

Loss of *N*-Glycolylneuraminic Acid in Human Evolution

IMPLICATIONS FOR SIALIC ACID RECOGNITION BY SIGLECS*

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The common sialic acids of mammalian cells are *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc). Humans are an exception, because of a mutation in CMP-sialic acid hydroxylase, which occurred after our common ancestor with great apes. We asked if the resulting loss of Neu5Gc and increase in Neu5Ac in humans alters the biology of the siglecs, which are Ig superfamily members that recognize sialic acids. Human siglec-1 (sialoadhesin) strongly prefers Neu5Ac over Neu5Gc. Thus, humans have a higher density of siglec-1 ligands than great apes. Siglec-1-positive macrophages in humans are found primarily in the perifollicular zone, whereas in chimpanzees they also occur in the marginal zone and surrounding the periarteriolar lymphocyte sheaths. Although only a subset of chimpanzee macrophages express siglec-1, most human macrophages are positive. A known evolutionary difference is the strong preference of mouse siglec-2 (CD22) for Neu5Gc, contrasting with human siglec-2, which binds Neu5Ac equally well. To ask when the preference for Neu5Gc was adjusted in the human lineage, we cloned the first three extracellular domains of siglec-2 from all of the great apes and examined their preference. In fact, siglec-2 had evolved a higher degree of recognition flexibility before Neu5Gc was lost in humans. Human siglec-3 (CD33) and siglec-6 (obesity-binding protein 1) also recognize both Neu5Ac and Neu5Gc, and siglec-5 may have some preference for Neu5Gc. Others showed that siglec-4a (myelin-associated glycoprotein) prefers Neu5Ac over Neu5Gc. Thus, the human loss of Neu5Gc may alter biological processes involving siglec-1, and possibly, siglec-4a or -5.

The sialic acids are a family of acidic sugars found in all vertebrates and are frequently located at the outer end of glycoconjugates on cell surfaces or on secreted glycoconjugates (1–4). This location explains the prominent roles of sialic acids

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as ligands in recognition phenomena, which can take place via either exogenous or endogenous lectins. Exogenous recognition occurs, for example, when lectins/hemagglutinins/adhesins expressed by a microorganism recognize sialic acids on the cell surface of another organism, thereby permitting invasion of the latter (4–8). In contrast, endogenous receptors can recognize sialic acids on the surfaces of cells within one and the same organism. Prominent examples of such endogenous receptors are members of the selectin and siglec families of vertebrate lectins (2–4, 9–16).

One of the major types of sialic acids is *N*-acetylneuraminic acid (Neu5Ac), which is the biosynthetic precursor for most of the other types (2–4). The addition of a single oxygen atom to this sialic acid gives rise to a very common variation, *N*-glycolylneuraminic acid (Neu5Gc). This irreversible conversion is catalyzed by a specific hydroxylase that converts CMP-Neu5Ac to CMP-Neu5Gc (17–20). These sugar nucleotides are the high energy donors necessary for the addition of sialic acid to glycoconjugates in the Golgi apparatus. Neu5Gc is found in large amounts in all mammals except humans, in whom it is even antigenic (21–23). Humans are evolutionarily most closely related to the African great apes, sharing nearly 99% genetic identity with *Pan troglodytes* (the chimpanzee) and *Pan paniscus* (the bonobo) and a somewhat lesser identity with *Gorilla gorilla* (the gorilla) and *Pongo pygmaeus* (the orangutan) (24–30). In striking contrast to humans, all great apes express Neu5Gc in large amounts; indeed it is the dominant sialic acid, in several tissues (31). This difference is caused by an exon deletion/frame shift mutation in the human gene encoding CMP-Neu5Ac hydroxylase, resulting in a highly truncated protein that appears to have no hydroxylase activity (32, 33). Thus, the lineage leading to modern humans suffered a mutation sometime after the common ancestor with the most closely related great apes, the chimpanzee and the bonobo. This loss of Neu5Gc could potentially affect recognition processes involving a variety of endogenous and exogenous sialic acid-binding lectins (2–8).

The selective pressure of exogenous recognition by a pathogen may have been responsible for the loss of Neu5Gc expression in humans. It has been postulated that evading glycan recognition by exogenous agents without losing functional endogenous recognition may require some "looseness of fit" by lectins (8). Here we ask if the loss of Neu5Gc expression in humans had any consequences for the biology of the siglecs¹ (sialic acid-binding immunoglobulin superfamily lectins). Six

¹ The abbreviations used are: siglecs, sialic acid-binding immunoglobulin superfamily lectins; FCS, fetal calf serum; EBV, Epstein-Barr virus; DMB, 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride; HPLC, high performance liquid chromatography; Sn, sialoadhesin; PCR, polymerase chain reaction; OB-BP1, obesity-binding protein 1.

different siglecs have been characterized to date (13, 16, 34–39), and a recent paper describes a potential seventh member (40). Besides recognizing sialic acids, they all share structural similarities, including a type I membrane topology, and an amino-terminal Ig V-set domain followed by a varying number of C2-set domains (16). By virtue of their differential tissue expression and sialic acid recognition specificities, the siglecs are thought to play roles in a wide array of recognition and signaling phenomena (41–54). Here we asked if the evolutionary loss of Neu5Gc expression in humans resulted in a significant loss or gain of potential ligands for this family of endogenous sialic acid binding receptors. The answers are of potential relevance in understanding differences in biological and pathological processes that exist between humans and great apes, despite their close genetic similarity.

EXPERIMENTAL PROCEDURES

Materials—Most of the general materials used were from Sigma Chemical Co. or Fisher Scientific. The following materials were purchased from other sources: protein A-Sepharose, Amersham Pharmacia Biotech; phycoerythrin-conjugated goat F(ab')₂ anti-human IgG (Fc-specific), CalTag Laboratories; *Arthrobacter ureafaciens* sialidase, Calbiochem; peroxidase-conjugated goat anti-mouse IgG and MB-3 resin, Bio-Rad; and 3-amino-9-ethylcarbazole, Vector. Other immunohistochemistry reagents were from Dako and Jackson. Molecular Biology reagents were from Boehringer Mannheim, Invitrogen, Life Technologies, New England Biolabs, and Qiagen. Products for cell culture were from Life Technologies.

