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Research Article

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Sialoadhesin Binds Preferentially to Cells of the Granulocytic Lineage

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Abstract

Sialoadhesin is a macrophage-restricted, sialic acid–dependent receptor of 185 kD that binds to the oligosaccharide sequence NeuAcα2,3Gal on cell surface glycoconjugates. Recent cDNA cloning has shown that sialoadhesin is a new member of the immunoglobulin superfamily with sequence similarity to CD22, a sialic acid–dependent receptor of B lymphocytes. Sialoadhesin has been implicated in cellular interactions of stromal macrophages with developing myeloid cells. In this study, direct evidence for this interaction was obtained in cell–cell binding assays using both native and recombinant forms of the protein. In all assays, sialoadhesin exhibited specific, differential binding to various murine cell populations of hemopoietic origin. In rank order, sialoadhesin bound neutrophils > bone marrow cells = blood leukocytes > lymphocytes > thymocytes. Single-cell analyses confirmed that sialoadhesin selectively bound myeloid cells in complex cell mixtures obtained from the bone marrow and blood. In comparison, a recombinant Fc-chimeric form of murine CD22 showed high binding to B and T lymphocytes, but very low binding to immature and mature myeloid cells. These results are consistent with the notion that sialoadhesin is involved in interactions with granulocytes at different stages of their life histories. (*J. Clin. Invest.* 1995. 95:635–643.) Key words: hemopoiesis • neutrophils • inflammation • sialic acid • bone marrow

Introduction

Carbohydrate-binding proteins are important in a wide variety of biological processes involving specific cell–cell interactions. In mammals, the best characterized carbohydrate-binding proteins are the selectins, which are involved in leukocyte–endothelial cell interactions and contain a C-type lectin domain at the amino terminus (1).

Members of the immunoglobulin superfamily (IgSF)[1] mediate diverse recognition functions at the cell surface via both homotypic and heterotypic interactions. In general, these molecular interactions are considered to be protein–protein in nature, involving in each case a relatively restricted range of "ligand" counter receptors. Recently, however, it has become apparent that certain members of the IgSF can also function as lectin-like receptors by binding to specific carbohydrate determinants present on a potentially wide range of counter receptors (2–4). The best established of these is the human B cell–restricted antigen, CD22, which has seven Ig-like domains and can function as a sialic acid–dependent adhesion molecule (4, 5). Upon transient expression in monkey COS cells, human CD22 has been shown capable of binding T cells, B cells, monocytes, granulocytes, and erythrocytes (RBCs) in a sialic acid–dependent manner, via recognition of NeuAcα2,6Galβ1,4Glc(NAc) in N-linked glycans on a wide range of cell surface glycoproteins (6–8).

Sialoadhesin is a macrophage (Mϕ)-restricted plasma membrane receptor with an apparent M_r of 185K. It has been characterized as a sialic acid–binding protein that specifically recognizes the oligosaccharide sequences NeuAcα2,3Galβ1,3-GalNAc and NeuAcα2,3Galβ1,3(4)GlcNAc in either glycoproteins or glycolipids (9, 10). Recent cDNA cloning has revealed that sialoadhesin is a new member of the IgSF (3). With 17 extracellular Ig-like domains, it is the largest cell surface member of this family so far described. Importantly, the first four amino-terminal Ig-like domains of sialoadhesin share ~ 50% sequence similarity with a distinct subgroup of the IgSF that includes CD22. This establishes the fact that members of the IgSF can mediate cellular interactions through recognition of specific cell surface glycoconjugates (11).

The distribution and properties of sialoadhesin indicate that the receptor functions in vivo as a Mϕ-restricted cellular interaction molecule that binds appropriate sialylated ligands on developing myeloid cells in the bone marrow (BM) and lymphocyte subpopulations in lymphoid tissues (12, 13). To address directly the question of whether sialoadhesin can function as a cellular interaction molecule for granulocytic cells and other cells of hemopoietic origin, we have studied its binding properties in a range of binding assays with both native and recombinant forms of the receptor. We demonstrate that sialoadhesin can mediate differential, sialic acid–dependent binding to murine hemopoietic cells. Importantly, cells of the granulocytic lineage consistently show the highest levels of binding in all assay systems. The implications of these findings for the possible functions of sialoadhesin are discussed.

