the activity of Siglec-1 on native macrophages is measured as follows. Rat lymph node cells (2–3 × 10^6) are mixed with the [^{125}I]GT1b-polymer (2–5 n*M*) in 30 μl of 1% FCS–PBS containing 0.05% sodium azide (incubation buffer). After a 90-min incubation at room temperature, the mixture is overlaid in a plastic tube (3 mm i.d. × 45 mm) onto 200 μl of an oil mixture [dibutyl phthalate/bis(2-ethylhexyl)phthalate] (3:2, by volume) and centrifuged for 2 min at 7000 rpm. The plastic tube is cut at the center of the oil layer to isolate the lower part of the tube containing pelleted cells. Radioactivity associated with the pelleted cells is measured with a γ counter. Nonspecific binding is determined by adding an excess amount of either GT1b ganglioside (300–500 μM) or a specific antibody against Siglec-1. Macrophage content is determined by flow cytometry using appropriate marker antibodies. The GT1b-polymer exhibits saturable binding; the apparent K_d and B_{max} values are 1–2 n*M* and 1.4 f mol/1 × 10^4 macrophages, respectively (Fig. 4), allowing successful detection of the activity of Siglec-1 on native macrophages.

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[9] Probing for Masked and Unmasked Siglecs on Cell Surfaces

By ELS C. M. BRINKMAN-VAN DER LINDEN and AJIT VARKI

Introduction

Sialic acid-binding immunoglobulin superfamily lectins (Siglecs) are sialic acid binding lectins sharing certain structural features.[1–4] All are single pass type 1 transmembrane proteins with similar extracellular domains containing an N-terminal V-set Ig-like domain with the sialic acid binding site, followed by variable numbers of C2-set Ig-like domains. All

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Siglecs have a conserved arginine residue on the F strand of the V-set domain essential for optimal sialic acid recognition. Siglecs contain an unusual arrangement of conserved cysteine residues in the V-set domain and adjacent C2-set domains that probably results in a conventional intra-sheet disulfide bond in each domain, as well as an unusual interdomain disulfide bond.[5] Because each Siglec has a very distinct expression pattern in different cell types, it is thought that they perform highly specific functions. Furthermore, many Siglecs have immunoreceptor tyrosine-based motifs (ITIMs) in the intracellular domain, suggesting a role for Siglecs in inhibitory signaling. All human Siglec-encoding genes are found on human chromosome 19q and in the syntenic regions of murine chromosome 7 [except for Sialoadhesin (Sn)/Siglec-1]. The gene structures are very similar and seem to have arisen by duplication and possibly by some degree of exon shuffling. Eleven human Siglecs have been cloned and characterized,[1–4] as well as one Siglec-like molecule (Siglec-L1).[6] The more recently discovered Siglecs happen to be most similar to CD33/Siglec-3 and thus form a subset called CD33/Siglec-3-related Siglecs.[1,2]

Siglecs recognize forms and linkages of sialic acid commonly expressed on the cell surface of a wide variety of cell types (see Angata and Brinkman-Van der Linden[3] for details). Cell surface sialic acids can occupy the binding site of a Siglec molecule on the same plasma membrane (a *cis* interaction), resulting in a "masked" form of the Siglec.[7–13] Possible exceptions to this rule are Sn/Siglec-1[14] and Siglec-6, which recognizes an epitope uncommon on cell surfaces, i.e., sialyl-Tn (Neu5Acα2-6GalNAcα-).[15] Figure 1 shows a schematic representation of the masking of Siglecs on a cell surface, which complicates studies of their functions in intact cells. This chapter describes methods to probe for unmasked Siglecs as well as masked Siglecs on cell surfaces.

