Siglec-9, a Novel Sialic Acid Binding Member of the Immunoglobulin Superfamily Expressed Broadly on Human Blood Leukocytes*

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Here we characterize the properties and expression pattern of Siglec-9 (sialic acid-binding Ig-like lectin-9), a new member of the Siglec subgroup of the immunoglobulin superfamily. A full-length cDNA encoding Siglec-9 was isolated from a dibutyryl cAMP-treated HL-60 cell cDNA library. Siglec-9 is predicted to contain three extracellular immunoglobulin-like domains that comprise an N-terminal V-set domain and two C2-set domains, a transmembrane region and a cytoplasmic tail containing two putative tyrosine-based signaling motifs. Overall, Siglec-9 is ~80% identical in amino acid sequence to Siglec-7, suggesting that the genes encoding these two proteins arose relatively recently by gene duplication. Binding assays showed that, similar to Siglec-7, Siglec-9 recognized sialic acid in either the α2,3- or α2,6-galactosidic linkage to galactose. Using a specific mAb, Siglec-9 was found to be expressed at high or intermediate levels by monocytes, neutrophils, and a minor population of CD16+, CD56+ cells. Weaker expression was observed on ~50% of B cells and NK cells and minor subsets of CD8+ T cells and CD4+ T cells. These results show that despite their high degree of sequence similarity, Siglec-7 and Siglec-9 have distinct expression profiles.

Sialic acid-binding immunoglobulin-like lectins (Siglecs) are transmembrane sialic acid-binding proteins of the immunoglobulin (Ig) superfamily characterized by the presence of an N-terminal V-set Ig-like domain and variable numbers of C2-set domains (1). The first Siglecs to be characterized were sialoadhesin/Siglec-1, CD22/Siglec-2, CD33/Siglec-3 and myelin-associated glycoprotein/Siglec-4 which share ~25–30% sequence identity within the extracellular regions (2). Recent studies (3–8) have uncovered the existence of a cluster of genes on human chromosome 19q13.3-4 that encode novel Siglecs highly related to CD33. This CD33-related subgroup includes Siglec-3, -5, -6, -7, and -8, each of which share ~50–70% sequence identity, suggesting that the genes encoding them have arisen relatively recently by gene duplication and exon shuffling. Despite their sequence similarity, all novel Siglecs characterized to date are expressed on distinct subsets of hemopoietic cells, such as neutrophils (Siglec-5) (4), B cells (Siglec-6) (8), natural killer (NK) cells (Siglec-7) (5, 6), and eosinophils (Siglec-8) (7). Each of these Siglecs also exhibits distinct carbohydrate binding properties (4, 5, 7–10). These differences in expression and ligand binding suggest that each Siglec mediates a specific, nonredundant function in hemopoietic cell biology.

The cytoplasmic tails of most CD33-related Siglecs contain two homologous tyrosine-based motifs, one of which fits the consensus for immune receptor tyrosine-based inhibitory motifs (ITIMs) (11). The presence of one or more ITIMs has been described in a growing number of other leukocyte membrane receptors, many of which are tightly linked to CD33-related Siglecs on chromosome 19q13.4, in a region known as the leukocyte receptor cluster (12). The consensus that has emerged is that receptors bearing ITIMs mediate inhibitory functions when cross-linked with activating receptors bearing tyrosine-based activatory motifs (reviewed in Ref. 11). This has been shown to be due to tyrosine phosphorylation of the ITIMs, leading to recruitment and activation of intracellular phosphatases, either the tyrosine phosphatases SHP-1 and SHP-2, or the inositol phosphatase, SHIP (reviewed in Ref. 11). From the limited studies that have been carried out so far, CD33 and related Siglecs appear to behave similarly in mediating inhibitory signals. Taylor and co-workers (13) showed that the tyrosine residues of both motifs in CD33 can be phosphorylated by Src-like kinases following antibody-induced cross-linking and that this leads to recruitment of SHP-1 and SHP-2. The potential functional significance of tyrosine phosphorylation and SHP-1 recruitment by these novel Siglecs was first demonstrated by Falco and colleagues (6) who identified Siglec-7 as p75/AIRM1, a receptor that could inhibit NK cell cytotoxicity. In a separate study of mononuclear phagocytes, it was shown that co-cross-linking of CD33 with the CD64 high affinity Fc receptor led to reduced CD64-dependent calcium fluxing as a result of SHP-1 recruitment and activation (14).