Methods

Materials. Unless specified otherwise, all reagents and chemicals were purchased from Sigma Chemical Co. (Poole, UK, or St. Louis, MO). Carrier-free $Na^{125}I$ and $Na_2{}^{51}CrO_4$ were purchased from Amersham Int. (Slough, UK). Sialidase from *Vibrio cholerae* or *Arthobacter ureafa-*

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1. *Abbreviations used in this paper:* BM, bone marrow; IgSF, immunoglobulin superfamily; Mϕ, macrophage(s); PBA, PBS + 0.25% BSA; RBC, red blood cell.

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ciens and octylglucoside were purchased from Calbiochem-Behring Corp. (La Jolla, CA). Dextran T-500, Sephacryl S-300, and Percoll were obtained from Pharmacia Fine Chemicals Ltd. (Uppsala, Sweden). Dulbecco's modification of Eagle's medium and PBS without calcium and magnesium were purchased from Gibco Biocult Ltd. (Paisley, Scotland).

Animals. Specific pathogen-free male or female C57Bl/6 mice were bred at the Pasteur Institute, the Sir William Dunn School of Pathology, or Clare Hall, ICRF, and used at 6–12 wk of age. Female AO rats at 8–16 wk of age were bred at the Sir William Dunn School of Pathology. Albino rabbits were provided by Oxford University Park Farm, Northmoor, UK.

Purification of sialoadhesin. Sialoadhesin was purified as described previously (9), with the following modifications: 2% CHAPS was used instead of sodium deoxycholate as the detergent, and the total spleen lysate was cleared of nuclei and insoluble material by centrifugation at 20,000 *g* for 30 min. The yield of sialoadhesin was ~ 700 μg per 100 g of spleens, and purity was estimated at 95% by SDS-PAGE.

Antibodies. IgG, $F(ab')_2$, and Fab fragments of SER-4 (IgG2a), a rat mAb directed against sialoadhesin, were prepared as described previously (14). Purified IgGs from 5C1, a rat IgG2c mAb against the murine Mϕ-restricted F4/80 antigen, and 5C6, a rat IgG2b against murine CD11a (15), were used as negative controls in some experiments. GK1.5 (anti-CD4), TIB 165 (anti-CD8), and TIB 146 (anti-B220) rat mAbs were generously provided by Dr. J.M. Austyn (Oxford University, UK). NIMP-R14 rat anti-mouse neutrophil mAb (16) was a kind gift of Dr. C.J. Sanderson (University of Western Australia). The rat myeloma cell, Y3, was provided by Dr. C. Milstein (Cambridge University, UK).

To prepare a new panel of mAbs directed to different epitopes of sialoadhesin, an AO rat was immunized with purified sialoadhesin, using a regime described previously (14) except that the final injection was intrasplenic. Hybridoma supernatants were screened for their ability to block sheep RBC binding to mouse serum-induced peritoneal Mϕ (14). 10 stable hybridomas were cloned. Their designations and isotypes are as follows: 1C2 (IgG2a), 1C3 (IgG1), 2D5 (IgG1), 2F8 (IgG1), 3B3 (IgG1), 3B8 (IgG2a), 3D6 (IgG2a), 4D5 (IgG1), 4F7 (IgG1), and 5D8 (IgG1).

IgGs from the panel of mAbs were purified from ascites by sodium sulfate precipitation and FPLC ion exchange chromatography using a Mono-Q column. Fab and $F(ab')_2$ fragments were prepared as described previously for SER-4 (14), except that FPLC Mono-Q ion exchange chromatography was used instead of DEAE-Sephacel.