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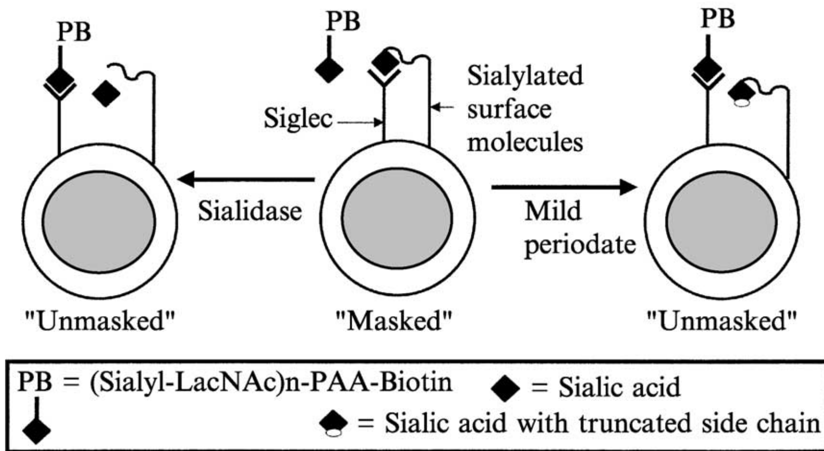

FIG. 1. Schematic representation of the masking and unmasking of Siglec activity on cell surfaces using sialidase treatment or mild periodate oxidation.

Reagents and Other Materials

- *Arthrobacter ureafaciens* sialidase (AUS, Calbiochem, La Jolla, CA, or Sigma, St. Louis, MO)
- 20 m*M* HEPES, 140 m*M* NaCl, pH 6.9 (AUS reaction buffer for cells)
- 2 m*M* sodium metaperiodate ($NaIO_4$, Sigma) freshly prepared in ice-cold phosphate-buffered saline (PBS)
- 20% glycerol (Sigma)
- 1% bovine serum albumin (BSA, fraction V, approximately 99%, Sigma) in PBS
- Fluorescein isothiocyanate (FITC)- or biotin-conjugated polyacrylamide substituted with Neu5Acα2-6Galβ1-4Glc-(6′SLL-PAA-FITC and 6′SLL-PAA-bio) or with Neu5Acα2-3Galβ1-4Glc-(3′SLL-PAA-FITC and 3′SLL-PAA-bio), the nonsialylated form (Lac-PAA) and nonconjugated forms of these PAA probes (Glycotech, Rockville, MD)
- Streptavidin–phycoerythrin (SA-PE) (Jackson ImmunoResearch Laboratories, West Grove, PA)
- RPMI-1640 medium (Gibco-InVitrogen Co., Carlsbad, CA) supplemented with 10% fetal calf serum (FCS)
- Cell activators, such as PMA (phorbol ester, Gibco), ionomycin (Calbiochem), pokeweed mitogen (Gibco), lipopolysaccharide (Fluka, Milwaukee, WI), mouse monoclonal antihuman IgM $(Fab')_2$ fragments, and antihuman CD40 (Pharmingen, La Jolla, CA)

Antibodies specific for the various Siglecs (Dako, Carpinteria, CA, BD Pharmingen, or academic laboratories)
Goat antimouse Ig antibodies with the appropriate fluorochrome (Caltag Laboratories, Burlingame, CA, or Pharmingen)
Centrifuge at 4°
FACscan machine (Beckton-Dickinson, Franklin Lakes, NJ)

Unmasking of Masked Siglecs on Cell Surfaces

Masked Siglecs on cell surfaces can be unmasked by treatment with either sialidase or mild periodate. For sialidase treatment the cells are first washed in 20 m*M* HEPES, 140 m*M* NaCl, pH 6.9, resuspended ($1–5 \times 10^6$ cellsp/ml) in this buffer, and then incubated with 20 mU AUS for 15 min at room temperature. Excess AUS is removed by washing several times with PBS/1% BSA. For mild periodate treatment (specifically truncating the glycerol side chain of sialic acid), cells are first washed with PBS, resuspended ($1–5 \times 10^6$ cells/ml) in PBS containing freshly dissolved 2 m*M* $NaIO_4$, and incubated for 30 min at 4° in the dark. Excess periodate is destroyed by adding 10 μl of 20% glycerol followed by immediate washing with PBS/1% BSA.

