

## Cryptic sialic acid binding lectins on human blood leukocytes can be unmasked by sialidase treatment or cellular activation

Nahid Razi and Ajit Varki<sup>1</sup>

Glycobiology Program and Cancer Center, Divisions of Hematology-Oncology, and Cellular and Molecular Medicine, University of California San Diego, La Jolla, CA, USA

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**We recently reported that the sialic acid-specific binding sites of CD22 molecules on B cells are masked by endogenous ligands, and can be unmasked by sialidase treatment or cellular activation. Here, we show that many other human blood leukocyte types have endogenous sialic acid binding sites that can be unmasked by sialidase treatment. Truncation of sialic acid side chains on the soluble probes used for detection abolishes all binding, indicating the specificity of the interaction for the details of sialic acid structure. There is limited overlap between  $\alpha$ 2-6- and  $\alpha$ 2-3-sialic acid-specific binding sites, which are unmasked on monocytes, natural killer cells, a minority of mature T cells, neutrophils, and some cultured human leukemic cell lines. Activation with phorbol ester and calcium ionophore causes spontaneous exposure of some of the binding sites, occurring over a period of minutes on neutrophils and several hours on monocytes and U937 leukemia cells. Activation is accompanied by some evidence for desialylation of cell surface molecules. Thus, many human blood cells have specific binding sites for sialic acids, masked by endogenous sialylated ligands. Cellular activation can unmask these sites, possibly by the action of an endogenous sialidase. The nearly universal masking of such sites in unactivated blood cells could explain why many of these sialic acid-binding lectins have not been previously discovered. Similar considerations may apply to sialic acid binding lectins of other cell types and tissues.**

*Key words:* human/blood/cell-to-cell interactions/cellular activation/neutrophils

### Introduction

Sialic acids are a family of 9-carbon acidic sugars that are ubiquitously expressed on vertebrate cell surfaces (Varki, 1992; Kelm and Schauer, 1997). Given their location, their remarkable structural diversity and the variety of their linkages to underlying sugar chains, it is not surprising that sialic acids are recognized with exquisite specificity by many viral hemag-

glutinins and bacterial adhesins (Karlsson, 1995; Varki, 1997b). It is reasonable to suppose that this diversity might also be utilized to generate biological ligands for endogenous sialic acid-binding lectins. A decade ago, the only known vertebrate sialic acid binding lectin was the H protein of the alternative complement pathway (Pangburn and Muller-Eberhard, 1978; Kazatchkine *et al.*, 1979; Ram *et al.*, 1998). The discovery and characterization of the selectins (Rosen and Bertozzi, 1994; Lasky, 1995; Nelson *et al.*, 1995; Tedder *et al.*, 1995; Butcher and Picker, 1996; Kansas, 1996; Lowe and Ward, 1997; McEver and Cummings, 1997) added three vertebrate “C-type” lectins that utilized sialic acids as part of their ligands. However, the only requirement for selectin recognition of sialic acids seems to be the negatively charged carboxylate at the 1-position, and the adjacent  $\alpha$ 2–3 linkage (Rosen and Bertozzi, 1994; Varki, 1994; Lasky, 1995; Nelson *et al.*, 1995; Crocker and Feizi, 1996; Kansas, 1996; Vestweber, 1996; McEver and Cummings, 1997; Varki, 1997a). The discovery of the sialic acid-binding property of human CD22 (SgROI *et al.*, 1993; Powell *et al.*, 1993) and the purification and cloning of mouse macrophage sialoadhesin (Crocker *et al.*, 1991, 1994) led to the realization that Ig-superfamily members other than immunoglobulins can specifically recognize vertebrate oligosaccharides (Powell and Varki, 1995). Amongst these “I-type” lectins, CD22 and sialoadhesin are part of a distinct subset that specifically bind sialic acids (Kelm *et al.*, 1994a,b; Powell and Varki, 1995; Crocker *et al.*, 1996), and have been recently renamed as the Siglecs (for sialic acid binding immunoglobulin superfamily lectins) (Crocker *et al.*, 1998). Apart from sialoadhesin (siglec-1) and CD22 (siglec-2), the other members are CD33 (siglec-3), myelin associated glycoprotein (siglec-4a), and Schwann cell myelin protein (siglec-4b). (Dulac *et al.*, 1992; Crocker *et al.*, 1994, 1995, 1997; Kelm *et al.*, 1994a,b; Powell and Varki, 1994, 1995; Sjöberg *et al.*, 1994; Tchilian *et al.*, 1994; Hanasaki *et al.*, 1995a,b; Freeman *et al.*, 1995; Powell *et al.*, 1995; Crocker and Feizi, 1996; Kelm *et al.*, 1996; Shi *et al.*, 1996; Yang *et al.*, 1996; Collins *et al.*, 1997a,b). An additional member of the family (siglec-5) has recently been discovered by database homology searching (Cornish *et al.*, 1998). Throughout this family of molecules, the sialic acid binding property appears to be mediated primarily by an amino-terminal V-set Ig domain, with some contribution by the next C2-set domain (Engel *et al.*, 1993, 1995; Kelm *et al.*, 1994b; Law *et al.*, 1995; Nath *et al.*, 1995; Van der Merwe *et al.*, 1996; Vinson *et al.*, 1996). Available data suggest that recognition involves the entire sialic acid molecule, including the carboxylate group at the 1-position, the linkage from the 2-position, the N-acyl group at the 5-position, and the exocyclic hydroxyl groups at the 7-, 8-, and 9-positions