A rabbit polyclonal anti-sialoadhesin antiserum (R6.2-4) was prepared by immunization of an albino rabbit with purified sialoadhesin. IgG was purified from the pooled serum by chromatography on protein A-Sepharose, and Fab and $F(ab')_2$ fragments were prepared by standard methods.

The purities of IgGs and fragments were consistently > 95%, as assessed by Coomassie blue-stained SDS-PAGE gels. Protein concentrations were determined by measuring absorbance at 280 nm, assuming an extinction coefficient of 1.4.

Epitope mapping. Thioglycollate-elicited peritoneal Mϕ induced to express sialoadhesin were used as targets for epitope mapping (14). Cells were fixed in methanol for 10 min immediately before the binding assay, and nonspecific sites were blocked in PBS + 1% BSA for 60 min at 37°C. mAbs were iodinated by the Iodogen method to specific activities of ~ 250 Bq/ng. Fixed Mϕ were preincubated for 60 min with unlabeled mAb at 20 μg/ml, and ^{125}I-labeled mAbs were added to the wells at a final concentration of 72 kBq/ml (~ 0.3 μg/ml), in the continued presence of the unlabeled mAb. After 60 min at room temperature, wells were rinsed five times with PBS + 0.1% BSA, and the bound radioactivity was solubilized by addition of 0.1 N NaOH and counted in a Berthold gamma counter.

Preparation of cells. Human RBCs (type A or AB) were prepared fresh from the same donors and stored in Alsever's solution. Sheep erythrocytes were purchased from Gibco Biocult Ltd. and used within 2 wk. Sialidase treatment of cells was performed as described previously (9). Resident peritoneal Mϕ were obtained as described (17). Mouse BM cells were flushed from excised femora and dissociated into a single-cell suspension by gentle pipetting in PBS followed by passage through a 25-gauge needle. Mesenteric lymph node cells and thymocytes were obtained by gentle dissociation of each tissue in PBS. Inflammatory neutrophils were obtained 18–24 h after subcutaneous injection of 1.5 ml of Bio-Gel P4 bead slurry, as previously described for Mϕ with Bio-Gel P-100 beads (18). This resulted in a population of cells (P4 neutrophils) that consisted of 85–95% neutrophils and 5–15% Mϕ, as assessed by May-Grünwald-Giemsa staining. Blood leukocytes were isolated from heparinized whole mouse blood by mixing 1:4 (vol/vol) with 1.5% Dextran T-500 in PBS and allowing RBCs to sediment for 45 min at room temperature. Residual RBCs were lysed in 10 mM Tris-buffered 0.85% ammonium chloride, pH 8.0. Enrichment of neutrophils from total blood leukocytes was performed on Percoll step gradients, using 2 ml of each of the following densities (g/ml) in each 15-ml tube: 1.10, 1.095, 1.09, 1.085, 1.08, 1.075. Leukocytes were loaded onto each Percoll gradient (2×10^7 per gradient) and centrifuged at 950 *g* for 30 min at 15°C. Cells at the interfaces were collected and pooled for each density step and washed three times in PBS prior to binding assays. The percentage of neutrophils in each fraction was determined from cytocentrifuge smears stained with May-Grüwald-Giemsa.

Radiobinding assays with native ^{125}I-sialoadhesin. Native sialoadhesin was iodinated as described previously (9) to give specific activities ranging from 30 to 120 Bq/fmol. Binding activity was retained when the purified iodinated receptor was diluted into PBS containing BSA in the absence of detergent. This meant that binding assays could be performed in the presence of negligible (< 0.01%) concentrations of CHAPS. The specificity of binding was assessed in all experiments by a 60-min preincubation of ^{125}I-sialoadhesin with 3D6 $F(ab')_2$ fragments at 20 μg/ml, or in some cases, by sialidase treatment of the cells. In some experiments, cells were fixed in 0.25% glutardialdehyde for 10 min at room temperature, washed, and quenched in PBS + 1% BSA for at least 60 min prior to binding assays. Cells were suspended at 2×10^6/ml in PBS + 1% BSA, and 25 μl containing 5×10^4 cells was added to replicate wells of 96-well microtiter plates. 25 μl of ^{125}I-sialoadhesin, preneutralized or not with 3D6 $F(ab')_2$, was added to each well and mixed. The plates were incubated for 60 min at 4°C for live cells or at room temperature for fixed cells and washed five times with 200 μl of PBS + 0.25% BSA (PBA) by centrifugation in plate carriers at 300 *g* for 2 min at room temperature.