Probing for Siglec Sialic Acid Binding Activity on Cell Surfaces

Treated (sialidase or mild periodate) or sham-treated (buffer alone) cells ($0.5–1 \times 10^6$) are incubated with 100 μl of PBS/1% BSA containing 1–1.5 μg of the sialylated–PAA–FITC probe of interest for 1 h at 4° (on ice), followed by washing once with 1 ml of PBS/1% BSA. To obtain a stronger signal, incubation with biotinylated sialylated–PAA probes can be performed, followed by washing once with 1 ml of PBS/1% BSA and incubation with PE-conjugated streptavidin (1:100) for 30 min at 4° (on ice) in the dark. After the last washing step, binding of the sialylated–PAA probes to the cells is analyzed by flow cytometry using a FACscan machine.

To optimize binding, it is important that all steps be carried out at 4° and that all buffers and the centrifuge be kept at 4°. Furthermore, incubation with PE-conjugated streptavidin is best performed sequentially to probe incubation because precomplexing may result in a lower signal unless a very careful optimization of ratios is done. In addition to the commercial sialylated PAA probes from Glycotech, other streptavidin-based multivalent glycoprobes[16,17] can be used to probe for Siglec masking and

[16] Y. Hashimoto *et al.*, *J. Biochem. (Tokyo)* **123,** 468 (1998).
[17] K. Nakamura *et al.*, *Glycobiology* **12,** 209 (2002).

unmasking using a similar assay. In a few cases, binding requires a form of sialic acid other than Neu5Ac (e.g., mouse CD22/Siglec-2 strongly prefers Neu5Gc). In these instances, Neu5Ac-based commercial probes are not suitable, and special probes bearing the right kind of sialic acids have to be synthesized.

Confirming Sialic Acid Dependency of the Binding

A nonsialylated probe (Lac-PAA) can be used as a control, as this will not bind to any Siglec on the cell surface. However, it is possible that other receptors specific for terminal lactose units will be recognized on some cell types. An alternate control takes advantage of the fact that sialic acids with a truncated glycerol side chain will not bind to most Siglecs. For this control, 10–15 μg of the sialylated PAA probe (enough for 10 incubations of $0.5–1 \times 10^6$ cells per incubation) is first treated with 2 m*M* $NaIO_4$ (fresh) in 100 μl PBS for 30 min on ice in the dark. Subsequently, the aldhehydes formed by $NaIO_4$ treatment are reduced with 10 m*M* $NaBH_4$ (by adding 1 μl of 1 *M* stock to the 100-μl reaction mixture) in PBS for 1 h in the dark on ice. The reaction mixtures are then diluted with PBS/1% BSA to 1 ml and used directly in the probing assay as described earlier. For a (sham treatment) control, 2 m*M* IO_4 and 10 m*M* $NaBH_4$ are premixed, incubated in 100 μl PBS for 1 h on ice, and diluted with PBS/1% BSA to 1 ml. The 10–15 μg of probe is added to this mixture just before use in the assay. HPLC analysis of fluorescent 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride (DMB)–sialic acid adducts can be used to check efficiency of the treatment. A shift of ~1 min in HPLC elution of DMB–sialic acid adducts from the treated probes compared with sham-treated probes confirms truncation of the side chains of all sialic acid. HPLC runs of sham- and periodate-treated probes will also show if there is any loss of total sialic acid due to the treatment. Figure 2 shows an example of the unmasking of Siglec activity on human peripheral blood mononuclear cells (PBMCs) after sialidase treatment of the cells. The sialic acid dependency of the binding is indicated by the abrogation of probe binding after mild periodate treatment of the probe. Competition of sialylated PAA probes with nonbiotinylated sialylated PAA probes or sialyllactose is yet another way to show the sialic acid dependency of the interaction.