<sup>1</sup>To whom correspondence should be addressed

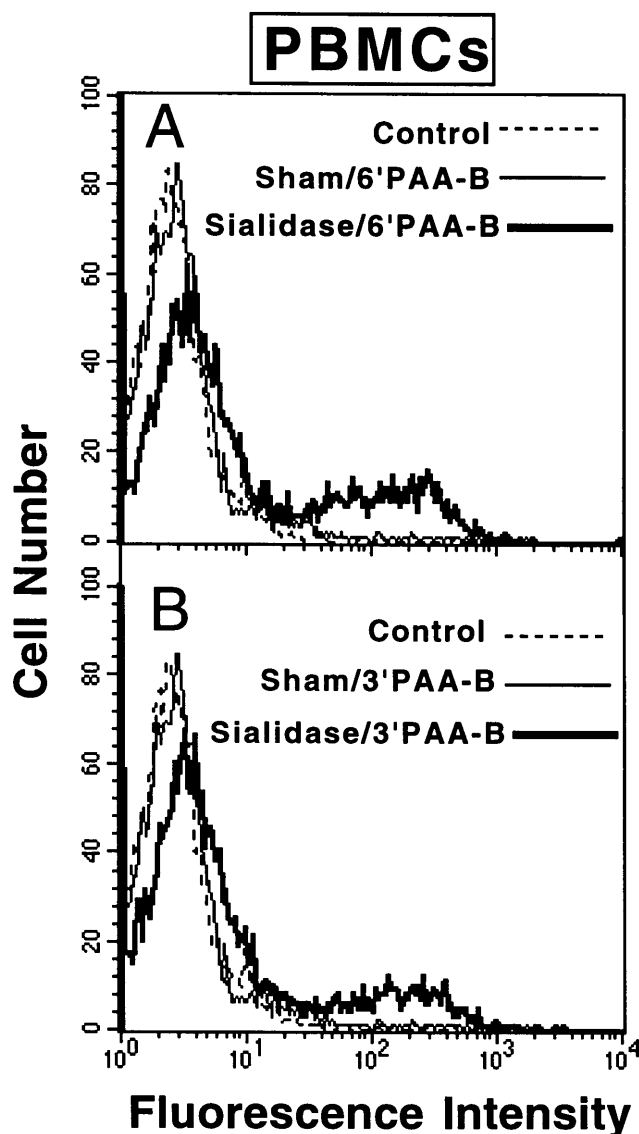
(Kelm *et al.*, 1994b, 1998; Powell and Varki, 1994; Sjoberg *et al.*, 1994; Hanasaki *et al.*, 1995a,b; Powell *et al.*, 1995; Shi *et al.*, 1996; Yang *et al.*, 1996; Collins *et al.*, 1997a,b; Crocker *et al.*, 1997; Kelm *et al.*, 1998) In the case of sialoadhesin (siglec-1), a crystal structure has been elucidated in the presence of bound ligand, confirming that all of these aspects of the sialic acid molecule are actually in direct contact with the binding site (May *et al.*, 1998).

The question arises as to why so few endogenous vertebrate sialic acid binding lectins have been discovered to date, despite exploration by many investigators over a long period of time. One possibility is that sialic acids are so common on cell surface and secreted glycoconjugates (Kitagawa and Paulson, 1994; Hanasaki *et al.*, 1995a) that they cannot be effectively used as discriminable biological ligands for endogenous functions. Another possibility is that, in order to prevent undesirable interactions with inappropriate targets, such lectins are kept in a masked state until they are ready to be used in specific situations. With regard to the latter possibility, masking by sialylated ligands on the same cell surface has been reported for the lectin activities of several siglecs when they were studied as recombinant forms expressed in cultured cells (Freeman *et al.*, 1995; Hanasaki *et al.*, 1995b; Sgroi *et al.*, 1996; Collins *et al.*, 1997b; Tropak and Roder, 1997). We recently extended this experimental paradigm to a more natural situation, showing that the CD22 lectin on resting B cells from normal human peripheral blood is naturally masked from detection by a soluble sialylated probe (Razi and Varki, 1998). The masking appears to be due to endogenous sialylated ligands that can be removed by treatment of the cell surface with a bacterial sialidase. Interestingly, partial unmasking of the lectin sites could also be achieved by pharmacological or physiological activation of the B cells. This provides a mechanism by which the CD22 lectin can regulate its exposure and function, within a milieu of natural glycoproteins that carry copies of the basic ligand structure, and are therefore low affinity ligands (Hanasaki *et al.*, 1995a). Thus, the receptor could expose its activity only when it is really needed, perhaps only in a protected environment, away from potential inhibitors. Here we show that the situation with CD22 can be extended to sialic acid binding lectins on many blood cell types, several of which were not previously known to express such lectins.

## Results

### *Sialidase treatment unmasks binding sites for sialyl-6'- and 3'-lactose probes on human PBMCs*

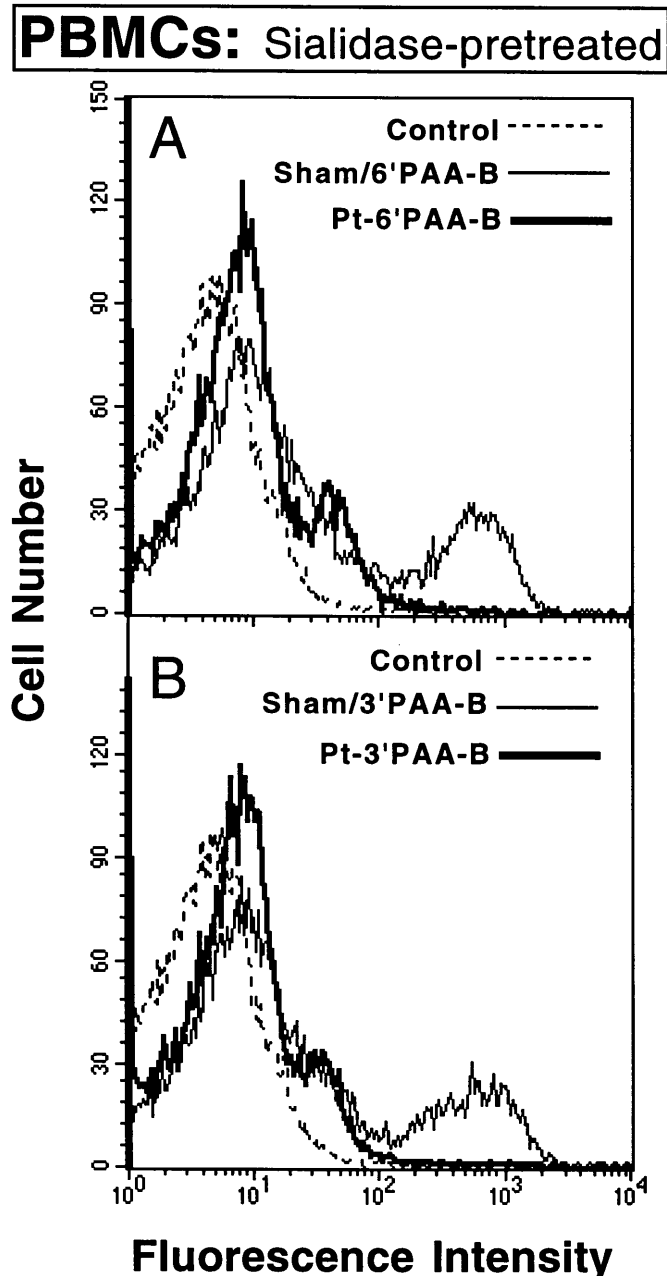
Mononuclear cells from the peripheral blood of normal human volunteers were washed, treated with sialidase, washed again, and then probed with biotinylated polyacrylamide molecules substituted with arrays of sialyl-6'- or 3'-lactose (hereafter called 6'PAA-B and 3'PAA-B, respectively). As can be seen from Figure 1, both the 6' and 3' probes detect binding sites on a subpopulation of the PBMCs, but only after sialidase treatment. We have previously shown that the 6'PAA-B can detect CD22 lectin binding sites that are unmasked on normal human peripheral blood B cells by sialidase treatment (Razi and Varki, 1998). The only other known sialic acid binding lectin on human peripheral blood cells is CD33. However, this lectin



**Fig. 1.** Sialidase pretreatment of PBMCs unmasks lectin activity recognizing  $\alpha 2$ -3 and  $\alpha 2$ -6-linked sialic acids. Freshly isolated PBMCs were washed, treated with sialidase, washed again, then probed with 3' or 6'-PAA-B followed by streptavidin-PE, and analyzed by single color flow cytometry as described under *Materials and methods*. Background control incubations were probed with the secondary reagent alone.