Production of Fc-adhesins. cDNAs encoding full-length sialoadhesin and murine CD22 were cloned into pcDNA I/Amp as described (3, 11). To produce Fc-adhesins, cDNA fragments encoding four (sialoadhesin) or three (CD22) extracellular domains were generated by PCR from the full-length cDNAs, cloned into the pIG vector, expressed in COS cells, and purified on protein A-Sepharose as described (11, 19). cDNA encoding human Fc-NCAM in pIG was kindly provided by Dr. D. Simmons (Oxford University, UK). This protein was used as a negative control in solid-phase binding assays.

Fluid-phase binding assays with complexed Fc-adhesins. To increase the valency of the dimeric Fc-adhesins, soluble complexes were formed using goat anti-human IgG (Fc specific) (Sigma Chemical Co.). ^{125}I-Fc-adhesins were labeled as described (9) to specific activities of 40–60 Bq/fmol, diluted to 0.4 μg/ml in PBA, and preincubated with an equimolar concentration of anti-human IgG for 60 min at room temperature. ^{125}I-Fc-adhesin-IgG complexes were then incubated with 25 μl of a glutardialdehyde-fixed cell suspension (1.5×10^5 cells) for 60 min at room temperature, the cells were washed in PBA, and the cell-associated radioactivity was counted. The sialic acid dependence of binding was determined by using sialidase-treated cells. Data shown are mean values of triplicate measurements.

Solid-phase binding assays with Fc-adhesins. Fc-adhesins at varying concentrations were adsorbed for 3 h at 37°C to wells of microtiter plates that had been coated overnight at 4°C with goat anti-human IgG at 15 μg/ml in 0.1 M bicarbonate buffer, pH 9.6. For quantitative binding assays, cells were washed three times in RPMI-1640 + 20 mM Hepes, incubated for 60 min at 37°C with 100 μCi of ^{51}Cr per 10^6 cells, and

washed three times in PBA. Cells were adjusted to 8×10^6 cells per ml in PBA containing 5 mM EDTA and 5 mM sodium azide, and 50-μl aliquots were added to wells of the microtiter plate containing 50 μl of the same buffer. After 30 min at room temperature, the nonadherent and loosely adherent cells were removed with three washes. For the single-cell, qualitative binding assays, the same conditions were used, except that the ^{51}Cr labeling step was omitted and the adherent cells were fixed at the end of the assay prior to immunocytochemistry. The specificity of binding to Fc-sialoadhesin was assessed in all experiments by a 60-min preincubation of coated wells with SER-4 and 3D6 F(ab')$_2$ fragments, each at 20 μg/ml. Nonspecific binding was also assessed using Fc-NCAM–coated wells.

COS cell binding assays. COS cells were transfected with plasmids encoding full-length sialoadhesin using DEAE–dextran (19). Binding assays were performed with BM cells as described (3). The cells were fixed for 10 min in 0.25% glutardialdehyde in PBS prior to immunoperoxidase or Giemsa staining.

Cell binding assays to sialoadhesin expressed by Mϕ. Resident peritoneal Mϕ were cultured for 3–5 d in RPMI-1640 containing 15% mouse serum, and binding assays were performed as described previously (20). To control for non-sialoadhesin-mediated binding, replicate wells were incubated in the same buffer containing 3D6 F(ab')$_2$ at 20 μg/ml for 15 min prior to addition of cells. Unbound cells were removed by washing, and the cells were fixed in 0.25% (vol/vol) glutardialdehyde. The percentage of Mϕ that formed rosettes (> 4 RBCs or > 1 nucleated cell bound) or the number of cells bound per 100 Mϕ were determined by scoring at least 200 Mϕ per coverslip from triplicate coverslips using phase-contrast microscopy.