Cells—All media used were supplemented with 10% FCS and L-glutamine. EBV-transformed lymphoblastoid cells derived from humans, chimpanzees, bonobos, gorillas, and orangutans (provided by Dr. Peter Parham, Stanford University) were cultured in RPMI medium 1640. COS-7 cells were maintained in Dulbecco's modified Eagle's medium. Recombinant soluble forms of the amino-terminal domains of siglec fused to the Fc region of human IgG₁ (siglec-Fcs) were produced in stably transfected Chinese hamster ovary cells or in transiently transfected COS-7 cells. Cells producing mouse siglec-2-Fc (CD22-Fc) and mouse siglec-1-Fc (Sn-Fc) were maintained in α -minimal essential medium with 1 mg/ml G418, and the Chinese hamster ovary cells producing human siglec-3-Fc (CD33-Fc) in F-12 nutrient mixture (Ham's). Before production the FCS in the media was diminished gradually from 10 to 3%.

Detection and Quantitation of Neu5Ac and Neu5Gc on EBV-transformed Lymphoblastoid Cells—Cells were washed twice in phosphate-buffered saline, resuspended in water, and lysed by sonication. Nuclear debris was removed by spinning at 100 \times g, and the supernatant was ultracentrifuged at 100,000 \times g for 1 h to pellet the membrane fraction. The content and profile of sialic acids were analyzed as described previously (31). Briefly, the sialic acids were released by mild acid treatment followed by derivatization with DMB, and the fluorescent adducts were detected by reverse phase HPLC.

Binding Studies for Comparing of Siglec-Fcs Preference for Neu5Ac or Neu5Gc—This was studied using EBV-transformed lymphoblastoid cells from humans (primarily Neu5Ac), from chimpanzees (Neu5Ac and Neu5Gc), and from orangutan (mainly Neu5Gc). Cultured cells (0.3–1 \times 10⁶) were washed and then incubated for 1 h at 4 $^{\circ}$ C with the various

siglec-Fcs (5 μ g/ml) that had been preincubated (at least 15 min at 4 $^{\circ}$ C) with 100 \times diluted phycoerythrin-conjugated goat F(ab')₂ anti-human IgG. Binding of the complex was analyzed using flow cytometry (using a Becton Dickinson FACscan machine). EBV-transformed lymphoblastoid cells mildly treated with periodate were used as negative controls. Mild periodate treatment was performed as described previously (52). For the inhibition assay, siglec-2-Fcs (0.3 μ g/ml) were preincubated with 0–800 μ M of sialylated diantennary glycans containing Neu5Ac or Neu5Gc for 15 min at 4 $^{\circ}$ C before adding the mixture to the EBV-transformed lymphoblastoid cells.

Synthesis of Sialylated Diantennary Glycans—Neu5Gc was reacted with CTP and recombinant CMP-sialic acid synthetase from *Escherichia coli* utilizing conditions described by Higa and Paulson (55). Reaction progress was monitored by C18 reverse phase HPLC using a Spherisorb 5 μ M C18 250 \times 4.6-mm column and 0.5 M K₂HPO₄ as mobile phase under isocratic conditions and a flow rate of 1 ml/min. Elution was monitored using a Beckman PDA detector scanning 200–300 nm wavelengths. After completion of the reaction, CMP-Neu5Gc was precipitated with 90% ethanol, the supernatant removed, and the pellet was dried under vacuum. Disialylated diantennary N-glycans (terminating with Neu5Ac) were prepared from hen's eggs (56). These were desialylated with *A. ureafaciens* sialidase in 50 mM ammonium acetate, pH 5.4, 37 $^{\circ}$ C for 12–24 h. Desialylated oligosaccharides were purified over a mixed bed resin, MB-3, which removed salts and essentially all sialidase activity (as judged the absence of [³H]Neu5Ac release upon incubation of 1% of the resulting material with oligosaccharides terminating in [³H]Neu5Ac). The asialodiantennary oligosaccharides were lyophilized and stored at –20 $^{\circ}$ C until resialylation. Diantennary N-glycans terminating with α 2–6-linked Neu5Gc were synthesized by reacting asialodiantennary N-glycans with rat ST6Gal-I and a 10-fold molar excess of CMP-Neu5Gc (based on galactose content). The reaction progress was analyzed by anion exchange HPLC monitoring the elution on a Beckman PDA detector scanning 200–300 nm wavelengths to detect both CMP-Neu5Gc and diantennary oligosaccharides. When the reaction was complete the disialylated oligosaccharides were desalted over a P-4 column using 20% ethanol as a mobile phase followed by purification over a semipreparative anion exchange column, using essentially the same procedure but increasing the flow rate to 2 ml/min. The disialylated diantennary oligosaccharides terminating with Neu5Gc were lyophilized, resuspended in water, and desalted on a P-4 size exclusion column using 20% ethanol in water as a mobile phase. The final product, carrying ~85% Neu5Gc (as checked by NMR), was lyophilized and stored at –20 $^{\circ}$ C prior to use.

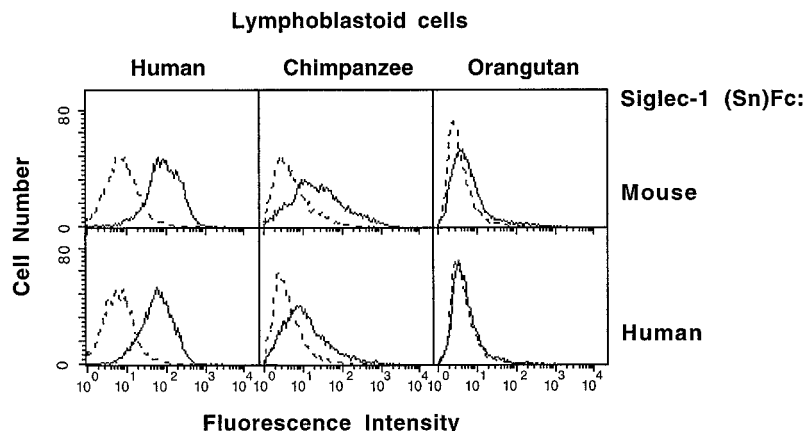
Staining of Spleen Sections with Anti-siglec-1 (Sn), Anti-CD19, and Anti-CD68—Human spleen samples were from the UCSD Cancer Center histology core, and chimpanzee spleens from the Yerkes Primate

TABLE I
Neu5Ac/Gc distribution on EBV-transformed lymphoblastoid cells from humans and great apes

Membrane fractions of EBV-transformed lymphoblastoid cells were analyzed for the distribution of Neu5Ac and Neu5Gc as described under "Experimental Procedures."