Unmasking of Siglecs by Activation of Cells

Various Siglecs on human blood leukocytes can be partially unmasked by cellular activation by some as yet unknown mechanism.[11,12] Cell lines such as U937 (a human monocytic leukemia) or freshly isolated human

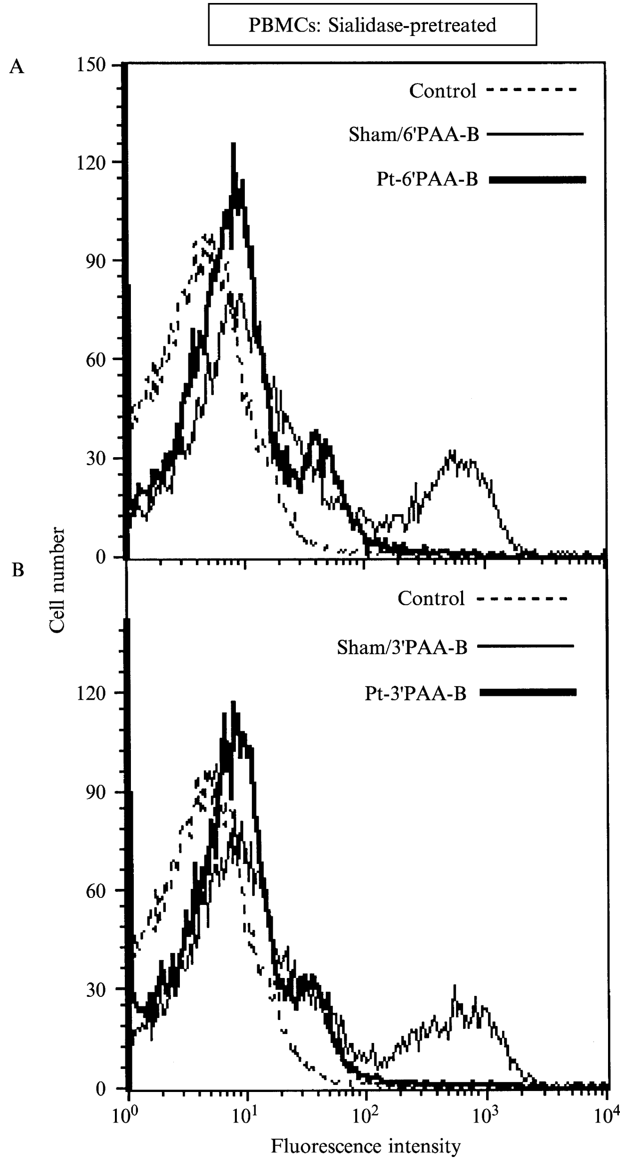

FIG. 2. Unmasking of Siglec activity on human PBMCs pretreated with sialidase. PBMCs were pretreated with sialidase, exposing binding sites for both 6′SLL–PAA (A) and 3′SLL–PAA (B). Mild periodate treatment (Pt) of the sialylated probes abolishes binding, showing the sialic acid dependency of the interaction. Detection of probe binding was by flow cytometry after incubation with SA–PE. Background control incubations were probed with SA–PE only (used with permission from Razi and Varki[12]). The probes do not bind to untreated cells (not shown).

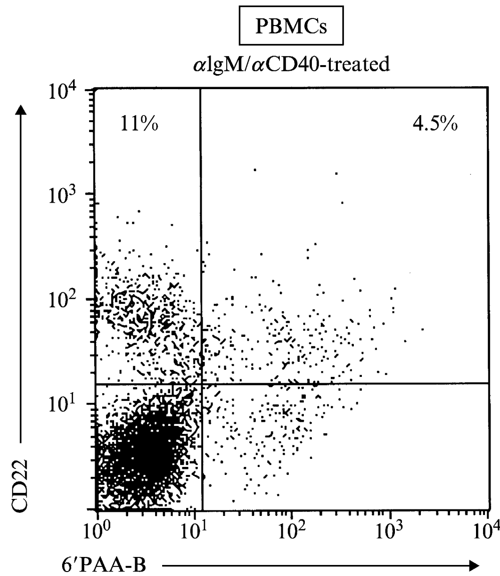

FIG. 3. Unmasking of Siglec-2/CD22 lectin sites on human PBMCs after activation of the cells. PBMCs were resuspended in RPMI-1640 supplemented with 10% FCS, incubated with anti-IgM and anti-CD40 antibodies (both at 10 μg/ml), and analyzed after 1.5 h by flow cytometry using anti-CD22–TC mAb and 6′SLL–FITC (used with permission from Razi and Varki[11]).