Single-cell analysis and immunocytochemistry. Cytocentrifuge preparations were fixed in acetone. Cells that had adhered specifically to Fc-adhesins in solid-phase assays on 96-well plates were fixed in a 1:1 mixture of acetone and methanol. Detection of cell surface antigens was then performed by immunoperoxidase labeling using a streptavidin–biotin–peroxidase kit (Vectastain; Vector Laboratories, Petersborough, UK). At least 200 cells were counted in each preparation.

Results

Characterization of mAbs against sialoadhesin. To characterize the binding of sialoadhesin to hemopoietic cells, it was important to use blocking anti-sialoadhesin mAbs in the form of Fab or F(ab')$_2$ fragments since hemopoietic cells express Fc receptors that could interact with intact mAbs. Previously it was shown that SER-4, the original mAb raised to sialoadhesin, inhibited binding of sheep RBCs as IgG but not as Fab fragments and only partially as F(ab')$_2$ fragments (14). 10 new mAbs against sialoadhesin were selected by their ability to inhibit sheep RBC binding to induced peritoneal Mϕ. All mAbs bound specifically to purified sialoadhesin in dot blot assays and stained discrete Mϕ populations in spleen and lymph nodes by immunocytochemistry, as described previously (14).

Epitope mapping. To determine whether different epitopes on the sialoadhesin molecule could be defined by the panel of mAbs, competition binding assays were performed using fixed Mϕ as targets (Table I). The majority of mAbs competed with SER-4 and therefore recognized the same epitope (epitope A). mAbs 3D6 and 1C2 defined a second, distinct epitope (epitope B), and 2F8 bound to a third epitope (epitope C) that seemed to overlap partially with that recognized by SER-4 but none of the other mAbs.

Inhibition of purified sialoadhesin binding by the mAb panel. The mAbs were next tested for their ability to inhibit binding of purified, iodinated sialoadhesin in cell binding assays. Purification of sialoadhesin in CHAPS results in oligomeric preparations of the receptor with a hemagglutinating activity similar to that described previously for octylglucoside (9). Human RBCs were used in initial experiments, since they were shown previously to be excellent ligands for sialoadhesin (9) and do not express Fc receptors that could interfere with the assay. 3D6 and 1C2 IgGs showed complete inhibition of ^{125}I-sialoadhesin binding at antibody concentrations as low as 0.1 μg/ml (Fig. 1 *a*). Only partial inhibition was observed with the other anti-sialoadhesin antibodies, even at concentrations as high as 10 μg/ml. This contrasts with their ability to inhibit completely binding of human RBCs to sialoadhesin expressed by Mϕ, suggesting that the blocking in cell–cell binding assays is due to the effects of steric hindrance. Fab fragments prepared from 1C2 and 3D6 as well as F(ab')$_2$ from 3D6 were totally inhibitory for binding of sialoadhesin to RBCs, whereas SER-4 F(ab')$_2$ and Fab fragments or Fab fragments from normal rat serum IgG had little or no effect (Fig. 1 *b*, Table II, and data not shown).

Table I. Epitope Mapping on Sialoadhesin by Competition Binding Assay of Iodinated mAbs to Induced Peritoneal Mϕ

	^{125}I-labeled mAb*		
Competing antibody‡	SER-4	3D6	2F8
SER-4	+++	–	+
1C2	–	+++	–
1C3	+++	–	–
2D5	+++	–	–
2F8	+	–	+++
3B3	+++	–	–
3B8	+++	–	–
3D6	–	+++	–
4D5	+++	–	–
4F7	+++	–	–
5D8	+++	–	–

(+++) Complete competition (>95% inhibition); (+) partial inhibition (40–60% inhibition); (–) No competition (<10% inhibition). * MAbs were labeled with ^{125}I to specific activities of ~260 Bq/ng and added directly to the methanol-fixed Mϕ at a final concentration of ~300 ng/ml. Bound ^{125}I-labeled mAb was estimated as described in Methods. ‡ Competing mAbs were added to Mϕ at 20 μg/ml and incubated for 1 h before addition of ^{125}I-mAb.