	% Neu5Ac	% Neu5Gc
Human	95	5
Chimpanzee	30	70
Orangutan	15	85

FIG. 1. Flow cytometry analysis of binding of mouse and human siglec-1 (Sn) to EBV-transformed lymphoblastoid cells from humans, chimpanzees, and orangutans. Mouse and human siglec-1-Fc were preincubated with 100 \times diluted phycoerythrin-conjugated goat F(ab')₂ anti-human IgG, and binding to various lymphoblastoid cells was studied as indicated. Solid lines show the results for cells with intact sialic acids (sham-treated), and the dashed lines show results for control cells that have sialic acids with a truncated glycerol side chain (mild periodate-treated).



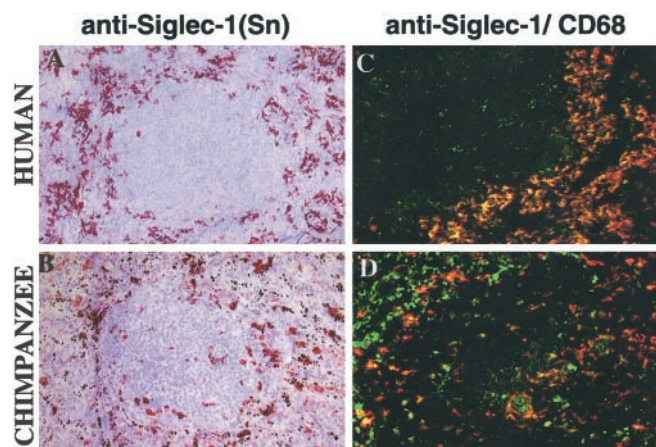


FIG. 2. Expression of siglec-1 on macrophages of human and chimpanzee spleen. Panels A and B, frozen sections of human and chimpanzee spleen were stained with anti-siglec-1 followed by horseradish peroxidase-conjugated goat anti-mouse IgG as described under "Experimental Procedures." The reddish brown color indicates binding of the antibody. Sections were counterstained with hematoxylin (blue). Panels C and D, anti-siglec-1 staining was visualized by biotinylated anti-mouse IgG and Cy-chrome™-conjugated streptavidin followed by anti-CD68 staining (pan macrophage marker) visualized by anti-mouse IgG-fluorescein isothiocyanate as described under "Experimental Procedures." Siglec-1-positive cells show red fluorescence in anti-siglec-1 single-stained sections (not shown). In the pictures shown, single-positive CD68 cells are green, and double-positive cells are orangelyellow.

Center in Atlanta, GA. Frozen human and chimpanzee spleen sections were fixed with acetone. Glucose oxidase inhibition of endogenous peroxidase activities was performed for 20 min at room temperature (57). Each subsequent step was preceded by washing three times with phosphate-buffered saline. All incubations were performed for 30–60 min at room temperature. The sections were blocked with 10% goat serum and 1% bovine serum albumin in phosphate-buffered saline and stained with 6.5 µg/ml (1:250) mouse monoclonal anti-human siglec-1 (7D2, production will be reported elsewhere), followed by horseradish peroxidase-conjugated goat anti-mouse IgG (1:50 in blocking-buffer with 5% normal human serum) and subsequent development with 3-amino-9-ethylcarbazole. For double staining with anti-CD19, staining with anti-siglec-1 was followed by goat anti-mouse IgG-fluorescein isothiocyanate (1:50) and then anti-CD19-phycoerythrin (1:50). For double staining with anti-CD68 the staining with anti-siglec-1 was followed by biotinylated horse anti-mouse IgG (prediluted) and Cy-chrome™-conjugated streptavidin (1:500) and subsequently with mouse anti-CD68 (prediluted) followed by goat anti-mouse IgG-fluorescein isothiocyanate (1:50). The Cy-chrome™-conjugated streptavidin blocked most of the binding sites on mouse IgG for the secondary antibody as confirmed by similar staining patterns in the single stain with each antibody. However, for this reason, the existence of cells positive for only siglec-1 cannot be excluded.

Reverse Transcriptase PCR to Obtain cDNA Encoding Domain 1–3 of Great Ape Siglec-2 (CD22)—Total RNA was isolated, using TRIzol, from EBV-transformed lymphoblastoid cells derived from each of the great apes. 10–20 µg of RNA was reverse transcribed using 1.5 µg of random hexamer, 200 units of Superscript II, 10 units of RNase inhibitor in a volume of 20 µl with first strand buffer, 10 mM dNTPs, and 10 mM dithiothreitol. 2.5–5.0 µl of the reverse reaction mixture was used for amplification by PCR at the following conditions: 94 °C for 5 min followed by 30 cycles of 94 °C, 30 s; 60 °C, 1 min; 72 °C, 1.5 min, ending with 72 °C for 5 min. *Taq* DNA polymerase was used with 20 pmol of each of the following primers specific for the first and third domain of human siglec-2 (58): CD22con1: 5'-CGC GGG CTC GAG ATG CAT CTC CTC GGC CCC TGG CTC-3' and CD22D3: 5'-CTC GAG ATC TTC CGG GGC ATA CTG CAC TTG CAG GAA-3'. *Xho*I and *Bgl*II sites were incorporated into these primers, respectively, for the construction of siglec-2-Fcs (58). PCR products were purified from 1% agarose gels with the QIAEX II gel extraction kit and ligated into the Topo TA cloning vector of the Invitrogen's original Tope TA cloning kit. Sequencing of both strands of two clones derived from each ape was done by the dideoxy chain termination method (performed by the MacConnell Research Corporation).

Construction and Production of Siglec Chimeras (Fcs)—The first

TABLE II

Amino acid differences between great ape and human siglec-2 (CD22)

The cDNA encoding the first three domains of siglec-2 (CD22) of the various apes was cloned and sequenced as described under "Experimental Procedures." Amino acid differences from humans shared by all four great apes are indicated in bold. Numbers refer to the position of the amino acid in the human sequence.