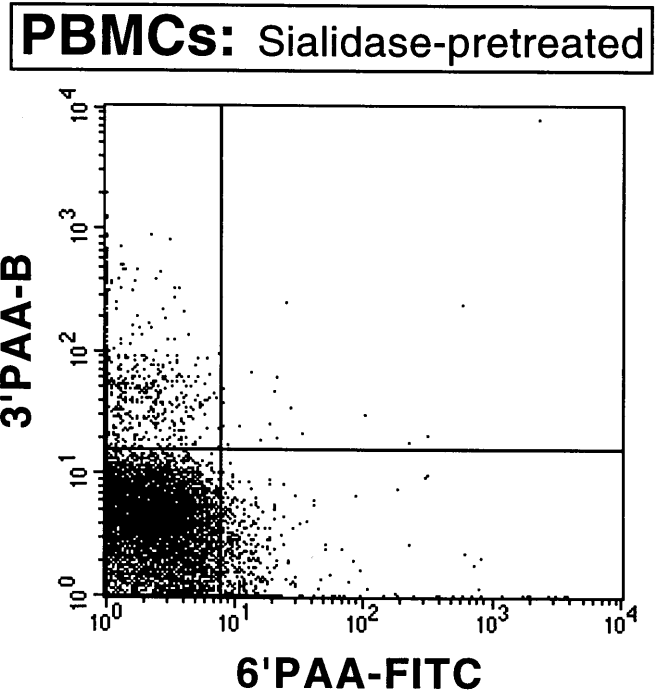
has a very low affinity, and cannot be detected with soluble probes even when overexpressed in recombinant form on sialidase-treated COS cells (Cornish *et al.*, 1998). Thus, the 3'PAA-B binding must be due to other previously unknown lectins. Also, since B cells represent a minority (typically ~10%) of these mixed populations, at least some sites detected by the 6'PAA-B probe must be due to novel lectins. Likewise, siglec 5 was found on monocytes (Cornish *et al.*, 1998), which also form a minor component of the PBMC mixture.

The sialidase preparation used has no known contamination with proteases or other glycosidases, and similar results were obtained using another sialidase from *Clostridium perfringens* (data not shown). However, the apparent unmasking of sialic



**Fig. 2.** Mild periodate oxidation of the sialylated probes abolishes binding to the sites exposed by sialidase treatment. Sialidase-treated PBMCs were probed with 3' or 6'-PAA-B that had been previously treated or sham-treated with mild periodate, as described under *Materials and methods*. Detection of probe binding was by flow cytometry after incubation with SA-PE. Background controls incubations were probed with the secondary reagent alone.

acid binding sites on the PBMCs could also be due to a general loss of negative charge repulsion resulting from the desialylation of the cell surface. To rule out this possibility, we pretreated aliquots of the PBMCs with mild periodate oxidation under conditions that are known to selectively oxidize and cleave the side chain of cell surface sialic acids, without affecting the negative charge of the sialic acids, or any of the underlying sugar chains (Van Lenten and Ashwell, 1971a,b).

