSSARY

CMP-Neu5Ac: Cytidine-monophosphate-Neu5Ac, the high-energy sugar nucleotide donor for enzymes that add Neu5Ac to glycosylated molecules. Also, the natural substrate for conversion to CMP-Neu5Gc.

Deuterostomes: coelomate animals which have radial instead of spiral cleavage at the eight-cell stage and forms the anus from the blastopore instead of the mouth (includes vertebrates and relatives, as well as certain higher invertebrates, such as sea urchins and starfish).

Glycosylation: attachment of sugar chains to other molecules like lipids and proteins, giving glycolipids and glycoproteins.

N-acetylneuraminic acid (Neu5Ac): the most common form of sialic acid in most mammals.

N-glycolylneuraminic acid (Neu5Gc): another common form of mammalian sialic acid, which is selectively missing in humans.

Pseudogene: an inactive but stable component of the genome derived from an ancestral active gene.

Sialic acids: a family of 9-carbon acidic sugars found attached to many glycosylated cell surface and secreted molecules.

Sialyltransferases: enzymes that use CMP-Neu5Ac or CMP-Neu5Gc as donors to add Neu5Ac or Neu5Gc to glycolipids and glycoproteins.

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The evolution of modern humans from a common ancestor with the great apes occurred in a series of steps, influenced by complex interactions among genetic, developmental, ecological, microbial, climatic, behavioral, cultural, social, and other factors. There are many scientifically valid approaches towards gaining an understanding of how we came to be human. Prominent among these approaches are: developmental, cognitive, social, and behavioral comparisons of humans with other primates; the paleontology and archeology of human ancestors; and the systematic genetic comparison of humans with other primates. Given the remarkable progress of molecular biology techniques, the deciphering of most of the human genome (Lander et al., 2001; Venter et al., 2001), and the relative ease of obtaining genomic DNA noninvasively from humans and other primates, the genetic approach should, in principle, be somewhat easier than the others mentioned. In fact, apart from the general realization that our genomic DNA sequences are remarkably similar to those of the great apes (King and Wilson, 1975; Sibley and Ahlquist, 1987; Caccone and Powell, 1989; Goodman et al., 1994; Ruvolo, 1997; Takahata and Satta, 1997; Kaessmann et al., 2001; Chen and Li, 2001), there have been relatively few specific genetic differences between humans and apes uncovered to date (reviewed in Gagneux and Varki, 2001). This has led some to call for a great ape/primate genome project (McConkey and Goodman, 1997; McConkey et al., 2000; McConkey and Varki, 2000), to accelerate progress in this area.

While morphologists have long sought to demonstrate the adaptive significance of divergent anatomical structures, this has generally not been the case for molecular anthropologists. The latter have, to a large degree, used comparative DNA (or amino acid) sequences for dating evolutionary events rather than for understanding the evolutionary processes that led to the sequence divergence. Thus, very little is known of the selective (or random) forces that led to the 1–2% sequence differences between humans and the African great apes, or about the adaptive molecular differences that emerged during this period of primate evolution.

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This review discusses one of the few known ape-human genetic differences with a clear-cut biochemical consequence, the selective inactivation of the CMP-N-acetylneuraminic acid (CMP-Neu5Ac) hydroxylase gene in the human lineage (Muchmore et al., 1998; Irie et al., 1998; Chou et al., 1998). The resultant loss of a specific cell-surface sugar on human cells has implications for issues as diverse as human susceptibility and resistance to pathogens, the consequences of human ingestion of animals foods, the human innate immune response, and the development of the human brain. As with most unexpected discoveries, this finding has raised more questions than answers. An attempt is made to address some of these questions, and to suggest directions for future research.

## GLYCOSYLATION OF CELL SURFACES

Every high school graduate should now know about DNA, RNA, and proteins, and most would understand that a universal DNA code defines the genes of all living things. These genes can be transcribed into their corresponding RNA sequences, which in turn are translated into proteins. This "central paradigm" of molecular biology, i.e., that DNA makes RNA makes protein, has dominated recent approaches to understanding how living things work. However, there are several reasons why this scientifically powerful reductionist approach cannot fully explain the structure and function of complex multicellular organisms like humans. One reason is that DNA itself is of little use unless the genes it encodes are expressed (transcribed to RNA, and hence translated into proteins)—and gene expression is profoundly influenced by the physical, ecological, biological, and social milieu in which an organism exists. Indeed, the social and cultural activities of humans must have a major impact on gene expression within the species, as well as in the many other species that humans interact with. A second reason is that besides DNA, RNA, and proteins, there are two other major classes of molecules that are required to create almost all life forms that we know of: lipids and sugars (glycans). Glycans fall into two general categories: the more familiar small sugars like glucose that are a major source of energy, and the less well-known glycan chains that are attached to many proteins and lipids, particularly on the surface of cells (Varki, 1999a). Indeed, there are no extant free-

living organisms whose cells are not each covered with a dense coating of these glycan chains, comprising a so-called "glycocalyx" that is no less obvious than the icing on a birthday cake. These complex cell-surface sugar chains are the products of a specialized intracellular machinery whose synthesis and organization are themselves dictated by the expression of several hundred genes (Varki and Marth, 1995; Varki, 1998). This machinery is supplemented to an unknown extent by the incorporation of sugar building blocks from dietary sources into endogenous biosynthetic pathways (Freeze, 1999). Given their ubiquitous occurrence in nature and their dominant presence on the surface of cells, it is not surprising that these sugar chains are intimately involved in the interactions between cells, both within an organism, and between organisms. A third limitation of the DNA-RNA-protein paradigm for explaining humans is that the human genome seems to have less than 35,000 genes (Lander et al., 2001; Venter et al., 2001). Thus, there cannot possibly be a "gene for" each specific biological entity or function. Rather, the genetic contribution to the structure and function of most aspects of an organism arise from the combinatorial effects of multiple genes, gene regulation mechanisms, and gene products that act upon one another in various ways, under the constant influence of environmental factors. The attachment of glycan chains to proteins and lipids (so-called glycosylation) is a prime example of such a "postgenomic" process, wherein new biological entities or functions result from the action of one set of genes on the products of other genes.

Despite these considerations, studies of the structure and biology of glycans have lagged far behind those of DNA, RNA, proteins, and lipids. There are many reasons for this, including the branching and complexity of these sugar chains, the lack of an easily recognizable template-driven functional "code," and technical limitations in studying their structure and function. Recent advances have substantially reduced this technical gap, opening up a new field now called glycobiology (Rademacher et al., 1988; Varki, 1999a). Thus, as with genomics (determining the total genomic DNA of an organism), transcriptomics (elucidation of the complete set of genes expressed in a given cell type in a given situation), and proteomics (the description of all proteins found in a given cell type in a given situation), we are just beginning to enter the era of glycomics (elucidation of the complete array of glycans found on a given cell type in a given situation).

## THE BIOCHEMISTRY OF SIALIC ACIDS

A microbial organism approaching a mammalian cell surface would likely first encounter members of a family of sugars called sialic acids, which tend to be the outermost units on the glycan chains attached to the proteins and lipids below (Fig. 1). This family of 9-carbon acidic sugars (Gottschalk, 1960; Rosenberg and Schengrund, 1976; Schauer, 1982;

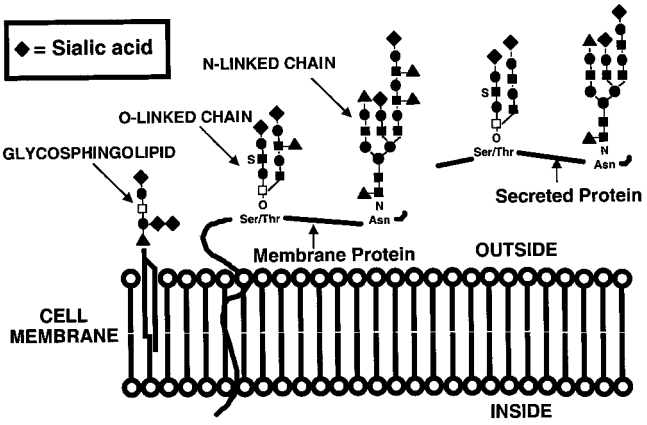


**Fig. 1.** Sialic acids on the surfaces of vertebrate cells. Sialic acids (shown as solid diamonds) are typically found at the outermost end of sugar chains attached to cell surface and secreted glycoproteins and glycolipids. See text for discussion.