	Chimpanzee	Bonobo	Gorilla	Orangutan
Domain 1	Val³ → Ala Gly ²⁹ → Arg	Val³ → Ala Gly ²⁹ → Arg	Val³ → Ala	Val³ → Ala Asp ³⁰ → Ala Lys ⁶² → Glu Asn ⁷⁷ → Ser Leu⁸⁹ → Val
Domain 2	Leu⁸⁹ → Val Glu ¹⁰⁴ → Ala	Leu⁸⁹ → Val Glu ¹⁰⁴ → Ala	Asn ⁷⁷ → Lys Leu⁸⁹ → Val	Leu ¹⁵⁵ → Phe Met ¹⁶¹ → Val Arg ¹⁶² → Gly Thr ¹⁶⁷ → Asn Thr ¹⁷² → Ala Ile ¹⁷³ → Thr Gln ²⁰⁰ → His Glu ²²³ → Lys Lys ²²⁵ → Glu Thr ²²⁷ → Asn
Domain 3	Asp ²³⁷ → Glu Val ²⁵⁵ → Ile	Asp ²³⁷ → Glu Val ²⁵⁵ → Ile	Glu ²⁵¹ → Met	Glu ²⁵¹ → Lys Ser ²⁶³ → Pro Thr ²⁶⁹ → Ala Phe²⁷⁰ → Leu Thr ²⁷¹ → Met Asn ²⁷³ → Thr Arg ²⁷⁵ → Gln
	Phe²⁷⁰ → Leu Thr ²⁷¹ → Met	Phe²⁷⁰ → Leu Thr ²⁷¹ → Met	Phe²⁷⁰ → Leu	Phe²⁷⁰ → Leu Thr ²⁷¹ → Met Asn ²⁷³ → Thr Arg ²⁷⁵ → Gln
	Arg ²⁷⁵ → His	Arg ²⁷⁵ → His	Arg ²⁷⁵ → Ser Cys ²⁸⁵ → Tyr Gly ²⁹⁶ → Glu Glu ²⁹⁹ → Ala	
	Glu ²⁹⁹ → Ala	Glu ²⁹⁹ → Ala	Glu ²⁹⁹ → Ala	

three domains of siglec-2 from the different apes and from human were cloned into a human Ig expression vector as described before (58, 59) using the *Xho*I and *Bgl*II restriction sites of the siglec-2 fragments. Stably transfected Chinese hamster ovary cells producing mouse siglec-1-Fc and mouse siglec-2-Fc (domains 1–3) (plasmid provided by Dr. Ivan Stamenkovic, Massachusetts General Hospital) were produced in the laboratory by Dr. Stephen Thiel. Chinese hamster ovary cells producing human siglec-3-Fc (domains 1 and 2) (36) and the plasmids encoding human siglec-5-Fc (domains 1–4) (38) and human siglec-6-Fc (domains 1–3) (OB-BP1-Fc) (39) were constructed as described before. Construction of human siglec-1-Fc (domains 1–4) will be described elsewhere. COS-7 cells were transiently transfected at 60–70% confluence using LipofectAMINE reagent in serum-free Opti-MEM medium. After 5 h the medium was diluted 2 × with 10% FCS containing Opti-MEM, and the next day the medium was changed to Dulbecco's modified Eagle's medium with 3% FCS. The COS-7 cell supernatants were collected 5–7 days after transfection, and various siglec-Fcs were purified on protein A-Sepharose. Prior to elution, siglec-3-Fc bound to protein A-Sepharose was treated with *A. ureafaciens* sialidase as described in the accompanying paper (60).

RESULTS AND DISCUSSION

Distribution of Neu5Ac and Neu5Gc on EBV-transformed Lymphoblastoid Cells from Humans and Apes—To determine if EBV-transformed lymphoblastoid cells from human, chimpanzee, and orangutan could be used to study the binding preference of siglecs for Neu5Ac or Neu5Gc, the distribution of these types of sialic acids was analyzed as described under "Experimental Procedures." The results (Table I) show that human EBV-transformed lymphoblastoid cells contain mainly Neu5Ac (>95%); the small amount of Neu5Gc on these cells was most probably incorporated from the 10% FCS in the culture medium (31). Chimpanzee EBV-transformed lymphoblastoid cells contain much more Neu5Gc (~70%), and orangutan EBV-transformed lymphoblastoid cells contain mainly Neu5Gc (~85%). These lymphoblastoid cells were therefore used to study the preference of various siglec-Fcs (soluble chimeric molecules of extracellular domains of the first six reported