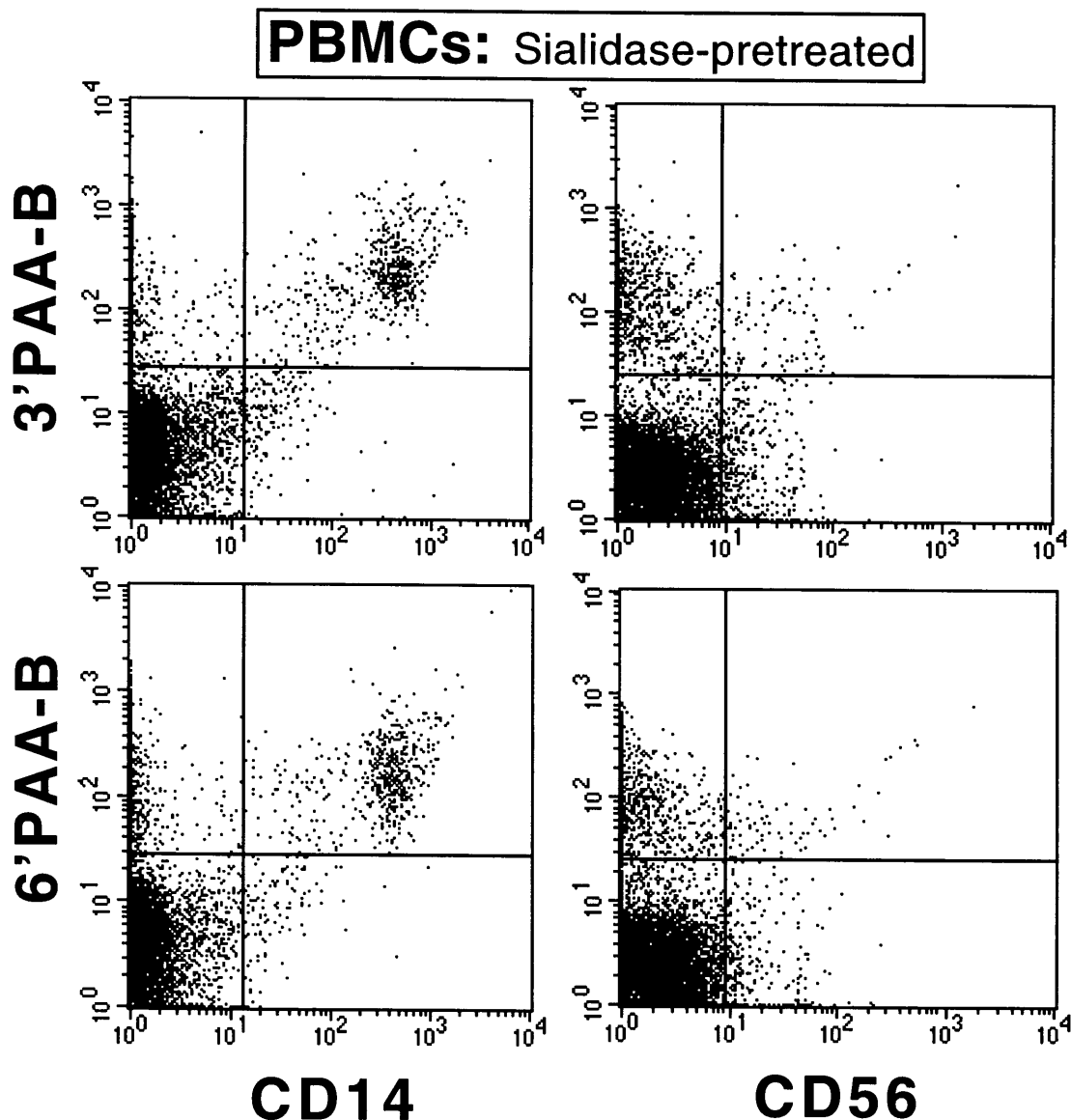


**Fig. 3.** Sialidase pretreated PBMCs show partial overlap between  $\alpha$ 2-3 and  $\alpha$ 2-6-linked sialic acid binding sites. Sialidase-treated PBMCs were simultaneously probed with 3'PAA-B and 6'PAA-FITC as described under *Materials and methods*. Detection of probe binding was by flow cytometry after incubation with SA-PE. Note that staining intensity with the directly conjugated 6'PAA-FITC probe is somewhat weaker than with 6'PAA-B.

Although this chemical approach was not as efficient as sialidase treatment, we found that binding sites were also exposed by this method (data not shown).

*Truncation of sialic acid side chains of the sialylated probes by mild periodate oxidation abolishes binding*

To confirm that probe binding to sialidase-treated cell surfaces involves more than just the negative charge of the probes, we pretreated the probes themselves with mild periodate under similar conditions as above, causing selective truncation of the sialic acid side chains on the sialyllactose units. After the treatment, the residual periodate was destroyed by an excess of glycerol. Sham treatment involved mixing of the periodate and glycerol prior to addition to the probe. As shown in Figure 2, prior periodate oxidation of either the 6' or 3'-PAA-B probes abolished the binding to desialylated PBMCs. These data indicate that all of the unmasked binding sites detected by these probes require their sialic acid side chain for the interaction. This finding rules out a non-specific interaction based on negative charge only, and indicates a binding phenotype typical of the siglec family (which require the sialic acid side chain for recognition) (Powell *et al.*, 1993; Kelm *et al.*, 1994a, 1996, 1998; Powell and Varki, 1994, 1994, 1995; Sjoberg *et al.*, 1994; Hanasaki *et al.*, 1995a,b; Powell *et al.*, 1995; Crocker and Feizi, 1996; Shi *et al.*, 1996; Yang *et al.*, 1996; Collins *et al.*, 1997a,b), rather than that of the selectins (wherein binding is unaffected, or even enhanced upon oxidation of the side chain) (Varki, 1994).



**Fig. 4.** Subpopulations of monocytes and NK cells carrying the unmasked sialic acid binding sites. Sialidase-treated PBMCs were probed with 3' or 6'-PAA-B and with Tricolor-labeled antibodies directed against CD14 or CD56 as described under *Materials and methods*. The 6'- or 3'-probe binding was detected with SA-PE. The figure shows examples of some of the double-color flow cytometry profiles obtained.

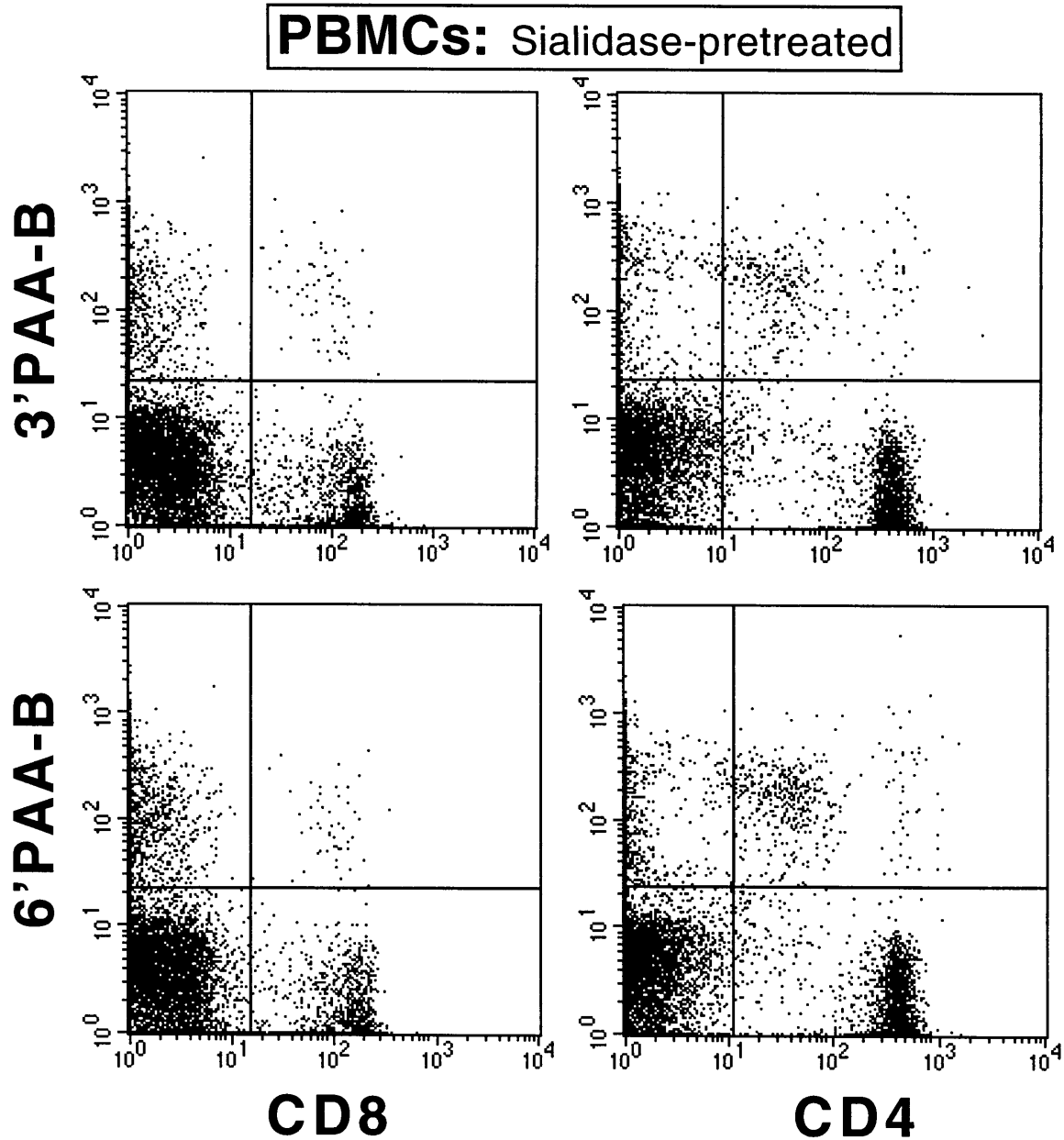
*There is limited overlap between the unmasked binding sites for 6' and 3'-PAA*