In this paper we describe the properties and expression pattern of Siglec-9, a new member of the Siglec family highly related to Siglec-7. When expressed at the cell surface, Siglec-9 exhibits sialic acid-dependent binding to human red blood cells and synthetic sialoconjugates. Using a specific monoclonal antibody, we demonstrate that, unlike other Siglecs character-
Characterization of Siglec-9

Isolation of Siglec-9 cDNA—Based on a Siglec-like genomic sequence deposited in GenBank (accession number AF135027), a dibutyryl cAMP-treated HL-60 cDNA library kindly provided by Dr. D. L. Simmons was used to isolate a full-length cDNA by polymerase chain reaction (PCR) using the following forward and reverse primers (5'-3').

Production of Fc Proteins—A construct encoding CD33-Fc was provided by Dr. D. L. Simmons. Constructs encoding Siglec-5-Fc and Siglec-8-Fc proteins were generated as described previously (4, 7). Constructs encoding Siglec-7-Fc and Siglec-9-Fc proteins were prepared using a modified version of the pEE14 vector (15), designated pEE14-33L-3C-Fc. cDNA encoding the extracellular region (not described previously) of human CD33 (17) was cloned into Hinc ll into the pEE14-3C-Fc vector. For Siglec-9-Fc, a specificity that is similar to other Siglects (see below), the homology extending throughout the extracellular, transmembrane, and intracellular regions (Fig. 1).

RESULTS AND DISCUSSION

Characterization and Features of Siglec-9—A full-length cDNA encoding a novel Siglec-like protein was isolated by PCR from a dibutyryl cAMP-treated HL-60 cDNA library using primers derived from the sequence of a Siglec-like gene (GenBank accession number AF135027). This gene was previously identified during characterization of novel human kallikrein-like genes located on chromosome 19q13.3-4 (21) and encodes a putative protein with high sequence similarity to Siglec-5, -6, -7, and -8 (5, 7). This protein has been designated Siglec-9 based on sequence similarity and its ability to bind sialic acid (see below).

Sequencing of independent PCR products from the HL-60 cDNA library confirmed the prediction (5, 7) that this novel Siglec contains three Ig-like domains made up of an N-terminal V-set domain and two C2-set domains (Fig. 1). However, a small number of differences were found in the coding sequence (not shown), only one of which resulted in a change in the protein sequence, with a conservative substitution of Arg132 to His132 (Fig. 1). There are 8 potential N-linked glycosylation sites and a cytoplasmic tail of 94 amino acids. Overall, the coding sequence of Siglec-9 is ~84% identical to that of Siglec-7, the homology extending throughout the extracellular, transmembrane, and intracellular regions (Fig. 1).

Siglec-9 contains most of the characteristic features of the Siglec subgroup of Ig superfamily proteins. These include the critical arginine at position 120 that interacts with the carboxyl group of sialic acid and the unusual pattern of cysteines in domains 1 and 2 that form intra-sheet and inter-domain disulfide bonds (22). Interestingly, unlike Siglec-7 described previously, Siglec-9 is predicted to lack the first of the two aromatic residues that has been shown in sialoadhesin to be important for interacting with the N-acetyl moiety at the C-5 position of sialic acid (22). Since Siglec-9 binds sialic acids with a specificity that is similar to other Siglects (see below), the aromatic residue on the A strand cannot be an obligatory requirement for sialic acid binding by Siglec. This would also be

22122
Characterization of Siglec-9

Consistent with studies showing that naturally occurring and artificial modifications of sialic acid at the C-5 position can result in marked differences in Siglec recognition (23–25). Within the cytoplasmic tail, there are two conserved, putative tyrosine-based signaling motifs typical of the majority of CD33-related Siglecs. The membrane proximal motif, LQYASL, fits the ITIM consensus, (L/I/V/S)XXYXX(L/V), and is similar to the corresponding motif in CD33, LHYASL, which has been shown to be dominant in tyrosine phosphorylation and recruitment of SHP-1 (13, 14) and SHP-2 (13). In comparison, the membrane distal motif, TETYSEV, which can also be tyrosine phosphorylated and interact weakly with SHP-1 and SHP-2 (13). These sequence similarities strongly suggest that Siglec-9 can also become phosphorylated and interact with tyrosine phosphatases, but further experiments are required to explore this possibility.