	Signal Peptide	Domain 1	
Hu:	MHLGWPWLLL-L-VLEYLAFSDSSKWA	FEHPETLYAWEGACVWIPCTYR-AL-DGDLESFILFHNPEYNKNT	46
Cp:	MHLGWPWLLL-L-VLEYLAFSDSSKWA	FEHPETLYAWEGACVWIPCTYR-AL-DRDLESFILFHNPEYNKNT	46
Bn:	MHLGWPWLLL-L-VLEYLAFSDSSKWA	FEHPETLYAWEGACVWIPCTYR-AL-DRDLESFILFHNPEYNKNT	46
Gr:	MHLGWPWLLL-L-VLEYLAFSDSSKWA	FEHPETLYAWEGACVWIPCTYR-AL-DGDLESFILFHNPEYNKNT	46
Ou:	MHLGWPWLLL-L-VLEYLAFSDSSKWA	FEHPETLYAWEGACVWIPCTYR-AL-DGALESFILFHNPEYNKNT	46
Mu:	MRVHYLWLLLILGHAASAQYSSANDWTV	DHPQTLFAWEGACIRIPCKYKTPLPKARLDNILLFQNYEFDKAT	48
		* * *	
Hu:	SKFDGTRLYESTKDGKVPSEQKRVQFLGDKNKNTLSIHPVH	VNDSGQLGLRMESKTEKWMERIHNLVNSERP	118
Cp:	SKFDGTRLYESTKDGKVPSEQKRVQFLGDKNKNTLSIHPVH	VNDSGQLGLRMESKTAKWMERIHNLVNSERP	118
Bn:	SKFDGTRLYESTKDGKVPSEQKRVQFLGDKNKNTLSIHPVH	VNDSGQLGLRMESKTAKWMERIHNLVNSERP	118
Gr:	SKFDGTRLYESTKDGKVPSEQKRVQFLGDKSKNTLSIHPVH	VNDSGQLGLRMESKTEKWMERIHNLVNSERP	118
Ou:	SKFDGTRLYESTKDGKVPSEQKRVQFLGDKSKNTLSIHPVH	VNDSGQLGLRMESKTEKWMERIHNLVNSERP	118
Mu:	KFKFGTVLYNKAEPELYPKQRRVTVFLGNSIDNCTLKIHP	IRANDSGNLGLRMTAGTERWMEPIHLNVSEKP	120
		* * *	
Domain 2			
Hu:	FPPHIQLPPEIQESQEVTLTCLLNFS	CGYPIQLQWLE-----GVPMRQAAVTST-SLTIKSVFTRSELKF	184
Cp:	FPPHIQLPPEIQESQEVTLTCLLNFS	CGYPIQLQWLE-----GVPMRQAAVTST-SLTIKSVFTRSELKF	184
Bn:	FPPHIQLPPEIQESQEVTLTCLLNFS	CGYPIQLQWLE-----GVPMRQAAVTST-SLTIKSVFTRSELKF	184
Gr:	FPPHIQLPPEIQESQEVTLTCLLNFS	CGYPIQLQWLE-----GVPMRQAAVTST-SLTIKSVFTRSELKF	184
Ou:	FPPHIQLPPEIQESQEVTLTCLLNFS	CGYPIQLQWLE-----GVPVQAAVNST-SLATKSVFTRSELKF	184
Mu:	FQPYIQMPSEIRESQSVTLTCCGLNF	SCFEYDILLQWFLEDSKITSVTPSVTSITSSVTSSIKNVYTESKLTFF	192
		* * *	
Domain 3			
Hu:	SPQWSHHGKIVTCQLQDADGKFLSNDTVQ	LNKHTPKLEIKVTPSDAIVREGDSVTMTCEVSSSNPEYTTV-	255
Cp:	SPQWSHHGKIVTCQLQDADGKFLSNDTVQ	LNKHTPKLEIKVTPSDAIVREGESVTMTCEVSSSNPEYTTI-	255
Bn:	SPQWSHHGKIVTCQLQDADGKFLSNDTVQ	LNKHTPKLEIKVTPSDAIVREGESVTMTCEVSSSNPEYTTI-	255
Gr:	SPQWSHHGKIVTCQLQDADGKFLSNDTVQ	LNKHTPKLEIKVTPSDAIVREGDSVTMTCEVSSSNPMYTTV-	255
Ou:	SPQWSHHGKIVTCQLHDADGKFLSNDTVQ	LNKHTPKLEIKVTPSDAIVREGDSVTMTCEVSSSNPKYTTV-	255
Mu:	QPKWTDHKGSKVCQVQHS-SEVLSERTVRLDVKY	TPKLEIKVNPT--VEKNNSVTMTCRVNNSNPKLRTVA	262
		*	
Hu:	--SWLKDGTSL--KK---QNTFTLNLREVT	KDQSGKYCCQVSNVDVGPGRSEEVFLQVQYAPE	310
Cp:	--SWLKDGTSL--KK---QNTLMLNLHEVT	KDQSGKYCCQVSNVDVGPGRSAEVFLQVQYAPE	310
Bn:	--SWLKDGTSL--KK---QNTLMLNLHEVT	KDQSGKYCCQVSNVDVGPGRSAEVFLQVQYAPE	310
Gr:	--SWLKDGTSL--KK---QNTLTLNLSEVT	KDQSGKYCCQVSNVDVGPERSAEVFLQVQYAPE	310
Ou:	--SWLKDGTSL--KK---QNALMLTLQEV	KDQSGKYCCQVSNVDVGPGRSEEVFLQVQYAPE	310
Mu:	VSWFKDGRPLEDQEQEQMSKLIILHSVTK	MDMRGKYRCQASNDIGPGESEVELTVHYAPE	324

FIG. 3. Predicted amino acid sequences encoding the first three domains of siglec-2 (CD22) of mouse, great apes, and humans. cDNAs encoding the first three domains of siglec-2 of the various apes were obtained by reverse transcriptase PCR using primers for human siglec-2, cloned, and sequenced as described under "Experimental Procedures." The sequence of human and mouse siglec-2 is included for comparison. X, conserved among mouse, apes, and human; X, change in amino acid the same in all apes compared with human; *, characteristic cysteine residues and key residues for sialic acid binding which are conserved among all siglecs reported to date (79).

siglecs fused with the Fc region of human IgG) to bind to either Neu5Ac or Neu5Gc.

Binding Preference of Siglec-1 (Sn) for Neu5Ac—Siglec-1 is expressed on (tissue) macrophages and is reported to recognize α 2-3-linked Neu5Ac residues (NeuAca2-3Gal β 1-3GalNAc and NeuAca2-3Gal β 1-3(4)GlcNAc) on glycoproteins and glycolipids (60, 61). The function of this siglec is unknown, but it has been suggested to be involved in the development of myeloid cells in the bone marrow because it is expressed at high levels at contact sites between macrophages and developing myeloid cells (41) and binds preferentially to cells of the granulocytic lineage (62). Other studies have indicated a potential role in the trafficking of leukocytes (48, 63). Mouse siglec-1 is known to strongly prefer Neu5Ac over Neu5Gc (44, 64). We tested human siglec-1-Fc binding to the lymphoblastoid cells derived from human, chimpanzee, and orangutan. Both human and mouse siglec-1-Fcs (first four domains of siglec-1 fused with the Fc region of human IgG) were found to bind much better to the human cells than to cells from chimpanzees (Fig. 1). No binding of this siglec was found to orangutan EBV-transformed lymphoblastoid cells that contain mainly Neu5Gc (Fig. 1). Thus, just as with the mouse molecule, human siglec-1 strongly prefers Neu5Ac over Neu5Gc. Siglec-1 has a low single-site affinity

for sialic acid, and effective binding probably requires multivalent arrays of the ligand. In the case of the mouse and chimpanzee, the normal endogenous mixture of Neu5Ac (a ligand) and Neu5Gc (not a ligand) would markedly restrict possibilities for generating multivalent arrays of the Neu5Ac. In contrast, the loss of Neu5Gc in humans increases markedly the overall density of Neu5Ac, thus generating much improved binding by this siglec.