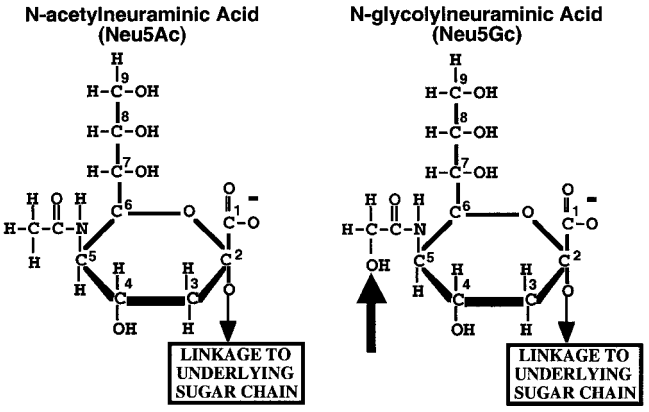


**Fig. 2.** Structure of the two most common sialic acids on mammalian cell surfaces. The 9-carbon backbone common to all sialic acids is numbered. Thick arrow points to the single oxygen atom that differentiates Neu5Gc from Neu5Ac. Humans are genetically defective in the gene encoding the enzyme responsible for adding the oxygen atom.

Ye et al., 1994; Inoue et al., 1996; Varki, 1992, 1999b) is found predominantly in the deuterostome lineage of animals (Warren, 1963; Traving and Schauer, 1998; Angata and Varki, 2001). There are more than 40 kinds of sialic acids known in nature, and most are derived via biosynthetic modifications of a parent molecule called N-acetylneuraminic acid (Neu5Ac) (Fig. 2). Further complexity arises from the fact that sialic acids can be attached to the underlying sugar chain in several different types of linkages (Tsuji et al., 1996). While many of these kinds and linkages of sialic acids can be found within a single species, there are also marked species-specific differences with regard to their relative amounts and/or distribution. Likewise, even within a single species, there can be substantial differences between different cell types in the pattern and composition of their sialic acids. The evolution of this remarkable structural complexity is probably related to the diverse biological roles of these sialic acids in different cell types.

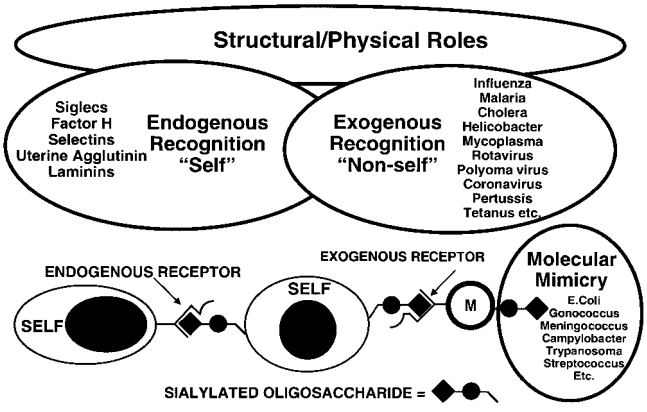


**Fig. 3.** Biological roles of sialic acids (Varki, 1999b; Traving and Schauer, 1998) can be divided into the general groupings indicated. M, microorganism or toxin. See text for discussion.

By virtue of their negative charge and surface location, sialic acids can mediate many biological roles in normal and pathological situations (Fig. 3). It is well-known that a wide variety of human and animal pathogens use cell-surface sialic acids to either gain an initial foothold on the cells they infect or to target toxic molecules that they secrete (Sandvig et al., 1991; Escalante et al., 1995; Sharon, 1996; Varki, 1997, 1999b; Gagneux and Varki, 1999; Karlsson, 1998, 2000; Fig. 3 for examples). If these were the sole functions of sialic acids, the detrimental consequences to deuterostome animals should have caused these sugars to be eliminated and/or replaced by others during the course of evolution. However, the absence of sialic acids is lethal during early mouse embryogenesis (W. Reutter, personal communication), and genetic modifications of sialic acid linkages in mice cause significant pathologies (Priatel et al., 2000; Hennet et al., 1998), indicating that these molecules also have critical endogenous functions. Some of these functions are primarily structural or physical, e.g., aiding the filtration function of the kidneys or providing negative charge repulsion between cells in the blood stream. In the brain, this type of negative charge repulsion becomes enhanced and specialized by the formation of long chains of sialic acids (called polysialic acids). These can serve to physically separate neurons and neuronal extensions and hence participate in what can be loosely called "brain plasticity" at the organizational level (Rutishauser and Landmesser, 1996). Over the last two decades, it has become clear that sialic acids are also recognized by specific receptors within the same animals that synthesize these sugars (Bevilacqua and Nelson, 1993; Kelm et al., 1994b; Varki, 1994, 1997; Powell and Varki, 1995; Crocker and Feizi, 1996; Kansas, 1996; Collins et al., 1997a; Kelm and Schauer, 1997; Crocker et al., 1998; Brinkman-Van der Linden et al., 2000; Crocker and Varki, 2001a,b; see Fig. 3 for examples). Emerging evidence indicates that these receptors, particularly a family called the Siglecs (Crocker and Varki, 2001a,b; Angata et al., 2001), are able to recognize the diversity in sialic acids, and their linkages to underlying sugars. An additional set of biological roles for sialic acids is found in certain microbial organisms that decorate their cell surfaces with sialic acids (Troy, 1992; Wessels et al., 1989; Bozue et al., 1999), thereby allowing them to evade recognition and destruction by certain vertebrate immune mechanisms. These examples occur despite the fact that sialic acids seem to be otherwise restricted to the deuterostome lineage of animals. The best explanation can be found in the fact that all of these microorganisms are vertebrate pathogens (see examples in Fig. 3). Since surface sialic acids limit activation of multiple functions of the immune system, these pathogenic organisms likely benefit from coating themselves with these sugars. This form of molecular mimicry may have occurred mostly via convergent evolution, wherein these microorganisms evolved a variety of ways either to make their own sialic acids or to procure them from their vertebrate hosts.

While there are many kinds of sialic acids in nature (Gottschalk, 1960; Rosenberg and Schengrund, 1976; Schauer, 1982; Varki, 1992; Ye et al., 1994; Inoue et al., 1996; Varki, 1999b; Angata and Varki, 2001), the surfaces of most cell types in the mammalian species studied to date tend to be dominated by two major kinds: N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc). Neu5Gc is different from Neu5Ac only by an additional oxygen atom (see arrow in Fig. 2). In order for sialic acids to get attached to glycoproteins and glycolipids, they must first be "activated" by conversion to their respective sugar nucleotide derivatives. Thus, the common sialic acid Neu5Ac is converted to cytidine-monophosphate-Neu5Ac (CMP-Neu5Ac), which is then used as a high-energy donor for attaching Neu5Ac to newly made glycoproteins and glycolipids that are on their way to the cell surface. The synthesis of the Neu5Gc form of sialic acid takes place initially at the level of this sugar nucleotide precursor (Shaw and Schauer, 1988; Bouhours and Bouhours, 1989; Muchmore et al., 1989; Kozutsumi et al., 1990; Shaw et al., 1992, 1994; Takematsu et al., 1994; Kawano et al., 1995; Schlenzka et al., 1996). Thus, as shown in Figure 4, an enzyme called CMP-Neu5Ac hydroxylase (hereafter referred to as CMAH) catalyzes the transfer of one oxygen atom to CMP-Neu5Ac, generating CMP-Neu5Gc. The latter can now also be used as a donor to add Neu5Gc to molecules destined for the cell surface from their sites of initial synthesis within the cell. The enzymes that actually transfer sialic acids to glycoproteins and glycolipids (called sialyltransferases) can typically use both CMP-Neu5Ac and CMP-Neu5Gc as donors (Higa and Paulson, 1985). Thus, the ratio of these two major sialic acids found on a given cell surface is likely to be largely determined by the ratio within the sugar nucleotide donor pool available in that cell type.