Siglec-9 Mediates Sialic Acid-dependent Binding to Human Red Blood Cells and Glycoconjugates—To investigate the potential sialic acid binding properties of Siglec-9, we initially performed binding assays in which native and sialidase-treated human red blood cells were added to transiently transfected COS cells. In contrast to Siglec-7 (5), no binding could be detected unless the COS cells were treated with sialidase before the binding assays (data not shown). Sialidase treatment is thought to remove potentially inhibitory sialic acids in the COS cell glycoalyx that interact with the Siglec-binding sites in cis. It is currently unclear how Siglec-7 expressed on COS or CHO cells mediates high levels of binding without a requirement for sialidase pretreatment, but this is a feature that is not apparently shared by Siglec-9, despite the high degree of sequence identity shared between the two molecules (Fig. 1). Although the binding site of Siglec-9 appears to be masked on transfected COS cells, a recent report (26) raises the possibility that unmasking could occur on activated cells that naturally express the receptor.

To determine the sialic acid linkage preference of Siglec-9, binding assays were carried out with synthetic polyacrylamide conjugates using CHO cells that had been sialidase-treated to remove the inhibitory sialic acids (Fig. 2). Under these conditions, sialidase-treated wild-type CHO cells showed no detectable binding to glycoconjugates whereas sialidase-treated CHO cells stably expressing Siglec-9 bound similarly to glycoconjugates linked to 2,3- or 2,6-linkages. Therefore, the binding specificity of Siglec-9 appears to be similar to that of Siglec-5 and Siglec-7 (4, 5).

Expression of Siglec-9 in Tissues and on Peripheral Blood Leukocytes—A human multiple tissue Northern blot was probed with a Siglec-9 specific cDNA probe that was predicted not to cross-hybridize with other Siglecs (Fig. 3). A clear signal at 2.3 kilobases was observed with spleen and placenta but Siglec-9 mRNA was low or undetectable in liver, colon, stomach, and testis (Fig. 3). The presence of readily detectable...
mRNA transcripts in spleen and placenta is consistent with the possibility that Siglec-9 is expressed on hematopoietic cells since these organs are rich in leukocytes.

To investigate expression of Siglec-9 at the cellular level, a Siglec-9-specific mAb, K8, was isolated. Given the high degree of sequence similarity between Siglec-9 and Siglec-7 it was important to rule out cross-reactivity of K8 with Siglec-7. Using stably transfected CHO cell lines expressing either Siglec-7 or Siglec-9, FACS assays showed clearly that K8 does not cross-react with Siglec-7 (Fig. 4). Likewise, the anti-Siglec-7 mAb, S7 described previously (5), does not cross-react with Siglec-9 expressed on CHO cells (Fig. 4). The potential cross-reactivity of K8 with Siglesc-3, -5, -7, and -8 was also investigated by enzyme-linked immunosorbent assay, using Siglec-Fc proteins. No reactivity of K8 with any other Siglecs was observed, despite high binding to the relevant mAbs (data not shown). Thus, K8 appears to be specific for Siglec-9.