To date, most reported siglecs and selectins have also shown a preference for the linkage of the cognate sialic acid residue to the underlying sugar chain (Powell *et al.*, 1993, 1995; Kelm *et al.*, 1994a, 1996, 1998; Powell and Varki, 1994, 1995; Sjoberg *et al.*, 1994; Hanasaki *et al.*, 1995a,b; Crocker and Feizi, 1996; Shi *et al.*, 1996; Yang *et al.*, 1996; Collins *et al.*, 1997a,b). Thus, while CD22 strongly prefers an  $\alpha$ 2-6 linkage, CD33, MAG, and Sn all prefer an  $\alpha$ 2-3 linkage (with sialoadhesin, an  $\alpha$ 2-8 linkage also permits some recognition, and siglec 5 may be able to bind both linkages). To examine this issue for the newly exposed binding sites on the sialidase-treated PBMCs, we incubated the desialylated PBMCs with a mixture of 6'-PAA-FITC and 3'-PAA-B probes. As shown in Figure 3, only

a limited overlap was seen between the cells binding the two kinds of probes. This indicates that most of the exposed binding sites are highly selective for the sialic acid linkage recognized.

*Binding sites are expressed on several leukocyte types not previously known to carry sialic acid binding lectins*

To examine which blood cell types express the unmasked sialic binding sites, we carried out two-color flow cytometry analysis using cell surface markers specific for various types of leukocytes, including CD19 (B cell-specific), CD14 (monocyte-specific), CD56 (natural killer cell-specific), and CD3 (T cell-specific). As expected, a subpopulation of the CD19 positive B cells have binding sites for the 6'-PAA-B probe (presumably those cells carrying CD22, data not shown). As seen

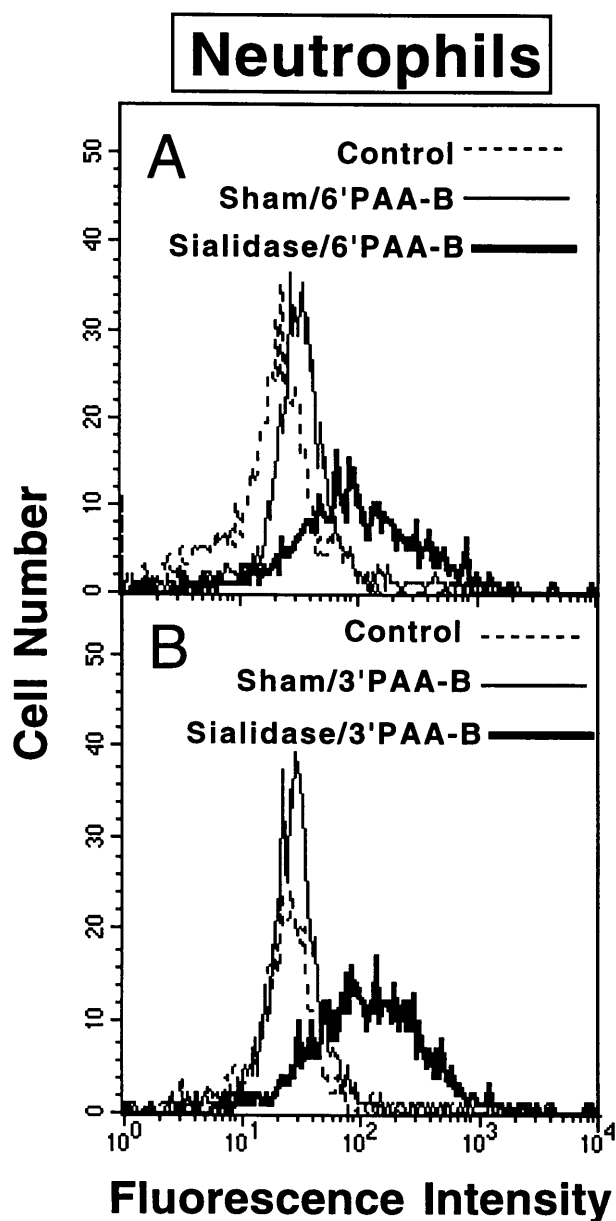


**Fig. 5.** Expression of CD4 and CD8 on cells carrying the unmasked sialic acid binding sites. Sialidase-treated PBMCs were probed with 3' or 6'-PAA-B and with Cyochrome-tagged antibody directed against CD4 or an FITC-tagged antibody against CD8 as described under *Materials and methods*. The 6'- and 3' probe binding were detected with SA-PE. The figure shows examples of the double-color flow cytometry profiles obtained.

in Figure 4, the 6'-PAA-B and 3'-PAA-B probes also showed binding to subpopulations of CD14<sup>+</sup> monocytes and CD56<sup>+</sup> NK cells. There was limited binding to a subset of CD3<sup>+</sup> positive T cells (data not shown). To further study the subsets of mature T cells, we explored the distribution of binding sites on CD4 and CD8-positive cells. As shown in Figure 5, the unmasked sialic acid binding sites were enriched on the CD4<sup>lo</sup> population (staining with anti-CD4 in the fluorescence intensity range  $10^1$ – $10^2$ ) and only to a limited extent on the mature CD4<sup>hi</sup> (fluorescence intensity range  $10^2$ – $10^3$ ) and CD8<sup>+</sup> T cells. In human peripheral blood, these CD4<sup>lo</sup> cells are known

to represent mostly monocytes, with a minor component of dendritic cells and immature T cells. Thus, the data indicate that only a small subpopulation of mature CD4<sup>hi</sup> and CD8<sup>+</sup> T cells carry these masked sialic acid binding sites. However, these binding sites appear to be specific, because these cells were not detected when using control anti-mouse antibodies (data not shown).