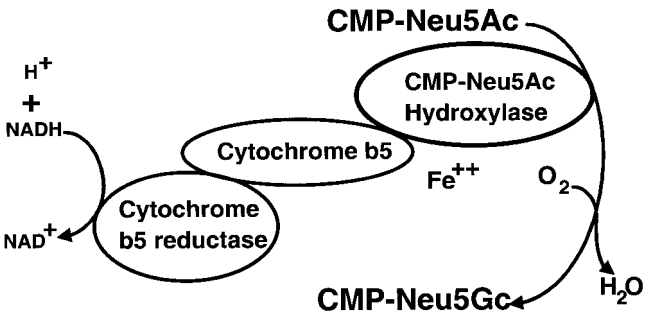


**Fig. 4.** Factors involved in biosynthesis of CMP-N-glycolylneuraminic acid (CMP-Neu5Gc). The enzymatic mechanism of the CMP-Neu5Ac hydroxylase that is primarily responsible for generating Neu5Gc in nonhuman animal cells is shown. The reaction takes place in the cytosolic compartment. The enzyme product CMP-Neu5Gc is the donor subsequently used for adding Neu5Gc to glycoproteins and glycolipids. CMP-Neu5Ac hydroxylase activity is present in great apes but not in humans. See text for discussion.

## HUMAN-SPECIFIC LOSS OF Neu5Gc EXPRESSION

### Modern comparative evidence

Some of the early pioneers who discovered the sialic acids noted that, in contrast to the situation in other mammals such as rodents and ungulates, Neu5Gc was very hard to find in human tissues (reviewed in Gottschalk, 1960; Rosenberg and Schengrund, 1976; Schauer, 1982). However, the methods they used could have missed small amounts of Neu5Gc in humans. An independent line of evidence suggesting that Neu5Gc is lacking in humans came from the medical field of hematology. For the clinical management of certain blood disorders, it becomes necessary to infuse serum from horses into human patients. Not surprisingly, these patients often generate an immune response against components of the infused animal serum and manifest a condition called "serum sickness reaction," which contraindicates further infusions. Some investigators who studied the serum sickness reaction noted that the immune response was being generated at least in part against Neu5Gc, which is present in abundance on horse serum glycoproteins and glycolipids (Kasukawa et al., 1976; Merrick et al., 1978; Higashi et al., 1977). This finding strengthened the notion that Neu5Gc is a foreign antigen to adult humans. However, several groups then reported (mostly using indirect methods such as antibody detection) that Neu5Gc could be found in human cancers and possibly in human fetal tissues (Kawachi and Saida, 1992; Ikuta et al., 1982; Higashi et al., 1984; Stacker et al., 1985; Hirabayashi et al., 1987a; Kawachi et al., 1988; Saida et al., 1990; Devine et al., 1991; Kawai et al., 1991; Marquina et al., 1996; Malykh and Schauer, 2001). Moreover, Neu5Gc was also reported in some cultured cell lines of human origin (Nakarai et al., 1987; Ohashi et al., 1983). Taken together, these data suggested that humans might have a functional CMAH gene, but simply suppress its expression sometime before the postnatal period when "immune tolerization" to self-antigens occurs.

Our recent studies done with more sensitive modern techniques (Muchmore et al., 1998) showed that while Neu5Gc is undetectable (<0.1% of total sialic acids) on the red blood cells and blood plasma proteins of adult humans, similar samples from all of the great apes have substantial amounts (Neu5Gc representing between ~20–90% of total sialic acids). Since the great apes are our closest evolutionary cousins (King and Wilson, 1975; Sibley and Ahlquist, 1987; Caccone and Powell, 1989; Goodman et al., 1994; Ruvolo, 1997; Takahata and Satta, 1997), the human loss of Neu5Gc expression must have occurred sometime after the ape-human common ancestor. A secondary consequence of this loss is that humans also have much higher levels of Neu5Ac, the precursor molecule to Neu5Gc.

Assays of CMAH enzyme (Fig. 4) showed easily detectable activity in great ape cells, but not in human cells (Muchmore et al., 1998). The next logical step was to examine the gene encoding the CMAH, to see if its promoter (regulatory) regions were mutated in some manner in humans, thereby altering its expression in adult humans. Indeed, two groups independently found that the CMAH gene in humans had suffered a mutation (Irie et al., 1998; Chou et al., 1998). Surprisingly the mutation was not in the regulatory regions of the gene that might have modified its expression patterns between fetal and adult states, but rather in the coding region which dictates the amino-acid sequence of the enzyme itself (Fig. 5). The mutational event had deleted 92 base pairs of a single stretch of the sequences coding for the protein (corresponding to exon 6 in the mouse gene). Since amino acids are dictated by triplets of DNA base pairs, the loss of 92 base pairs (bp) also resulted in a "frame-shift," thus markedly truncating the length of the final protein encoded by the human gene (Chou et al., 1998). Moreover, this truncated protein is missing certain amino acids that were known to be critical for the activity of the enzyme itself (Schlenzka et al., 1996). In contrast, examination of the CMAH gene from the chimpanzee and relevant portions from the corresponding genes from other apes (Chou et al., 1998) showed that they all encode an intact enzyme that is not very different from those originally cloned from mice and pigs (Kawano et al., 1995; Schlenzka et al., 1996). Thus, it is clear that humans lost Neu5Gc on their cell surfaces because of an inactivating mutation in the CMAH gene. The gene is localized to chromosome 6 band p23–p22 in both humans and great apes (Irie et al., 1998; Chou et al., 1998), which does not correspond to an area of known chromosomal rearrangement during hominoid evolution (Yunis and Prakash, 1982). Furthermore, the remaining human intronic region is very similar in size to that in the intact mouse genome (Irie et al., 1998), indicating that the deletion eliminating the