Next, a detailed analysis of the expression of Siglec-9 on human peripheral blood leukocytes was carried out by flow cytometry. Expression on granulocytes, monocytes, and lymphocytes was compared by gating cells according to their characteristic forward and side scatter properties (not shown). With granulocytes, ~97% of cells expressed intermediate levels of Siglec-9 (Fig. 5A). In comparison, 100% of monocytes were strongly positive. Since granulocytes contain mostly neutrophils, together with a small percentage of eosinophils, we asked whether Siglec-9 is absent from the eosinophils by carrying out double labeling in conjunction with anti-CD16, a low affinity Fc receptor that is expressed at low levels on eosinophils (7). This showed that, similar to Siglesc-5 (7), Siglec-9 is expressed on all neutrophils but is absent from eosinophils (data not shown). Interestingly, the expression of Siglesc-5 and -9 on granulocytes is reciprocal to that of Siglec-8 which is only found on eosinophils (7). With lymphocytes, two distinct labeled populations could be identified. ~26% of cells in the lymphocyte gate were weakly labeled with anti-Siglec-9, while ~2.5% were strongly labeled (Fig. 5A). To characterize the lymphocyte-reactive subsets in more detail, double labeling was carried out by combining staining for Siglec-9 with staining for CD3 (pan T cell), CD4 (T cell subset), CD8 (T cell subsets and NK cells), CD19 (pan B cell), CD16 (NK cells), and CD56 (NK cells) (Fig. 5B). Depending on the donor, the weakly labeled cells contained ~2% of the CD4-high T cells, ~5% of the CD8-high T cells, ~50% of the CD8-mid NK cells, ~50% of the CD19 B cells, ~50% of the CD56 NK cells, and ~50% of the CD16 NK cells. The strongly labeled cells in the lymphocyte gate were mostly made up of CD16-high cells (Fig. 5B). Surprisingly, these cells also expressed low levels of CD4, but they do not appear to be contaminating CD4+ monocytes because a monocyte marker, CD14, was absent from these cells (data not shown). Furthermore, this Siglec-9+, CD16-high subset of cells was also negative for Siglec-7 (data not shown) which is expressed by monocytes (5). Currently, the nature of these cells is not known. However, they were not labeled with CD56 or CD8 (Fig. 5B) and may correspond to a minor population of CD16+, CD56- cells (27) that were shown previously to have morphological features of NK cells and exhibit low levels of natural cytotoxicity (28).

Finally, FACS staining of various human leukemic cell lines was performed. Weak positive labeling was observed with the U937 promonocytic cell line (data not shown). No staining was seen with the other cell lines studied: KG1b (immature myeloid), HL-60 (myelomonocytic), MonoMac-6 (monocytic), THP-1 (monocytic), K562 (erythroleukemia), YT (NK-like), Daudi (B cell), Ramos (B cell), JY (B cell), HUT78 (T cell), and Jurkat (T cell) (data not shown). The failure of the myeloid cell lines, HL-60, MonoMac-6, and THP-1, to express Siglec-9 was surprising given the high levels on blood monocytes and neutrophils. Since the Siglec-9 cDNA was isolated from a dibutyryl cAMP-treated HL-60 cell library, it is possible that terminal differentiation of myeloid cells is required for Siglec-9 gene expression. An alternative possibility is suggested by the recent finding that treatment of normal hematopoietic progenitors and leukemic myeloid cells with anti-Siglec-3 or anti-Siglec-7 mAbs resulted in inhibition of cell growth (29). If naturally occurring ligation of Siglec-9 expressed by leukemic cells also inhibits cell growth, this could lead to selective expansion of
variant cells that express low, non-inhibitory levels of Siglec-9. Further experiments are needed to investigate these possibilities.

The results presented here with Siglec-9 extend the theme established previously, that the CD33-related Siglecs are expressed on distinct subsets of hemopoietic cells. It is striking that the ITIM-containing members of this subgroup are found at highest levels on effector cells of the innate immune system, namely neutrophils, monocytes, and NK cells. Siglec-9 is the first example of a Siglec being expressed on all three cell populations. Given that other related leukocyte receptors with ITIMs are important in negative regulation of cellular activation events (11), it is possible that the ITIM-containing Siglecs mediate similar functions via sialic acid recognition. It is thought that sialic acids appeared relatively late in evolution, being absent from many potential pathogens (30–32). In addition to their well recognized roles in cell-cell repulsion and masking of subterminal sugars (30–32), it is conceivable that sialic acids have evolved to function as molecular determinants of “self.” Thus, sialic acid-dependent ligation of Siglecs could provide a mechanism that contributes to the setting of appropriate thresholds for cellular activation. This could help prevent undesirable self-reactivity and tissue damage, while at the same time permitting effective killing of non-sialylated pathogens.

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Note Added in Proof—The results reported here are in good agreement with another paper on Siglec-9 published by Angata and Varki (Angata, T., and Varki, A. (2000) J. Biol. Chem. 275, 22127–22135) in this issue of the Journal.

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