Up to this point, all of our studies had excluded one other type of peripheral blood leukocyte, the neutrophil, which is not enriched for in the standard PBMC preparation. We therefore turned to the use of a modified Ficoll gradient that allows the



**Fig. 6.** Sialidase treatment of neutrophils unmasks lectin activity for  $\alpha$ 2–3 and  $\alpha$ 2–6-linked sialic acids. Freshly isolated neutrophils were washed, treated with or without sialidase, washed again, and then probed with 3' or 6'-PAA-B followed by streptavidin-PE, and analyzed by single color flow cytometry as described under *Materials and methods*. Background control incubations were probed with the secondary reagent alone.

simultaneous isolation of neutrophils in a separate band. As shown in Figure 6, neutrophils also carry binding sites for both the 6'- and 3'-PAA-B probes, that are unmasked upon sialidase treatment.

#### *Some cultured human leukemia cell lines also have sialic acid binding sites*

It is cumbersome to routinely isolate large quantities of peripheral blood mononuclear cells from normal humans for detailed studies of these sialic acid binding sites. We therefore asked if established malignant human leukemic cell lines express such

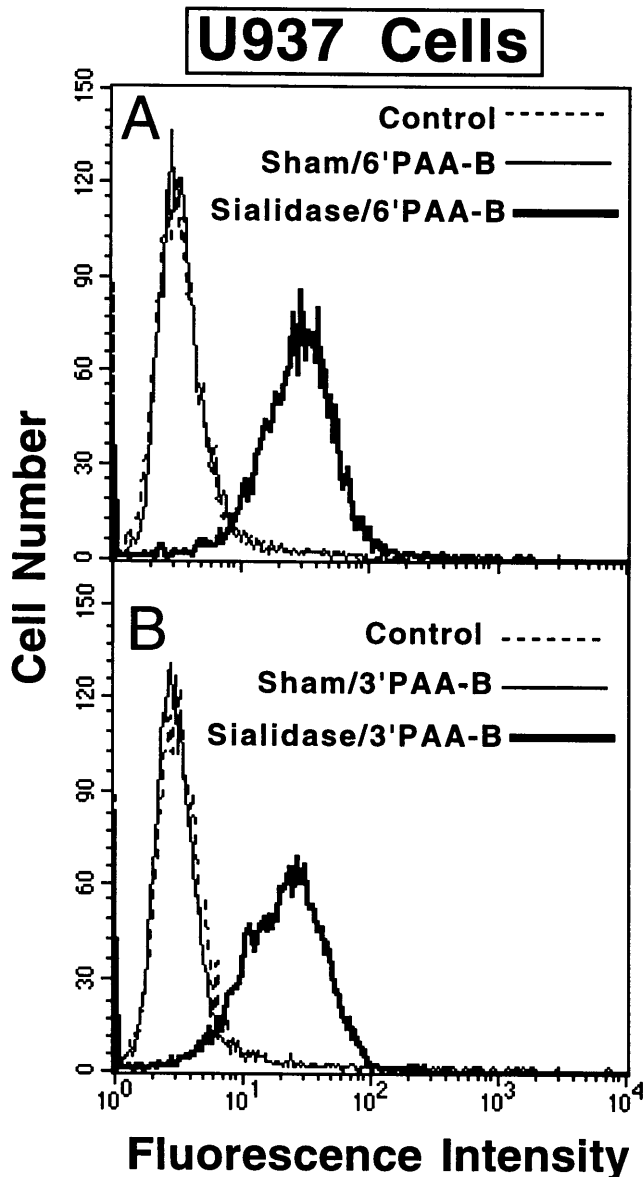
sites. Based on the results obtained above from the normal cells, we chose to study Jurkat, MOLT-4, and CEM CD4+ human T cell leukemia cells, and U937 cells, a human monocytic leukemia line. Among these cell types, sialic acid binding sites were found on U937 and CEM cells. As shown in Figure 7, U937 cells had easily detectable binding sites for both the 6'- and 3' probes, which were unmasked by sialidase. Among the T cell lines, only CEM cells expressed low levels of unmasked binding sites that were expressed whether or not prior sialidase treatment was done (data not shown). However, this low level of binding to CEM cells was only present when the cells were grown in serum-free media, and the matter was not pursued further.

#### *Cellular activation causes spontaneous exposure of lectin binding sites*

With the exception of the low levels of exposed binding sites found in CEM cells, all other sites detected in both normal and leukemic cells were masked, and needed to be exposed by exogenous addition of sialidase. We next asked if these binding sites could be spontaneously exposed upon cellular activation. Pharmacological activation of normal PBMCs or U937 cells with PMA/ionomycin exposed some binding sites for both the 6'- and 3' probes (data not shown). The kinetics of this unmasking occurred over a period of hours, with substantial further exposure occurring in the U937 cells after several days (data not shown). Since this unmasking is occurring within a mixture of multiple cell types in the PBMCs and over a long period of time in both cultures, we did not pursue the mechanistic details further at this point. The same treatment gave partial exposure of both 6'- and 3'-PAA-B binding sites on neutrophils over a very short period of time (see Figure 8, maximum exposure in 15 min). These experiments indicate that the phenomenon of unmasking of sialic acid binding sites upon activation that we first observed in B cells (Razi and Varki, 1998) can be extended to some other blood cell types.

#### *Does an endogenous sialidase activity explain the unmasking of binding sites?*

There are several prior reports indicating the activation of cell surface sialidases and/or the shedding of cell surface sialic acids upon activation of T cells, B cells, and neutrophils (see *Discussion*). To pursue the role of such sialidases in the unmasking of sialic acid binding sites, we choose to focus on neutrophils, since unmasking is very rapid, and cannot be ascribed to new synthesis of receptor molecules. Activation of neutrophils was associated with a variable but small increase in binding sites for peanut agglutinin (PNA, which binds nonsialylated Gal $\beta$ 1–3GalNAc units) and a small decrease in binding of *Maackia amurensis* lectin II (MAL-II, which binds molecules with terminal  $\alpha$ 2–3-linked sialic acids). These data (not shown) suggest the rapid action of an endogenous sialidase that appears to be acting at the cell surface. To examine if the unmasking of sialic acid binding sites is mediated by this sialidase, we carried out the activation experiments in the presence of the potent bacterial and mammalian sialidase inhibitor 2,3 dehydro-2,6 anhydro-N-acetyl-neuraminic acid (Neu2en5Ac) (Warner *et al.*, 1991,1993). However, even when adding this inhibitor at 5 mM concentration, there was no effect on the appearance of the PNA-binding sites, nor the loss of the MAL-II binding sites. The inhibitor also did not suppress the