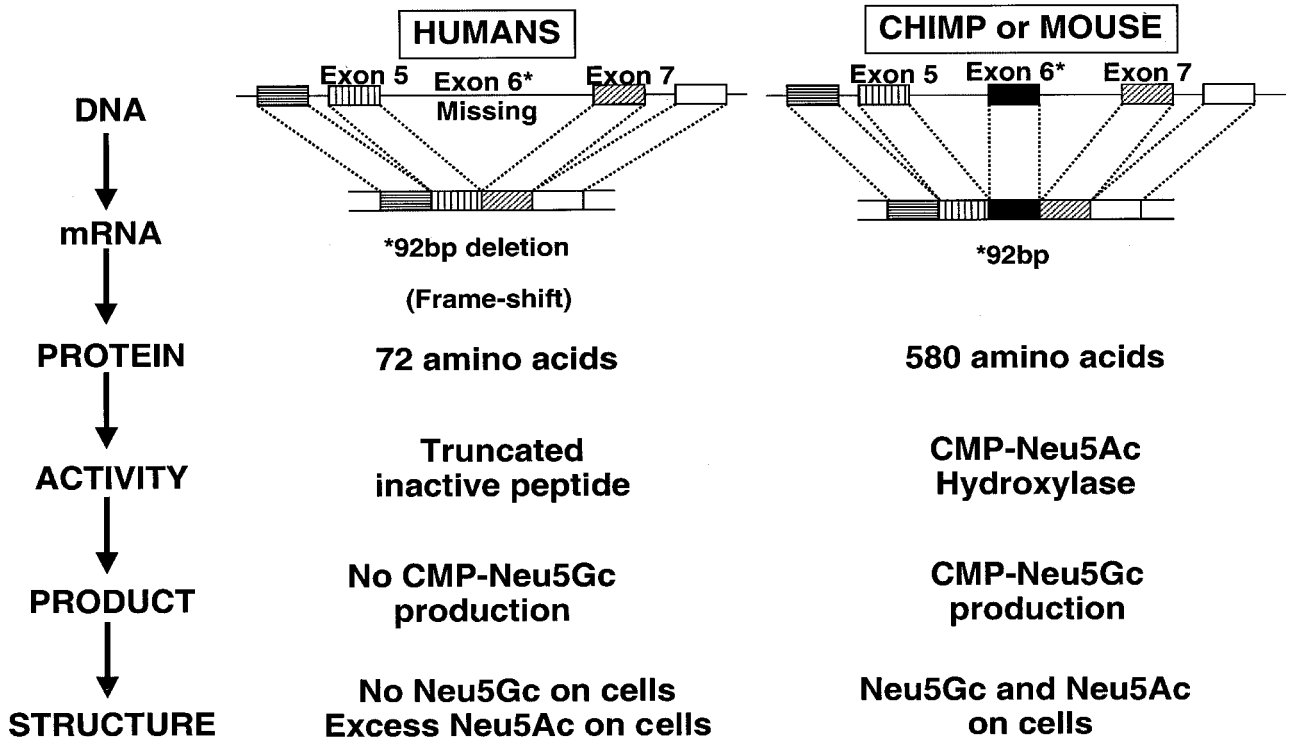


**Fig. 5.** Human-specific genomic mutation in CMP-Neu5Ac hydroxylase. The CMAH gene contains 18 exons that span more than 100 Kb of genomic DNA in humans, mice, and apes. A schematic version of a portion of the gene is shown, with boxes indicating amino acid-coding exons. Only the region mutated in humans is shown, and the downstream consequences of the mutation at each level are indicated. Only the spliced form of the messenger RNA is shown, again indicating only the region of the mutation. See text for further discussion.

92-bp exon in humans must have been quite small. The corresponding genomic regions from the chimpanzee and other nonhuman primates were recently sequenced (Hayakawa et al., 2001). The region containing a 92-bp exon of the CMAH gene turns out to have an adjacent *AluSq* element in all nonhuman primates studied, and both were replaced by a newly disseminated *AluY* element that is specific to humans (*Alu*s are parasitic repetitive DNA elements found in large numbers throughout the primate genome). Thus, it is possible to propose a mechanistic model whereby an *Alu*-mediated replacement event coincidentally deleted the 92-bp exon and inactivated the CMAH gene sometime in the human lineage (Hayakawa et al., 2001).

### Evolution and the loss of Neu5Gc production

Why did humans lose Neu5Gc production? It is difficult to be certain about why a particular genetic change became fixed in a particular species at some time in the evolutionary past. However, based on current knowledge of the functions of sialic acids (see above), one can propose some possible scenarios to explain the human loss of Neu5Gc. The most likely one is selection of a randomly occurring CMAH gene mutation by a lethal microbial pathogen that required cell-surface Neu5Gc for effective infection (see below for some examples of such current-day pathogens). Once such a mutation had thus become common in a human ancestral population, it could have undergone further selection because it conferred some additional benefits, or have simply drifted to fixation in the absence of any further selection. A more intriguing possibility is that the inactivated allele of the CMAH gene was favorably selected because it conferred valuable new endogenous functions upon individuals who became homozygous for it.

When did this mutation occur in relation to the various steps in human evolution? The identical mutation was found in genomic DNA in >40 individuals representing many different geographic regions, including African Kung bushmen and Khwe pigmies (among whom the greatest genetic diversity is known to be present; Takahata and Satta, 1997; Kaessmann et al., 2001). This, together with the lack of detectable Neu5Gc in blood samples from many additional humans studied by us, indicates that the mutation is universal to modern humans. Thus, the inactivation of the CMAH gene must have occurred sometime after our common ancestor with the bonobo/chimpanzee clade (~6 million years ago; King and Wilson, 1975; Sibley and Ahlquist, 1987; Caccone and Powell, 1989; Goodman et al., 1994; Ruvolo, 1997; Takahata and Satta, 1997), but prior to (or possibly coincident with) the common origin of modern humans (variably estimated by different authors, with the most recent possible date of about

~100,000 years ago; Takahata and Satta, 1997; Krings et al., 1997).

A more accurate estimate of the timing of the gene inactivation would be of value for generating hypotheses about the potential consequences of Neu5Gc loss in humans. For example, if it took place about 2 million years ago, this might suggest a possible contribution towards the brain size increase that began with the emergence of genus *Homo* (Wood and Collard, 1999). Sequenceable autosomal DNA has not been successfully recovered from fossilized mammalian bones that are more than about 50,000 years old (Greenwood et al., 1999; Hofreiter et al., 2001). Even the few successes tend to occur when using samples from northern latitudes. Indeed, the probability of recovering DNA is known to be worse for samples exposed to warmer temperatures (Smith et al., 2001). The occasional successes in obtaining mitochondrial DNA from fossil hominids >50,000 years old (Krings et al., 1997) are explained by the much higher copy number of mitochondrial DNA within each cell. Overall, it is not possible (at least with current technology), to obtain and directly study CMAH gene sequences from hominid fossils that predated the common origin of modern humans.

Two other approaches are therefore currently being pursued to try timing the inactivation of the CMAH gene. The first relies on sequence comparisons of the great ape and other primate CMAH genes with those of the remaining portions of the inactivated human gene. The assumption is that once the human gene became nonfunctional (i.e., became a pseudogene), it was no longer under selection pressure, and would thus accumulate both synonymous and nonsynonymous mutations at similar rates. Such data are currently being used to estimate an approximate inactivation date (collaboration with N. Takahata). The second approach relies on the direct study of residual sialic acids found in fossils (unpublished observations, in collaboration with S. Paabo). These preliminary analyses suggest a dating of a little over 2 million years ago.

It should be noted that there are reported strain variations in red blood cell expression of Neu5Gc among dogs (Hashimoto et al., 1984; Yasue et al., 1978) and cats (Ando and Yamakawa, 1982; Furukawa et al., 1988a), and in the latter instance, the presence or absence of Neu5Gc on red cells can act as a blood group system (Andrews et al., 1992). However, systematic studies of other tissues of these dog and cat strains have not been reported. Thus, we do not know if it is only red blood cells that show differential expression. Chickens are the only species besides humans that have been found to generate a generalized immune response to Neu5Gc when infused with animal serum (Fujii et al., 1982). In fact, polyclonal and monoclonal antibodies generated by chickens immunized with horse serum or horse red cell glycolipids have been very useful as tools in the study of Neu5Gc expression (Hirabayashi et al., 1987b; Higashi et al., 1988). Again, a systematic study of chicken tissues has not been carried out to see if the presumed lack of Neu5Gc is true for all organs. Furthermore, the presence of Neu5Gc in ducks (Ito et al., 2000) indicates that this is not a general feature of birds. Further studies of the CMAH gene in a wide selection of birds and carnivores seem warranted. However, a recent study showed that Neu5Gc is present on the serum immunoglobulins of cows, sheep, goats, horses, mice, dogs, guinea pigs, rats, and rabbits, as well as rhesus monkeys (Raju et al., 2000). Thus, even if genetic variations in Neu5Gc expression are found among members of some of these species, the 92-bp exon deletion responsible for the human loss of CMAH activity remains a unique genetic event that occurred after our common ancestor with the great apes, and is now universal among modern humans.