Siglec-1 Localization in Human and Chimpanzee Spleen—Earlier studies described an overall difference between the histologic architecture of rat and human spleen (65). In that study, the localization of siglec-1 on macrophages in the spleen was also shown to be different between rats and humans. In humans siglec-1 was found mainly on cells outside the follicles, whereas in rats it was also found in the marginal zone and on cells surrounding the periarteriolar sheaths (65). We found that the chimpanzee spleen resembles the human spleen in overall histologic architecture as assessed by hematoxylin and eosin staining (data not shown). To study if the loss of Neu5Gc may have affected the localization of siglec-1 expression, human and chimpanzee spleen sections were stained with anti-human siglec-1. In human spleen, siglec-1-positive macrophages are found primarily outside the follicles in the

perifollicular zone (Fig. 2, A and C) and also clustered around the sheathed capillaries in this area. However, such siglec-1-positive sheathed capillaries are not evident in chimpanzees. Furthermore, siglec-1 in chimpanzees is found not only in the perifollicular zone, but also in the marginal zone and around the intrafollicular arterioles (Fig. 2, B and D). These findings were confirmed with double staining for CD19 (defining the follicles by staining the B cells in the follicles) and siglec-1 (not shown). Double staining for siglec-1 and CD68 (a pan macrophage marker) revealed another striking difference: almost all of the CD68⁺ macrophages in the human spleen are siglec-1-positive, whereas in the chimpanzee spleen only a subpopulation of the macrophages expresses siglec-1 (Fig. 2, C and D). The rare macrophages (CD68⁺) in the human spleen which are negative for siglec-1 are all found within the follicles (Fig. 2C). Similar findings were obtained for four additional samples each of spleens of chimpanzees and humans (data not shown). Thus, despite the resemblance between humans and chimpanzees in overall histologic architecture of the spleen, the localization of siglec-1 in the chimpanzee spleen is similar to that reported previously in the rat spleen (65). Humans show a uniquely different pattern as well as striking expression of siglec-1 on almost all macrophages in the spleen.

Taken together, these data indicate that the localization and expression of siglec-1 in humans have been altered coincident with evolutionary loss of Neu5Gc. In view of prior suggestions about a role of siglec-1 in myeloid development, it is interesting to note that the normal white blood cell count in chimpanzees (and all the great apes) is, in general, substantially higher than the normal levels in humans (66, 67). One possibility is that in humans the myeloid precursors with a high amount of ligands for siglec-1 are sequestered by this siglec in sites such as the spleen, lymph nodes, or bone marrow. Because the function of siglec-1 is still unknown, it is for now impossible to point to a definitive physiological effect of the changes in siglec-1 biology in humans. The phenotypes of siglec-1 and CMP-Neu5Ac hydroxylase null mice may provide us with more information on this matter.

Sequencing of cDNA Encoding the First Three Domains of Siglec-2 (CD22) of the Various Apes—Another member of the siglec family, siglec-2, a B cell surface receptor, is capable of specifically recognizing α 2-6-linked sialic acid residues on its potential counterreceptors on plasma proteins and leukocytes (34, 60, 68–70). Several studies suggest a role for siglec-2 in cell adhesion and in modulating signaling through the B cell receptor (42, 47, 51, 52, 54, 71–76). A striking evolutionary difference in siglec-2 recognition properties is the strong preference of mouse siglec-2 for Neu5Gc (44, 64, 77) which stands in contrast to human siglec-2, which can bind both Neu5Ac and Neu5Gc (78). Because humans do not express Neu5Gc, and the closely related great apes do so in large amounts, the question arises as to how during mammalian evolution the preference of siglec-2 for Neu5Gc was adjusted in concert with the loss of Neu5Gc expression in humans. To address this question we used EBV-transformed lymphoblastoid cells to obtain cDNAs encoding domains 1–3 of the siglec-2 molecules of these great apes. Reverse transcriptase PCR with primers specific for human siglec-2 could be used because of the high genetic similarity between humans and the great apes. The derived amino acid sequences of the first three domains of siglec-2 of the various great apes are compared with human siglec-2 in Table II, and a comparison of these domains for humans, apes, and mice is shown in Fig. 3. The homology between human and chimpanzee siglec-2 (domain 1–3) is 97%, slightly lower than the ~99% found for most proteins (25, 27–30). The homology between siglec-2 of the other great apes and human siglec-2

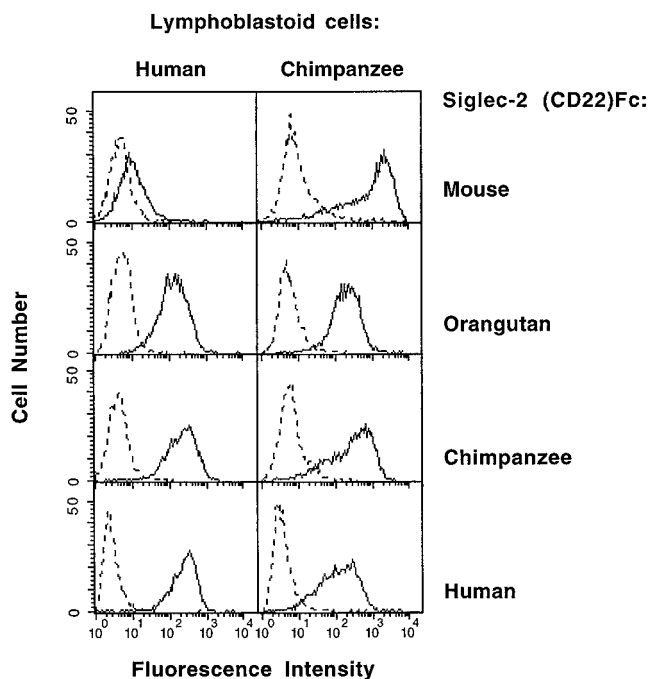


FIG. 4. Flow cytometry analysis of binding of mouse, ape, and human siglec-2-Fcs to EBV-transformed lymphoblastoid cells. Mouse, orangutan, chimpanzee, and human siglec-2-Fcs (constructed as described under "Experimental Procedures") were preincubated with $100 \times$ diluted phycoerythrin-conjugated goat F(ab')₂ anti-human IgG, and the binding ability was studied to various lymphoblastoid cells. Solid lines show the results for cells with intact sialic acids (sham-treated), and the dashed lines show results for control cells that have sialic acids with a truncated glycerol side chain (mild periodate-treated).

(domain 1–3) was as follows: bonobo, 96%; gorilla, 96%; and orangutan, 93%. A few changes were unique for each ape. The following changes were noted in the first domain of siglec-2 (which contains the sialic acid binding site) (77), of all the great apes compared with human siglec-2: Val³ → Ala and Leu⁸⁹ → Val. The change Leu⁸⁹ → Val is close to an Arg residue known to be critical for sialic acid binding (77, 79), and the change Val³ → Ala is close to a Trp residue conserved between siglec-2 and siglec-1, in which this residue is known to play a key role in sialic acid binding (79). In view of these differences we continued with the construction of chimpanzee and orangutan siglec-2-Fcs to study their respective binding preference for Neu5Ac or Neu5Gc in cell binding assays.