Taken together, these results demonstrate that 1C2 and 3D6 recognize an epitope that is closer to the binding site of sialoadhesin than that recognized by SER-4 and the other mAbs. 3D6 F(ab')$_2$ fragments were used in additional studies as specific inhibitors of sialoadhesin binding.

Characteristics of ^{125}I-sialoadhesin binding to murine BM cells. Binding assays were performed with murine BM cells and purified native ^{125}I-sialoadhesin to determine whether these cells carried appropriate sialylated ligands (Table II). Similarly to human RBCs, sialoadhesin binding to BM cells was largely sialic acid dependent (Table II) and inhibited strongly by Fab fragments of 3D6 and 1C2. Inhibition by Fab fragments of SER-4 was only partial (Table II). Since the binding of sialoadhesin to carbohydrate structures on cells was resistant to glutardialdehyde fixation (Table II), cells could be prepared in advance of binding assays.

Comparison of different murine cell populations for binding to ^{125}I-sialoadhesin. It was important to determine whether the

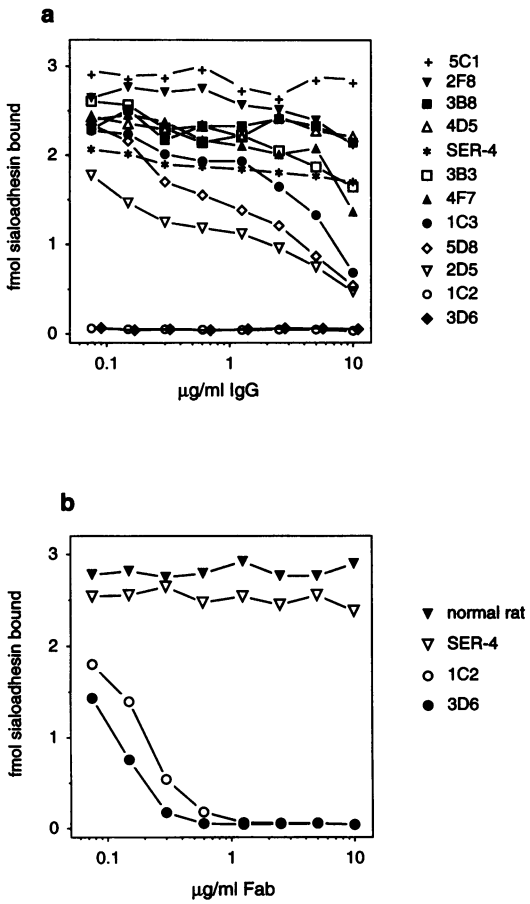

Figure 1. Inhibition of purified sialoadhesin binding to human RBCs by IgGs (*a*) or Fab fragments (*b*), purified from the panel of anti-sialoadhesin mAbs. 31-fmol aliquots of ^{125}I-sialoadhesin at a specific activity of 100 Bq/fmol were incubated for 60 min at room temperature with serial dilutions of the different mAbs. Human RBCs were added at 0.25% final concentration, and after 60 min at room temperature, they were washed and ^{125}I-sialoadhesin binding was determined. The negative control in *a* was 5C1, a mAb of the IgG2c subclass that binds to the murine Mϕ antigen F4/80. The negative control in *b* was Fab fragments purified from normal rat serum. Data show values from single wells at each concentration.