## FUNCTIONAL CONSEQUENCES OF Neu5Gc LOSS IN HUMANS

### Infection and disease

As indicated above, many major pathogens and their toxins gain access to their mammalian hosts by binding to cell-surface sialic acids. The microbial binding proteins involved in such interactions can show exquisite specificity for the precise structure and linkage of the sialic-acid target (Sharon, 1996; Karlsson, 1998; Varki, 1997, 1999b). Thus, the human loss of Neu5Gc would have conferred protection from animal pathogens that prefer to bind to this sialic acid, while enhancing the success of pathogens that prefer to bind to Neu5Ac. As shown in Table 1, this issue has not been thoroughly studied for most human pathogens. It is clear that certain microbes causing serious diarrheal diseases in farm animals like cows and pigs have a strong preference for Neu5Gc (Kyogashima et al., 1989; Ouadia et al., 1992; Willemsen and de Graaf, 1993; Lanne et al., 1995; Delorme et al., 2001; Schwegmann et al., 2001), and humans are thus immune to infection. It is reasonable to speculate that this mechanism of resistance may have facilitated the domestication of some animal species, by limiting transfer of their pathogens to human caretakers. An even more speculative possibility is that this difference aided the worldwide migrations that brought humans into contact with diverse pathogen regimes of novel species of wild animals they encountered. In this regard, more information is needed about the sialic acid binding specificity of pathogens affecting animals that now live in very close contact with humans, such as dogs and cats. Perhaps they will turn out to prefer Neu5Gc, explaining the relatively low rate of pathogen transfer from these domesticated pets to humans. Another intriguing issue is that of the influenza A virus, the agent of epidemic and pandemic influenza in humans (Wilson et al., 1981; Ito and Kawaoka, 2000; Taubenberger et al., 2000). It appears that these viruses originate from wild

*TABLE 1. Examples of effects of Neu5Gc vs. Neu5Ac on recognition of mammalian cells by microbial pathogens and toxins*

| Sialic acid-binding pathogen | Influence of Neu5Gc on recognition |
|---|---|
| *E. coli* K99 adhesin (diarrhea) | Required for binding (humans resistant) |
| Transmissible gastroenteritis *Coronavirus* (diarrhea) | Preferred for binding (humans resistant) |
| *Rotavirus* (diarrhea) | Human virus prefers Ac, bovine virus prefers Gc |
| *Influenza* A and B virus (influenza) | Depends upon strain; Gc sometimes preferred in nonhuman influenza viruses |
| *P. falciparum* merozoite (malaria) | Neu5Gc not studied (chimps not susceptible?) |
| *Vibrio cholerae* toxin (cholera) | Neu5Gc not studied in detail |
| *Streptococcus sanguis* (dental caries) | Neu5Gc not studied |
| *Helicobacter pylori* (ulcers) | Neu5Gc not studied |
| *Tetanus* toxin (tetanus) | Neu5Gc not studied |
| Coronaviruses (common cold) | Neu5Gc not studied |
| *Mycoplasma pneumoniae* (pneumonia) | Neu5Gc not studied |
| Polyoma virus (tumors) | Neu5Gc not studied |

water fowl and make their way to humans via domesticated livestock animals such as pigs (Suzuki et al., 1997; Ito and Kawaoka, 2000). Studies indicate that some animal forms of influenza A virus preferentially bind to Neu5Gc, while human forms can have some preference for Neu5Ac (Higa et al., 1985; Weis et al., 1988; Ito et al., 1997, 2000; Suzuki et al., 1986, 1997). Thus, a switch in specificity from Neu5Gc to Neu5Ac might be a required or facilitatory step in animal-to-human transmission of some influenza strains.

On the other hand, if there are microbes that selectively prefer Neu5Ac, these should be more pathogenic in humans. While no clear-cut examples of this situation are known, most of the possibilities have yet to be explored. One potential example is *Plasmodium falciparum*, the causative agent of the most serious form of human malaria that afflicts millions worldwide. The merozoite form of this organism that invades red blood cells uses cell-surface Neu5Ac as one of its targets for initial binding (Klotz et al., 1992; DeLuca et al., 1996; Reed et al., 2000). Since chimpanzees appear to be resistant to this form of malaria (Ollomo et al., 1997), it is possible that the *P. falciparum* merozoite receptor proteins do not recognize Neu5Gc. Conversely, the merozoite stage of the phylogenetically related *Plasmodium reichenowi* that infects chimpanzees (Qari et al., 1996; Escalante and Ayala, 1994) might recognize Neu5Gc preferentially. Functional comparison of the *P. falciparum* and *P. reichenowi* merozoite receptors seems to be worthwhile. Overall, it is clear from Table 1 that much further work is needed to pursue the consequences of Neu5Gc loss for resistance and susceptibility to infectious diseases in humans.

The antibodies generated by a normal adult human exposed to infusion of horse serum (called Hanganatziu-Diecher or HD antibodies) can agglutinate the red blood cells of various animals, such as horses, pigs, and cows, by virtue of the fact that they all carry surface Neu5Gc. Using the same kind of red-cell agglutination assay, spontaneously occurring HD antibodies have also been reported in patients who have never had exposure to animal serum infusion. While such serum reactivities are rare in normal human adults, they are found in a significant proportion of patients with cancer, as well as in diseases such as leprosy, rheumatoid arthritis, liver disease, and infectious mononucleosis (Morito et al., 1982, 1986; Nishimaki et al., 1979; Takiguchi et al., 1984). At least two explanations can be considered for these phenomena. The first possibility is that as in cancer, some of these diseases involve proliferation of vascularized tissues (e.g., the granulomas of leprosy and rheumatoid arthritis). Thus, a low-grade immune response might be occurring against Neu5Gc that is being gradually incorporated into such tissues from dietary sources over time (see discussion below). The second possibility is that these antibodies emerge as a nonspecific consequence of the disregulation of the immune system that occurs in some of these disease states. Individuals with prior exposure to Neu5Gc in the diet may have rare preexisting memory B cells capable of producing antibodies directed against Neu5Gc. Such B cells could undergo nonspecific expansion under conditions of generalized immune disregulation. The first possibility could be tested by directly assaying the affected tissues for the presence of Neu5Gc.

Despite the complete inactivation of the CMAH gene in humans, there have been reports of traces of Neu5Gc in normal human tissues (Muchmore et al., 1998) and in cultured human cell lines (Nakarai et al., 1987; Ohashi et al., 1983). The latter finding is easily explained by the fact that human cells are typically cultured in fetal bovine serum, which is rich in glycoproteins carrying Neu5Gc. The fluid-phase uptake of such glycoproteins into the lysosomes of cultured cells would eventually result in incorporation of the foreign Neu5Gc into human cellular glycoproteins and glycolipids (see pathway A in Fig. 6). The best evidence for this route of incorporation is that growing the cells in the absence of animal serum results in the eventual disappearance of Neu5Gc (Furukawa et al., 1988b; Muchmore et al., 1998). However, this pathway cannot explain the traces of Neu5Gc found in tissues from normal humans who are not directly exposed to animal serum (Muchmore et al., 1998). On the other hand, the nature of the CMAH mutation in humans (an exon deletion eliminating critical amino-acid residues)