Binding Preference of Great Ape Siglec-2 for Neu5Ac or Neu5Gc Is Similar to That of Humans—Recombinant soluble chimeric molecules of mouse, human, chimpanzee, and orangutan siglec-2 (fused with the Fc region of human IgG) were constructed and used to study their preference for Neu5Ac or Neu5Gc using cell binding assays as described above for siglec-1. Human, chimpanzee, and orangutan siglec-2-Fcs bound well to both human and chimpanzee EBV-transformed lymphoblastoid cells. In contrast, mouse siglec-2-Fc bound only to chimpanzee EBV-transformed lymphoblastoid cells (Fig. 4). These results show that in contrast to mouse siglec-2, the siglec-2 of the great apes can bind to Neu5Ac as well as to Neu5Gc. This finding was confirmed by inhibition studies using sialylated diantennary glycans containing only α 2-6Neu5Ac or ~85% α 2-6Neu5Gc. As shown in Fig. 5, the glycan with 85% α 2-6Neu5Gc was a much better inhibitor of the binding of mouse siglec-2 to orangutan EBV-transformed lymphoblastoid cells than the glycan with only Neu5Ac. In contrast, both glycans equally inhibited binding of orangutan, chimpanzee, and human siglec-2 to orangutan cells (Fig. 5). These data using pure glycans as inhibitors also serves to

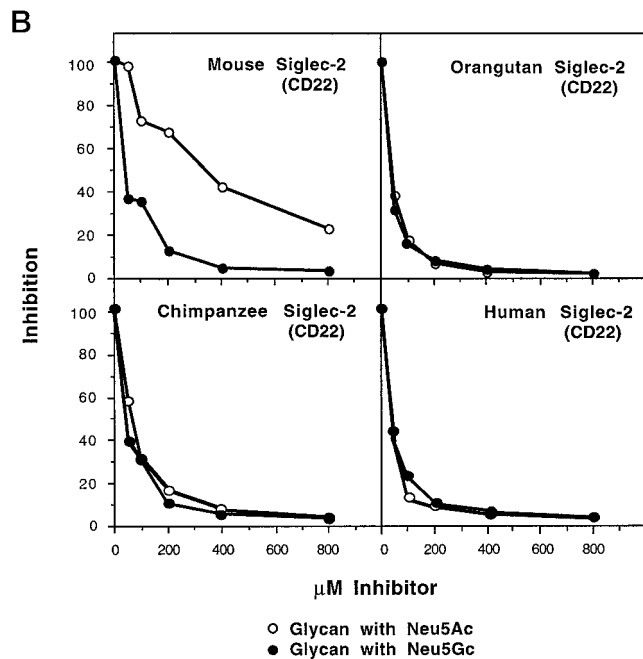
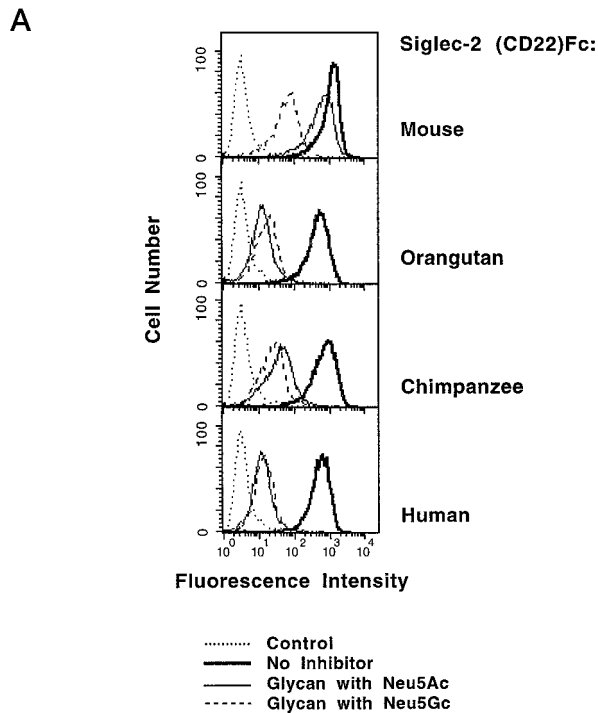


FIG. 5. Inhibition of siglec-2-Fc binding to orangutan EBV-transformed lymphoblastoid cells by α 2-6-Neu5Ac- or α 2-6-Neu5Gc-containing diantennary glycans. Panel A, flow cytometry analysis of binding (continuous line) of mouse, orangutan, chimpanzee, and human siglec-2-Fcs to orangutan EBV-transformed lymphoblastoid cells (containing mainly Neu5Gc) and inhibition of this binding by 400 μ M α 2-6-Neu5Ac-containing diantennary glycan (thin continuous lines) or α 2-6-Neu5Gc-containing diantennary glycan (thin dashed lines). Unstained cells are shown by the dotted lines. Panel B, concentration-dependent inhibition of binding of the various Fcs to orangutan EBV-transformed lymphoblastoid cells. Open circles, α 2-6-Neu5Ac-containing diantennary glycan; closed circles, α 2-6-Neu5Gc-containing diantennary glycans.

validate the use of the EBV-transformed lymphoblastoid cells as binding targets to determine preferences for Neu5Ac and Neu5Gc.

Binding Ability of Siglec-3, Siglec-5, and Siglec-6 for Neu5Ac or Neu5Gc—Three other human siglecs were examined for