blood leukocytes (1×10^6 cells/ml) in RPMI-1640 medium supplemented with 10% FCS can be activated with 10–100 ng/ml PMA and 1 μM ionomycin.[12] B cells can be activated using 5 ng/ml PMA and 2 μM ionomycin, pokeweed mitogen at 10 μl/ml, lipopolysaccaharide at 15–150 μg/ml, or by mouse monoclonal antihuman IgM $(Fab')_2$ fragments at 10 μg/ml with or without mouse monoclonal antihuman CD40 at 10 μg/ml. Aliquots of cells can be removed at various time points (minutes to hours) after activation and analyzed for spontaneous Siglec unmasking using the assay described earlier. Figure 3 shows as an example the unmasking of Siglec lectin sites on human PBMCs after activation with anti-IgM/anti-CD40. It should be noted that this unmasking never achieves the levels obtained by sialidase or mild periodate treatment of cells.

Determining which Siglec Activity Is Found on the Cell Surface

Due to overlapping sialic acid linkage specificities between the Siglecs (see Angata and Brinkman-Van der Linden[3]) binding of the sialylated PAA probes cannot resolve which Siglec is revealed on the cell surface.

Furthermore, some cells have more than one Siglec on the cell surface. To establish which Siglec is being unmasked, PAA probe binding needs to be studied in the presence of blocking or nonblocking antibodies specific for each of the Siglecs.

Some anti-Siglec antibodies are blocking (i.e., they interfere with sialic acid recognition in standardized *in vitro* assays). Such antibodies can be used in unlabeled form to directly define what portion of the sialylated PAA probe binding to a cell surface is due to the Siglec in question. The sialidase-or periodate-treated cells are probed with the biotin-conjugated sialylated PAAs as described earlier in the presence or absence of an adequate concentration of the unlabeled blocking antibody. The difference in binding between the two samples indicates what fraction of the sialylated PAA probe binding is accounted for by the Siglec in question. The best control for this is an isotype-specific antibody that recognizes the same Siglec without interfering with sialic acid recognition.

Such nonblocking antibodies can also be used to determine what fraction of the cells showing the sialylated PAA probe binding also express a given Siglec. Because the probing with biotin-conjugated sialylated PAAs is visualized using flow cytometry with SA conjugated with PE, the fluorochrome used to visualize the antibody staining by flow cytometry should be anything different than PE, such as FITC or Tricolor (TC). When FITC-conjugated sialylated PAAs are used, the antibody staining should use either PE or TC as fluorochrome. The cells are first stained with a nonblocking antibody specific for the Siglec of interest, either directly conjugated to FITC, TC, or PE or unconjugated. If the primary antibody is unconjugated, this step is followed by staining with a secondary antibody conjugated to an appropriate fluorochrome. Subsequently, the probing with biotin-conjugated sialylated PAAs can be performed as described earlier. The antibody stainings are done in 100-μl aliquots containing 0.5–1×10^6 cells in PBS/1% BSA for at least 30 min at 4° (on ice). In between the incubations cells are washed once with 1 ml PBS/1% BSA. First, the optimal antibody concentrations must be determined by single stains using a titration range of the primary (mouse antihuman–Siglec) and secondary (goat antimouse–IgG) antibodies. The double stain using both anti-Siglec-2/CD22 and the 6′SLL–PAA probe indicates that part of the 6′SLL–PAA interaction to PBMCs is due to the unmasking of Siglec-2/CD22 (see Fig. 3), as induced by activation with Anti-IgM/Anti-CD40.