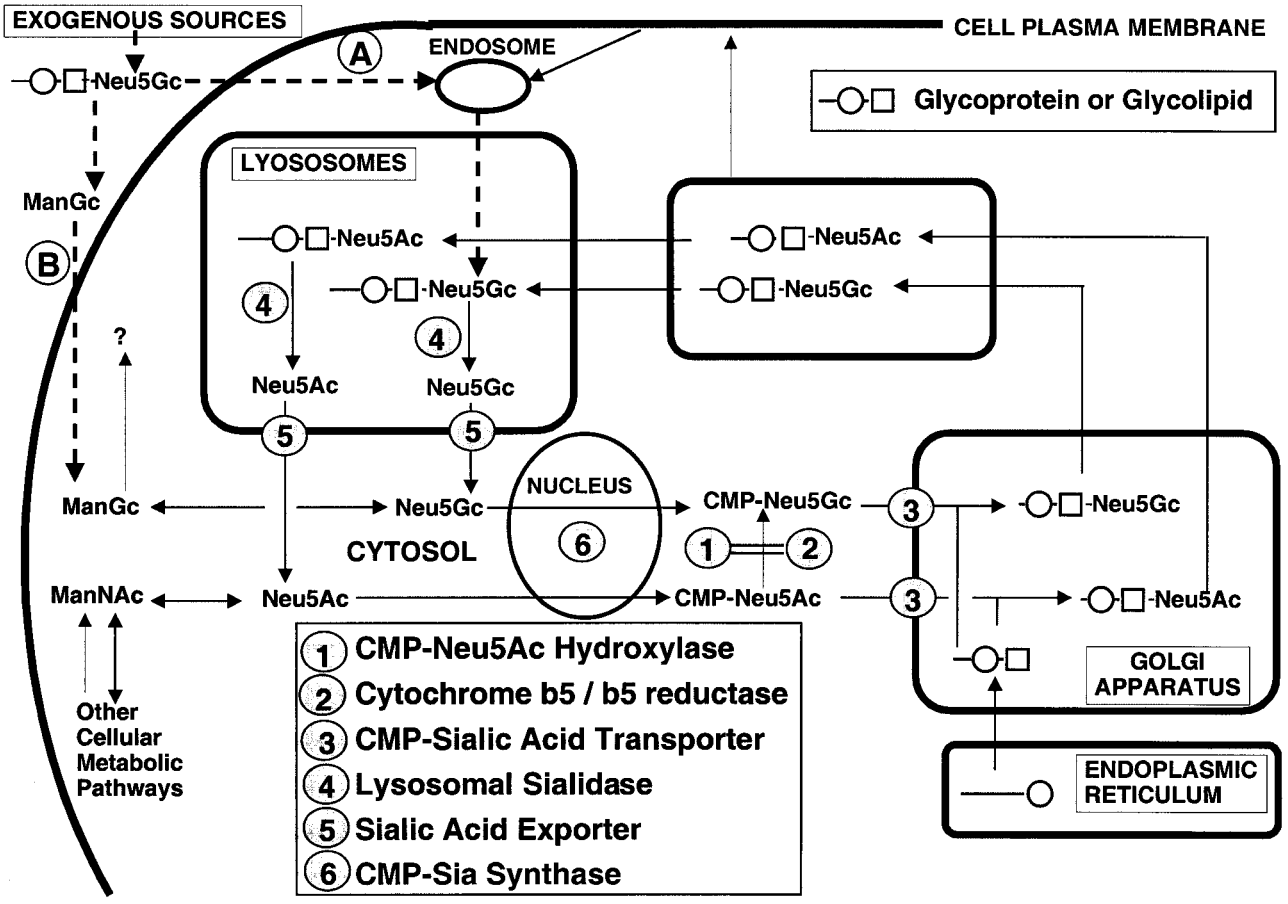


**Fig. 6.** Uptake, metabolism, and turnover of Neu5Ac and Neu5Gc in mammalian cells. The general pathways for production and utilization of sialic acids in mammalian cells are shown. Thick lines represent cellular membranes, or intracellular membranous compartments, as indicated. The common sialic acid Neu5Ac is synthesized from a neutral precursor N-acetylmannosamine (ManNAc) in the cytosolic compartment, activated into CMP-Neu5Ac in the nucleus, and eventually pumped into the Golgi apparatus by a specific transporter (Lepers et al., 1989). There it serves as a donor for adding sialic acids to newly synthesized glycolipids and glycoproteins that originate from the endoplasmic reticulum, and are en route to the cell surface or to be secreted from the cell. Sialic acids are eventually released in the lysosomal compartment as part of the overall degradation of glycolipids and glycoproteins and then pumped back into the cytosol (Verheijen et al., 1999), to be reutilized as shown. Conversion of CMP-Neu5Ac to CMP-Neu5Gc occurs in nonhuman cells, and the subsequent fate of Neu5Gc is similar to that of Neu5Ac-recycling and varying degrees of reutilization. Double line indicates block in conversion of CMP-Neu5Ac to CMP-Neu5Gc in human cells. Two possible pathways (A and B) for incorporation of Neu5Gc of exogenous into human cells are indicated with dotted lines. Pathway A involves uptake of glycolipids or glycoproteins carrying Neu5Gc into the lysosomal compartment. This pathway could only operate in cultured cell lines, or in humans given intravenous infusions of animal proteins. Pathway B involves uptake of neutral precursor N-glycolylmannosamine (ManNGc) by passive diffusion across the plasma membrane, with subsequent conversion into Neu5Gc. ManNGc could originate from breakdown of dietary Neu5Gc in the gut. Both these pathways would effectively bypass the enzymatic defect in humans. Note that Neu5Ac and Neu5Gc can also be degraded back to ManNAc and ManNGc. While there are many known interconverting pathways involving ManNAc, the cellular fate of ManNGc is unknown, in human and nonhuman cells. There is also no known pathway for converting Neu5Gc back to Neu5Ac. Hence Neu5Gc tends to recycle and accumulate to high levels in cells that express the hydroxylase (Muchmore et al., 1989). See text for further discussion.

makes it hard to postulate a genetic mechanism to restore its function in malignant cells. Indeed, the complete sequencing of the corresponding intronic regions of the CMAH gene from human and chimpanzee genomes (Irie et al., 1998; Hayakawa et al., 2001) reveals no obvious avenues for such a repair mechanism. Meanwhile, searches of gene databases show no evidence for any related proteins that might have a similar activity. Two other possibilities must therefore be considered to explain the traces of Neu5Gc found in normal human tissues. The first is that there could be an additional completely different biochemical pathway for the biosynthesis of Neu5Gc in mammalian cells. There is so far no strong evidence for such a pathway being active in humans. The suggestion has been made that the glycolyl group of Neu5Gc (which replaces the acetyl group in Neu5Ac) could also originate from an unusual high-energy donor called glycolyl-coenzyme A (CoA). Such a potential donor can in fact be formed via two known biochemical pathways: beta-oxidation of 4-hydroxybutyrate in mitochondria, or from 3-hydroxypyruvate by the action of pyruvate dehydrogenase (Vamecq and Poupaert, 1990; Vamecq et

*TABLE 2. Effects of Neu5Gc vs. Neu5Ac in recognition by intrinsic animal lectins*

| Animal lectin | Location | Influence of Neu5Gc on binding |
|---|---|---|
| Selectins | Blood cells<br>Endothelium | Not studied carefully; probably no effect |
| Complement factor H | Blood plasma | Not studied |
| Uterine agglutinin | Endometrium | Enhanced binding |
| Laminin | Extracellular matrix | Not studied |
| Sialoadhesin (Siglec-1) | Macrophages | Blocks binding |
| CD22 (Siglec-2) | B cells | Ac and Gc okay in humans (only Gc in mice) |
| CD33 (Siglec-3) | Myeloid cells | No effect |
| MAG (Siglec-4a) | Myelin | Blocks binding |
| Siglec-5 | Myeloid cells | Slight decrease in binding? |
| Siglec-6 | B cells, placenta | No effect? |
| Siglecs 7–10 | Blood cells | Under study |
| Siglec "11"[1] | Macrophages? | Under study |
| Siglec-L1 | Epithelium | Enhanced binding |