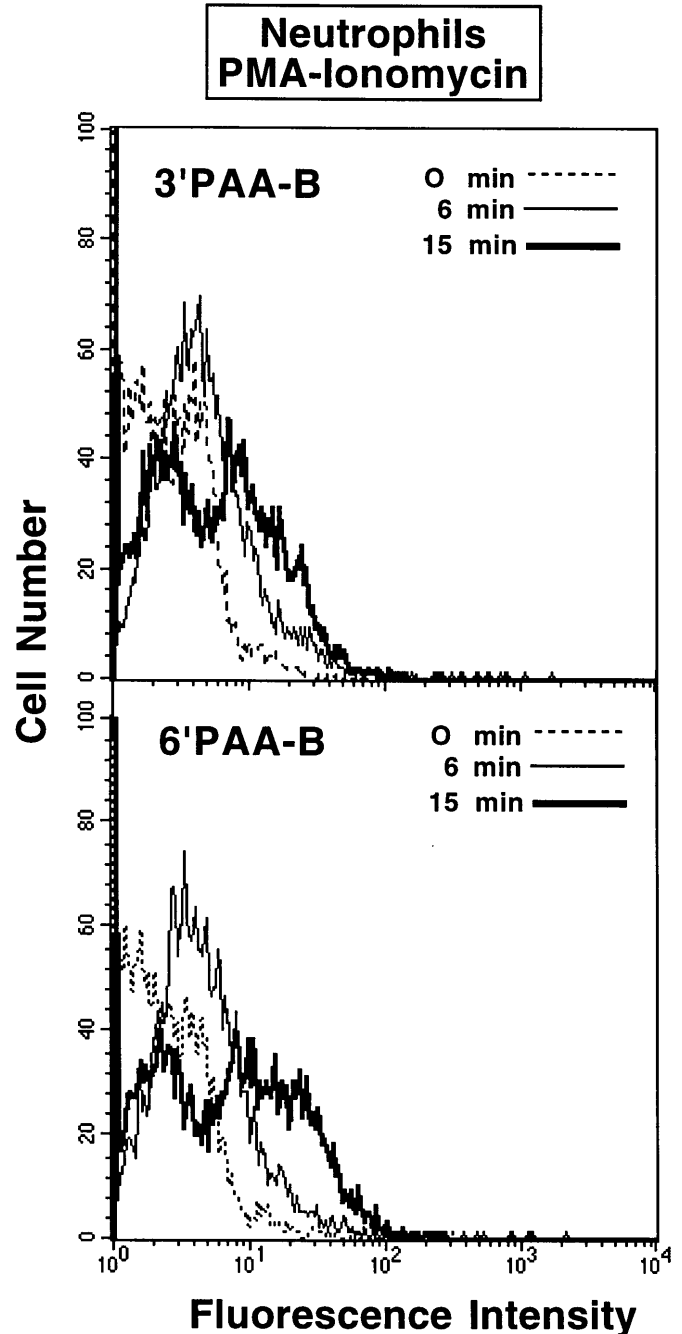


**Fig. 7.** Expression of sialic acid binding sites in U937 human leukemia cells. Cultured U937 leukemia cells were washed, treated with or without sialidase, washed again, then probed with 3' or 6'-PAA-B followed by streptavidin-PE and analyzed by single color flow cytometry as described under *Materials and methods*. Background control incubations were probed with the secondary reagent alone.

unmasking process in U937 cells or PBMCs (data not shown). Thus, if a sialidase is responsible for the unmasking, it is not susceptible to this inhibitor.

### Discussion

We have previously shown that biotinylated  $\alpha 2-6$  sialyllactose-substituted polyacrylamide arrays can be used to specifically probe the lectin binding site of cell surface CD22 on normal and leukemic B cells. Detection of this activity required prior unmasking by sialidase treatment or cellular



**Fig. 8.** Unmasking of lectin sites on neutrophils by treatment with PMA and Ionomycin. Neutrophils were resuspended in complete RPMI, incubated with PMA and Ionomycin, probed at different time points with 3' or 6'-PAA-B followed by streptavidin-PE, and analyzed by single color flow cytometry as described under *Materials and methods*. The single-color flow cytometry profiles shown are examples from selected time points. Exposure tended to be maximal at 15 min. A control incubation without PMA and Ionomycin at 0 min is shown, similar profiles were obtained with streptavidin-PE alone at all time points (data not shown).

activation (Razi and Varki, 1998). Here we use biotinylated  $\alpha 2-6$  and  $\alpha 2-3$  sialyllactose-substituted polyacrylamide probes to detect additional masked binding sites on a variety of human blood cell types. In all cases, binding was abolished simply by truncating the side chain of the sialic acid residues

on the probe, indicating that recognition involves not just the acidic charge of the sialic acid carboxylate, but also the rest of the molecule. This requirement for the exocyclic side chain of sialic acids is typical for most siglec family members described so far, as well as for the Complement H protein. The only two previously reported sialic binding lectins on circulating human blood cells are CD22 and CD33. The latter has a very poor affinity, and cannot be detected using these types of probes, instead requiring the use of intact cell assays (Cornish *et al.*, 1998). Thus, all of the binding sites uncovered in this study on NK cells, neutrophils, monocytes (and possibly a few mature T cells) must represent previously unknown sialic acid binding lectins. In this regard, another group just reported the cloning of a new siglec-5 that is present on some monocytic cells and on neutrophils (Cornish *et al.*, 1998). This lectin may account for some but not all of the binding sites that we have uncovered. The binding sites on some NK cells could reflect activity of the previously described NK cell lectins (Bezouska, 1996; Lanier, 1998), whose natural ligands are unknown. The small number of mature T cells with specific binding sites also remains unexplained.

Because of the potentially multivalent nature of the interactions on both sides (probe and cell surfaces) we cannot estimate the true affinity of binding. The existence of binding sites for both  $\alpha 2-6$  and  $\alpha 2-3$  binding sites on such a variety of different blood cell types might also raise some initial concerns about the significance of the result. However, the double label and competition experiments as well as the loss of binding upon mild periodate oxidation of the probes indicate substantial specificity of the interactions. In almost all instances, we also found that the sialic acid binding sites were masked by endogenous ligands and could be unmasked by sialidase treatment. We also show that cellular activation results in spontaneous unmasking of a portion of such binding sites. The kinetics of this unmasking were slow (over a period of hours) for monocytic cells and PBMCs and very rapid (over a period of minutes) for neutrophils. The question arises as to how the unmasking takes place. New synthesis of sialic acid binding lectin molecules cannot explain the findings in neutrophils, where the response occurs within minutes. In the case of PBMCs and the monocytic leukemia cell line, maximum exposure occurs over a period of many hours, and therefore could represent new synthesis. However, the starting cells already have a substantial amount of masked sites, making it unnecessary to invoke this mechanism. The most logical possibility is that unmasking is due to the action of an endogenous sialidase. Indeed, prior studies have suggested the existence of lymphocyte sialidases that can affect activation and cell:cell interactions (Guthridge *et al.*, 1994; Chen *et al.*, 1997) and a neutrophil sialidase (Cross and Wright, 1991) that is released upon activation. We were able to show that some cell surface desialylation is indeed taking place rapidly upon activation of neutrophils. However, our attempts to prevent the unmasking of monocytes, neutrophils, and U937 cells by a well-known inhibitor of sialidases were not successful. Based on changes in cell surface lectin binding, it appears that this inhibitor is not very active against this particular sialidase. Better inhibitors and/or sialidase-deficient cells are required to pursue this matter further. There could of course be additional cooperating mechanisms, such as a conformational change in the lectin molecules resulting in improved binding, an induced clustering of lectin molecules generating enhanced binding of the multivalent