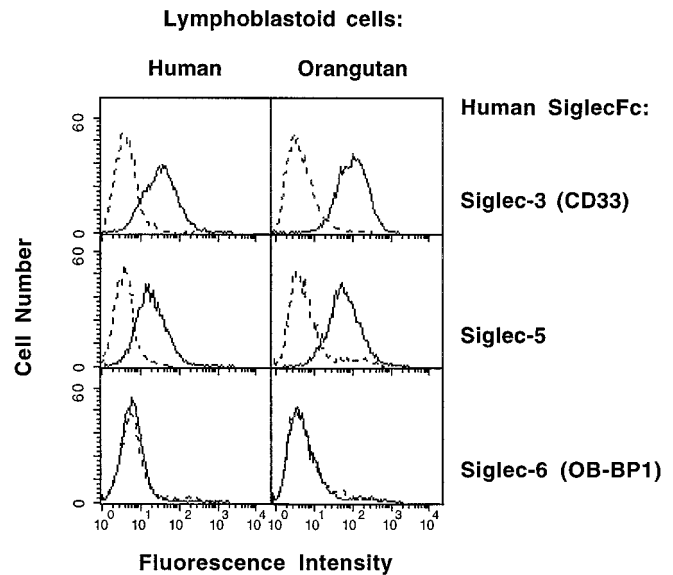


FIG. 6. Flow cytometry analysis of binding of human siglec-3-Fc (CD33-Fc), siglec-5-Fc, and siglec-6-Fc (OB-BP1-Fc) to EBV-transformed lymphoblastoid cells. Human siglec-Fcs (siglec-3-Fc pretreated with *A. ureafaciens* sialidase as described under "Experimental Procedures" of accompanying paper (60)) were preincubated with 100 \times diluted phycoerythrin-conjugated goat F(ab')₂ anti-human IgG, and binding ability was studied to lymphoblastoid cells. Solid lines show the results for cells with intact sialic acids (sham-treated), and the dashed lines show results for control cells that have sialic acids with a truncated glycerol side chain (mild periodate-treated).

their ability to recognize Neu5Ac and Neu5Gc. Siglec-3 (CD33) is found on myeloid cells and binds both α 2-6- and α 2-3-linked sialic acids (see accompanying paper (60); this is in contrast to what has been reported in earlier studies) (36). Siglec-5 is expressed mainly on neutrophils and recognizes α 2-3- α 2-6- and α 2-8-linked sialic acids (38, 60). Siglec-6 (OB-BP1) recognizes sialy-Tn, and is expressed on B cells and placental trophoblasts (39). We found that human siglec-3 and human siglec-5 bound to both human and orangutan EBV-transformed lymphoblastoid cells, indicating that these siglecs can recognize both types of sialic acid (Fig. 6). The use of the sialylated diantennary glycans as inhibitors of binding could not be applied in these cases because the IC₅₀ was much higher compared with siglec-2 (data not shown). Regardless, as mentioned above, the use of these glycans in assays with siglec-2 validated the EBV-transformed lymphoblastoid cells as a good system for studying Neu5Ac or Neu5Gc preference. For siglec-5 there may be a slight preference for Neu5Gc because the binding to orangutan EBV-transformed lymphoblastoid cells seems to be somewhat stronger than to human cells. No ligands were present on these cells for siglec-6 (Fig. 6). However, the accompanying paper (60) describes binding of this siglec to synthetic probes with only Neu5Ac and to a mucin (porcine submaxillary mucin) with almost exclusively Neu5Gc, indicating that siglec-6 can recognize both types of sialic acid. Similar data were obtained for human siglecs-2, -3, and -5, confirming their ability to bind both Neu5Ac as well as Neu5Gc.

Conclusions and Perspectives—Because of the high degree of DNA sequence identity between humans and great apes it is likely that the number of significant genetic differences between these species are relatively few. We have examined potential evolutionary implications of the loss of Neu5Gc expression in humans on the recognition specificity of mammalian siglecs for the *N*-acyl group of sialic acid. From the data presented it is clear that the evolutionary loss of Neu5Gc expression results in a marked increase in ligand availability for siglec-1, which coincides with a major increase in macrophages

TABLE III
Ability of the siglecs to recognize Neu5Ac or Neu5Gc

This summary is compiled from data of this study and from data in the literature.

No.	Siglec Other name	Species	Ability to recognize		Based on data from
			Neu5Ac	Neu5Gc	
1	Sialoadhesin	Mouse	+	–	This study and Refs. 44 and 64
		Human	+	–	
2	CD22	Mouse	–	+	This study and Refs. 44, 64, and 77
		Orangutan	+	+	
		Chimpanzee	+	+	
		Human	+	+	
3	CD33	Human	+	+	This study and Refs. 64 and 78
4a	MAG ^a	Rat/mouse	+	–	Refs. 50 and 64
5		Human	+	+	This study
6	OB-BP1	Human	+	+	Accompanying paper, Ref 60

^a MAG, myelin-associated glycoprotein.

expressing the lectin and a change in localization of these macrophages. It is reasonable to suggest that these events are linked to one another. Although we cannot of course be certain, we postulate that the marked increase in ligand availability for siglec-1 caused by the loss of Neu5Gc in humans may have modified the functional state of this lectin *in vivo*. Such a modified state may have selected for an increased percentage of macrophages positive for siglec-1 in human spleen compared with chimpanzee spleen as well as for a different localization of siglec-1-positive cells in humans. The biological consequences of this difference need to be explored.

While there are clear differences between human and mouse siglec-2 in the recognition of Neu5Ac and Neu5Gc, the great ape siglec-2 molecules behave similarly to those of the human. Thus, during the coevolution of this mammalian lectin and its endogenous ligand, the binding flexibility of the lectin (for both Neu5Ac and Neu5Gc) had evolved before a preferred carbohydrate ligand was lost. This indicates that siglec-1 and not siglec-2 may contribute to the biological differences between humans and great apes. Likewise, because human siglecs-3, -5, and -6 can also recognize both types of sialic acids, lack of Neu5Gc in humans may not affect the biology of these siglecs (although siglec-5 may have some preference for Neu5Gc). According to studies by others, siglec-4a (myelin-associated glycoprotein) can only recognize Neu5Ac (50, 64). For siglec-4a the natural ligands are most likely expressed in the nervous system. However, Neu5Gc is very rare in this tissue (1, 31). It is therefore unlikely that the loss of Neu5Gc in the other tissues is of significance for the function of siglec-4a in humans. Table III summarizes the ability of these six siglec family members to recognize Neu5Ac or Neu5Gc.

It is reasonable to speculate that recognition of Neu5Gc by a major pathogen may have initially selected for the loss of its expression sometime prior to the common origin of all modern humans. This study supports the idea of a “looseness of fit” of endogenous receptors as a means to adjust to the evolutionary loss of a carbohydrate ligand (8), which seems to apply for all siglecs except for siglec-1. Thus, among all siglecs here considered (Table III) siglec-1 stands out as the one for which a major change in biology most likely occurred concurrently with the loss of expression of Neu5Gc in humans. More information on the exact function of siglec-1 is needed to understand the full significance of this evolutionary change. Subtle effects of differences in the biology of siglecs-4a and -5 are also possible.

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