[1] Our unpublished observations.

al., 1992). The glycolyl-CoA would presumably donate its glycolyl group directed to a de-N-acetylated neuraminic acid (Zhou et al., 1994; Sjoberg et al., 1995; Mitsuoka et al., 1999), or to a potential precursor form of sialic acids called mannosamine. However, there is so far no evidence for the enzymatic activity of such a pathway in human cells. The other interesting possibility is the incorporation of Neu5Gc from dietary sources. Studies of rats fed with radiolabeled sialic acids showed that a large fraction can be absorbed, and while most is excreted unchanged in the urine, a small amount of the label does get incorporated into tissues (Nohle and Schauer, 1981, 1984; Nohle et al., 1982). The latter is thought to occur by intestinal conversion of the ingested sialic acids into the acylmannosamine derivative, which can be taken up into the circulation, absorbed into cells, and converted back into sialic acids (see pathway B in Fig. 5). We are currently studying the possibility that a similar pathway exists in intact humans. If so, it is possible that the traces of Neu5Gc found in normal human tissues are all derived from the ingestion of foods containing Neu5Gc. In this regard, it is of note that sialic acids are only found in animal foods, and that Neu5Gc seems to be very common in pigs, cows, goats, and sheep (Raju et al., 2000; Wang et al., 1990), while probably being much lower in poultry and fish. It remains to be seen whether this pathway has any relevance to diseases associated with the consumption of red meats. There also needs to be a detailed survey of the amounts of Neu5Gc present in common foods of animal origin. Regardless of the mechanism, the question also arises whether there is a weak immune response to these traces of Neu5Gc in adult humans, and if so, what the consequences of such a response might be.

As mentioned above, both direct and indirect studies have indicated that Neu5Gc is present in some human cancerous tissues and possibly in fetuses. These earlier reports used less sensitive methods that failed to detect the traces of Neu5Gc we subsequently noted in normal human tissues. Thus, it is likely that the levels of Neu5Gc present in cancers are simply higher. The possible explanations presented above for the traces of Neu5Gc in normal tissues can be extended to fetuses and cancers as well, with the added suggestion that these rapidly growing tissues might be more efficient at scavenging Neu5Gc (or its breakdown product N-glycolylmannosamine) from dietary sources. Of course, we still cannot rule out an alternative pathway for the synthesis of Neu5Gc that becomes specifically activated in tumors and fetuses. Regardless of the mechanism(s) involved, the question again arises as to whether the presence of a sugar that is typically immunogenic in humans can in some way affect the outcome of a pregnancy or a malignant disease. Perhaps it will be possible to take advantage of the presence of Neu5Gc on tumors to design some novel approach to the treatment or containment of cancer. Of course, all of these considerations do not apply to cancers in other mammals, since they naturally have large amounts of Neu5Gc to begin with. Thus, the animal model will have to be a mouse with a homozygous "knockout" of its CMAH gene. Such mice are currently being prepared.

### Intrinsic functions of sialic acids

It is of great interest to ask if the loss of Neu5Gc in humans resulted in some significant changes in the endogenous functions of sialic acids. Since Neu5Gc has a glycolyl group rather than an acetyl group, it is more hydrophilic, and this could result in changes in the physical or structural functions of sialic acids. However, this possibility and its potential consequences have yet to be investigated. As indicated above, the intrinsic receptors that bind sialic acids within animals have only recently been described, and there may be more to be discovered. Correspondingly, we also know relatively little about the impact of Neu5Gc loss on the functions of these intrinsic sialic-acid receptors in humans.

The limited information available concerning Neu5Gc and intrinsic sialic-acid receptors is summarized in Table 2. The best-studied examples to date are certain members of a family of proteins called the Siglecs (sialic acid-binding immunoglobu-

lin-like lectins; Sgroi et al., 1993; Crocker et al., 1994, 1998; Kelm et al., 1994a; Powell and Varki, 1995; Crocker and Feizi, 1996; Crocker and Varki, 2001a,b; Angata et al., 2001). Sialoadhesin (Siglec-1) is a large molecule found on tissue macrophage cells in various organs such as bone marrow, spleen, and lymph nodes. The sialic acid-recognizing function of sialoadhesin has been extensively studied, and it is clear that it binds well to Neu5Ac, but not to Neu5Gc (Brinkman-Van der Linden et al., 2000; Collins et al., 1997a; Kelm et al., 1994b). Thus, human cells (which have an excess of Neu5Ac) have an excess of binding sites for sialoadhesin (Brinkman-Van der Linden et al., 2000). Presumably as a secondary consequence, there is an obvious difference in the tissue distribution of sialoadhesin-positive spleen macrophages between humans and chimpanzees. Also, while only a subset of chimpanzee macrophages are sialoadhesin-positive, most human macrophages express this molecule (Brinkman-Van der Linden et al., 2000). Interestingly, in all the above respects chimpanzee spleens are more similar to those of rats than to humans. The biological significance of these facts will not be known until the functions of sialoadhesin itself are elucidated. Meanwhile, it is interesting to note that sialoadhesin-positive macrophages are found in large numbers in pathological tissue specimens from patients with diseases such as breast cancer (Nath et al., 1999) and rheumatoid arthritis (Hartnell et al., 2001), both of which appear to be rare conditions in the great apes (Varki, 2000). Another Siglec that strongly prefers Neu5Ac over Neu5Gc is myelin-associated glycoprotein (Siglec-4a), which is expressed on Schwann cells and oligodendrocytes, and appears to be involved in organizing and maintaining the myelin sheath that surrounds the axons in the white matter of the brain and the peripheral nerves (Kelm et al., 1994b, 1998; Collins et al., 1997b). Curiously, the brain has very low levels of Neu5Gc, even in nonhuman mammals (see below). Thus, it is presently difficult to make a hypothesis about how the loss of Neu5Gc expression in humans might have affected the function of myelin-associated glycoprotein. Initial evaluation suggests that Siglecs 2, 3, 5, and 6 are probably not affected by the human loss of Neu5Gc (Brinkman-Van der Linden et al., 2000; Collins et al., 1997a; Kelm et al., 1994b, 1998). Further studies are underway to evaluate of some of the newer Siglecs that were recently described.

Of particular interest is a recently discovered human Siglec-like molecule (Siglec-L1) that lacks a conserved amino acid (an arginine residue) which is known to be essential for optimal sialic-acid recognition by previously known Siglecs (Angata et al., 2001). We found that the loss of the arginine residue was caused by a single nucleotide substitution in human genomic DNA that occurred after the common ancestor of humans with the great apes but prior to the common origin of modern humans (Angata et al., 2001). The chimpanzee ortholog of Siglec-L1 has the arginine residue, remains fully functional in recognizing sialic acids, and turns out to preferentially recognize Neu5Gc over Neu5Ac. Reintroducing the ancestral arginine by "repairing" the cloned human molecule regenerated sialic acid binding, along with a similar preference for Neu5Gc (Angata et al., 2001). Thus, the single base-pair mutation that replaced the arginine residue on human Siglec-L1 seems likely to be evolutionarily related to the loss of Neu5Gc expression in the human lineage. However, we do not know which came first, and how exactly the two events are related. In the course of doing this work, we also examined the whole human genome for other Siglec-like genes. It turns out that the human genome contains many Siglec-like pseudogenes (currently inactive components of the genome derived from ancestral active genes). Interestingly, some of these pseudogenes have independent mutations that would have replaced the same conserved arginine residue that is required for optimal sialic acid recognition. Much further work needs to be done to know if any of these pseudogenes are derived from genes that are still active in other primates. Regardless, this work indicates that additional changes in the biology of sialic acids may have taken place during human evolution.