probe, the action of a selective protease that cleaves the endogenous ligands, conformational changes of endogenous masking ligands which lower their affinity, or a redistribution of the lectins to a privileged site on the cell surface (e.g., the filopodia).

Regardless of the mechanism involved, we can also ask when (during the life of each cell) this unmasking actually occurs *in vivo*, and what the biological consequences might be. Answering these questions will require the isolation, cloning and full characterization of each of the proteins with novel sialic acid binding sites that we have detected. Logical approaches include affinity purification and/or expression cloning after sialidase treatment to remove potentially masking sialic acid residues. Of course, some of these molecules may turn out to be previously cloned leukocyte cell surface proteins whose sialic acid binding properties were missed earlier, because of masking by endogenous ligands.

## Materials and methods

### *General chemicals and biologicals*

These were mostly from Sigma or Oxford GlycoSystems. The others were phorbol ester (PMA)<sup>2</sup> from GIBCO/BRL and Ionomycin from Calbiochem.

### *Antibodies and probes*

Tri-color-conjugated anti-human CD22, CD14, CD56, and CD19 mAbs, Caltag Laboratories; Cychrome-tagged anti human CD4 and FITC-tagged antihuman CD8, Pharmingen, and Phycoerythrin-conjugated streptavidin (SA-PE), Boehringer-Mannheim. The FITC or biotin-conjugated polyacrylamide substituted with  $\alpha 2-6$  sialyllactose (6'PAA-FITC and 6'PAA-B, respectively), its analog with  $\alpha 2-3$  Sia substitution, (3'PAA-B), a nonsialylated version (Lac-PAA), and non-biotinylated forms of all conjugates were from GlycoTech. The sialidase inhibitor 2,3 dehydro-2,6 anhydro-N-acetyl-neuraminic acid (Neu2en5Ac) was from Calbiochem.

### *Peripheral blood mononuclear preparations*

Fresh peripheral blood samples from normal human volunteers and mononuclear cells were separated by a standard Ficoll-Hypaque density gradient, diluted 1/1 with serum-free RPMI medium, followed by two washes with the same medium. In other studies, we used Mono-Poly Resolving Medium Ficoll-Hypaque (ICN Biomedical, Inc.) according to the manufacturer's instructions (except that centrifugation speed and time were adjusted for optimum separation), allowing the simultaneous separation of two bands, one containing mononuclear cells, and the other containing neutrophils.

### *Cell lines*

U937 cells were cultured in RPMI 1640 medium with 10% FCS and l-glutamine. T cell leukemia lines CEM, MOLT-4 and Jurkat were grown in serum-free medium supplemented with 1% ITS (insulin, transferrin, and selenium) supplement (Sigma).

### *Sialidase treatments*

Cells were resuspended in serum-free RPMI medium ( $1-5 \times 10^6$  cells/ml) containing 0.05 M HEPES, pH 6.9 and incubated for 15 min at room temperature (RT) with 20 mU of *Arthrobacter ureafaciens* sialidase (AUS). Excess sialidase



was removed by washing several times with serum-free RPMI, followed by washing with the staining buffer.

#### Mild periodate treatment

Cells were washed with PBS and resuspended ( $1-5 \times 10^6$  cells/ml) in phosphate buffer pH 7.4 containing freshly dissolved 2 mM NaIO<sub>4</sub>, and incubated for 30 min at 4°C in the dark. Excess periodate was destroyed by adding 10 µl of 20% glycerol followed by immediate washing with staining buffer. Treatment of the probes with mild periodate followed a similar protocol. In each case, the “sham” treatment consisted of premixing the periodate and the glycerol, and then adding them to the probe sample.

#### Activation of cells

PBMCs, neutrophils, or U937 cells ( $1 \times 10^6$ /ml) were resuspended in RPMI supplemented with 1% l-glutamine, 1% Pen-Strep (complete RPMI), and activated with 10–100 ng/ml PMA and 1 µM ionomycin (for prolonged activation studies, 10% FCS was added). Cells from each well ( $3 \times 10^6$ ) were removed in different times for the flow cytometry analysis to probe for the presence of sialic acid binding sites and/or other lectin binding sites.

#### Flow cytometric analysis

Flow cytometry was performed on a Becton Dickinson FACS-can machine. The binding activity of cell surface sialic binding lectins was examined after several washings with staining buffer by incubating the cells (intact or with sialidase or periodate treatment) in 100 µl staining buffer containing 1–1.5 µg 6'PAA-B probe for 1 h on ice (conditions of time and probe concentration were determined to be optimal). After washing once with 0.5 ml of the staining buffer, cells were incubated with phycoerythrin-conjugated streptavidin for 30 min to detect binding of the biotinylated probe.

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#### Abbreviations

PMA, phorbol ester; PAA, polyacrylamide; PAA-B, PAA substituted with biotin; 6'PAA, PAA, substituted with  $\alpha 2-6$  sialyllactose; 3'PAA, PAA substituted with  $\alpha 2-3$  sialyllactose; Sia, sialic acid; AUS, *Arthrobacter ureafaciens* sialidase; PBMCs, peripheral blood mononuclear cells; Tc, Tricolor fluorophore, FITC, fluorescein isothiocyanate; PE, phycoerythrin; SA-PE, streptavidin conjugated with PE.

#### Note Added in Proof

Since the original acceptance of this manuscript, we have discovered, characterized, and reported an additional member of

the human Siglec family, called OB-BP-1/Siglec-6 (Patel *et al.*, 1999). Amongst human leukocytes, this Siglec is found only on B cells, and does not appear to bind to the 6' or 3' sialyllactose probes used in this study. Thus, Siglec-6 is not responsible for any of the binding phenomena noted in the present study.

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