The consequence of Neu5Gc loss for most of the other known mammalian sialic acid-recognizing lectins is also not very clear (see Table 2), and further studies are needed. An intriguing case is that of a sialic acid-binding uterine agglutinin that was purified from the endometrium (the epithelial lining of the uterus) of both humans and rats, and that was shown to prefer Neu5Gc over Neu5Ac (Chatterji et al., 2000). It was also shown to recognize sialic acids on sperm. While the functions of this molecule are unknown, one can speculate that its preference for Neu5Gc might somehow be connected to anecdotal suggestions of differences between humans and great apes in matters such as menstrual blood loss and early fetal wastage (Varki, 2000).

### Implications for biotechnology and xenotransplantation

As mentioned above, a normal adult human directly exposed to Neu5Gc in the form of infused horse serum generates an immune response against this foreign sugar. Biotherapeutic molecules produced via recombinant DNA technology are typically produced in nonhuman cells, because human cells may allow transmission of human retroviruses and other infectious agents. As many of these products are glycoproteins, they can contain varying amounts of Neu5Gc originating from the nonhuman producer cells used. Fortunately, most of the animal cell lines commonly used to produce such agents (e.g., Chinese hamster ovary cells and baby hamster kidney cells) happen to produce very low levels of Neu5Gc. Furthermore, although erythropoietin (produced by Amgen) was later recognized to have very small

amounts of Neu5Gc (Hokke et al., 1995), microgram amounts of this therapeutic glycoprotein had already been injected repeatedly into many humans, without apparent evidence of an obvious immune reaction (Noguchi et al., 1996). It is possible that the immune system of most humans is partially tolerized to Neu5Gc because of prior exposure to dietary Neu5Gc. If so, the reaction might be dose-dependent, and there should still be concern over recently developed products from the milk of sheep and goats which have very high levels of Neu5Gc (Gagneux and Varki, unpublished findings). Another possible explanation for the lack of immune response to Neu5Gc on erythropoietin is that the primary antigens in horse serum are glycolipids (not glycoproteins) bearing Neu5Gc. Thus, there may be a lesser immune response to Neu5Gc on glycoprotein therapeutic agents. However, Neu5Gc injected in the form of glycoproteins could be directly taken up by human cells via pathway A of Figure 6, and eventually presented on the patient's own cell surfaces, attached to endogenously synthesized glycolipids. This could result in an immune response developing over time. This concern remains unresolved at the present time.

Similar considerations apply to the currently controversial attempts to transplant organs from other species into humans. All early attempts at such xenotransplantation of organs from other primates to humans failed for unknown reasons. These included attempts to transplant chimpanzee kidneys into humans (Deodhar, 1986; Reemtsma, 1989) which would nowadays be considered unethical. Rejections of the transplanted organs were typically delayed by several weeks, and the mechanism of rejection remained obscure. If posttransplant serum from such patients had been saved, one could have determined if at least part of the rejection response was directed against Neu5Gc. Regardless, it should be noted that the most popular model animal for xenotransplantation of organs into humans is currently the pig, which happens to be a species that expresses high levels of Neu5Gc in many tissues. No attention has yet been paid (in the published literature) to the potential for delayed immune response to Neu5Gc on such transplanted animal organs. It remains to be seen if this will pose a significant barrier to xenotransplantation.

### Implications for the human brain

As indicated above, Neu5Gc is common in tissues of nonhuman mammals and is the major sialic acid in most organs and cell types of the great apes. In striking contrast to this situation, Neu5Gc is hard to find in the brains of these animals. Indeed, early studies suggested that Neu5Gc was completely absent from the mammalian brain (Gottschalk, 1960; Rosenberg and Schengrund, 1976; Schauer, 1982), and it was only subsequent analyses with more sensitive techniques that showed that there were small but clearly detectable amounts present (Tettamanti et al., 1965; Nakao et al., 1991; Mikami et al., 1998; Muchmore et al., 1998). The mechanism for this differential expression in nonhuman animals appears to be the downregulation of CMAH gene expression in the brain. This represents a rather rare example of a gene that is widely expressed in many tissues and yet selectively downregulated only in the brain. The fact that this unusual situation has been conserved throughout mammalian evolution suggests that there is a strong selection pressure in its favor. In other words, there must be some reason why the mammalian brain has restricted its Neu5Gc content for >100 million years of evolution. In humans, of course, the last traces of Neu5Gc are eliminated from the brain by virtue of the genomic inactivation of the CMAH gene. A tantalizing possibility is that some significant positive consequence of this change for the human brain offset any negative consequences in other human organs. However, there is at present no direct evidence to support this hypothesis. Studies are currently underway in our laboratory to investigate the consequences of eliminating or overexpressing Neu5Gc in the brains of genetically modified mice. Of course, the difference between a mouse and a human brain is much greater than the difference between a great ape and a human brain. Thus, these experiments in mice may or may not provide final answers concerning this issue. On the other hand, genetic manipulation of the germline of humans or apes would be considered highly unethical. If the mouse experiments do not yield clear evidence, we may have to seek indirect clues towards understanding the implications of CMAH loss for the evolution of the human brain.

## CONCLUSIONS AND PERSPECTIVES

Reviewed here is a genetic, biochemical, and structural difference between human and great ape cells that has potential implications for a wide variety of issues related to human evolution, physiology, and pathology. The nature of the genetic mutation, their universality in modern humans, and its direct biochemical consequences to human cells and tissues are clear. There is also good evidence that it can affect the susceptibility or resistance of humans to certain pathogens. Circumstantial evidence is consistent with the possibility of human incorporation of traces of Neu5Gc from dietary origins. In addition, limited data show the effects of human Neu5Gc loss on cells of the human immune system such as macrophages. Finally, the universal and selective suppression of Neu5Gc expression in mammalian brains raises the possibility that the human loss of Neu5Gc had some beneficial effects in human brain evolution.

However, we have at present many more questions than answers. For example, when did this mutation first occur, and when did it become fixed as a universal feature of human ancestors? Are there other consequences for infection risk or resistance in humans? What are the functional consequences for

the intrinsic human sialic-acid receptors whose Neu5Gc preference has yet to be explored? Do the traces of Neu5Gc found in normal humans indeed come from dietary sources? If so, are there any pathological consequences to the human ingestion of Neu5Gc in foods of animal origin? Is the mechanism of Neu5Gc reexpression in tumors and fetuses simply an exaggeration of what happens in normal tissues? Or is there another biochemical pathway that becomes activated in these situations? Does it matter that animal organs intended for xenotransplantation and certain biotechnology therapeutics produced in animal cells can be quite rich in Neu5Gc? And last but not least, what are the consequences, if any, for the evolution and function of the human brain? Much diligent work by many investigators will be needed to answer these questions.

On a more general note, the discovery of a major biochemical and structural difference between humans and great apes and the elucidation of its underlying genetic basis tend to raise hopes that this molecular change played a major role in the evolution of uniquely human characteristics. Indeed, this is the issue likely to be of particular interest to the readership of this Journal. However, the fact that this happens to be the first such difference recognized should not be taken as any indication of its relative significance in human evolution. It is indeed possible that this genetic change came under strong positive selection pressure because it contributed towards the evolution of organs like the human brain. On the other hand, it may have simply aided in the ability of humans to evade certain pathogens of animal origin. A less likely possibility is that this gene inactivation was a fortuitous event that simply drifted to fixation in the immediate ancestors of modern humans, because of a population bottleneck caused by other unrelated factors. Further studies will eventually elucidate which of these possibilities is correct.

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