Table II. Characteristics of ^{125}I-Sialoadhesin Binding to Murine BM Cells Compared with Human RBCs

	Femtomoles of sialoadhesin bound			
	Human RBC		Murine BM cells	
Treatment	–	+3D6*	–	+3D6*
Experiment 1‡				
Control	16.39	0.03	6.90	0.53
Sialidase (*V. cholerae*)	0.15	0.09	1.85	0.73
Sialidase (*A. ureafaciens*)	ND	ND	2.20	0.94
Experiment 2§				
Control	13.93	0.17	ND	ND
5 mM EDTA	15.99	0.17	ND	ND
Experiment 3‖				
Control	15.43	0.16	8.82	0.57
0.25% Glutardialdehyde	16.88	0.99	8.68	0.90
Experiment 4¶				
Control	2.28	0.03	2.13	0.40
1C2 Fab	0.03	ND	0.36	ND
3D6 Fab	0.06	ND	0.35	ND
SER-4 Fab	1.67	ND	1.20	ND
Normal rat Fab	2.30	ND	2.09	ND

Data show mean values of three to four replicates. Standard deviations are not shown for clarity but were consistently within 10% of the mean. Binding assays were performed in 50 μl with human RBCs at 0.25% final concentration or 5×10^5 BM cells. Cells were incubated with 85 (experiment 1–3) or 25 (experiment 4) fmol of ^{125}I-sialoadhesin for 1 h at room temperature, washed, and counted. * 3D6 F(ab')$_2$ was added at 50 μg/ml to ^{125}I-sialoadhesin 1 h prior to its addition to cells. ‡ 10^7 cells were pretreated in 500 μl of PBS with 100 U/ml *V. cholerae* or *A. ureafaciens* sialidase for 60 min at 37°C. § ^{125}I-sialoadhesin was preincubated in 5 mM EDTA for 15 min prior to addition of RBCs. ‖ Cells were fixed in 0.25% glutardialdehyde for 10 min at room temperature, washed, and quenched for 1 h in PBS containing 1% BSA prior to the binding assay. ¶ ^{125}I-sialoadhesin was incubated with Fabs at 2 μg/ml for 1 h at 4°C prior to addition of cells.
ND, not determined.

receptor could discriminate between different murine hemopoietic cell types in vitro, since this would favor the possibility that sialoadhesin is able to mediate selective cellular interactions in vivo. Initially, BM cell binding to sialoadhesin was compared with inflammatory neutrophils recruited to the site of Bio-Gel P4 injection, mesenteric lymph node cells, and thymocytes (Fig. 2 *a*). For all cell types, binding was > 90% inhibitable by 3D6 and, where studied, by pretreatment of the cells with *V. cholerae* sialidase (data not shown). Unexpectedly, inflammatory neutrophils showed the highest level of binding, being approximately twofold higher than BM cells, fourfold higher than lymphocytes, and fivefold higher than thymocytes (Fig. 2 *a*).

The high binding observed with inflammatory neutrophils raised the possibility that normal, circulating neutrophils are strongly recognized by sialoadhesin. Alternatively, neutrophil activation during recruitment could result in high levels of binding due to increased surface expression of ligands. To distinguish between these two possibilities, neutrophils were enriched from total blood leukocytes by density separation in discontinuous Percoll gradients. Of the five fractions obtained, only the most dense fraction was significantly enriched in neutrophils (63%) compared with total blood leukocytes (21% neutrophils). Binding assays were performed to compare the different fractions with total leukocytes and P4 neutrophils (86% neutrophils) (Fig. 2 *b*). Binding of sialoadhesin to the neutrophil-enriched fraction was at the same level as P4 neutrophils and three- to fourfold higher than the other fractions, whose contents of neutrophils varied from 6 to 24%.

Binding of hemopoietic cells to sialoadhesin expressed by Mϕ. Peritoneal Mϕ cultured for 2–5 d in 10–20% mouse serum express high levels of sialoadhesin (17). This provides a useful system to ask whether sialoadhesin expressed on Mϕ was able to mediate selective binding of a type similar to that observed with the purified receptor. In initial experiments with BM cells, the overall level of binding varied among experiments as a result of different levels of sialoadhesin expression (compare experiments 1 and 2 in Table III). However, binding of BM cells was sialic acid dependent and did not require the presence of divalent cations (Table III). Binding assays could therefore

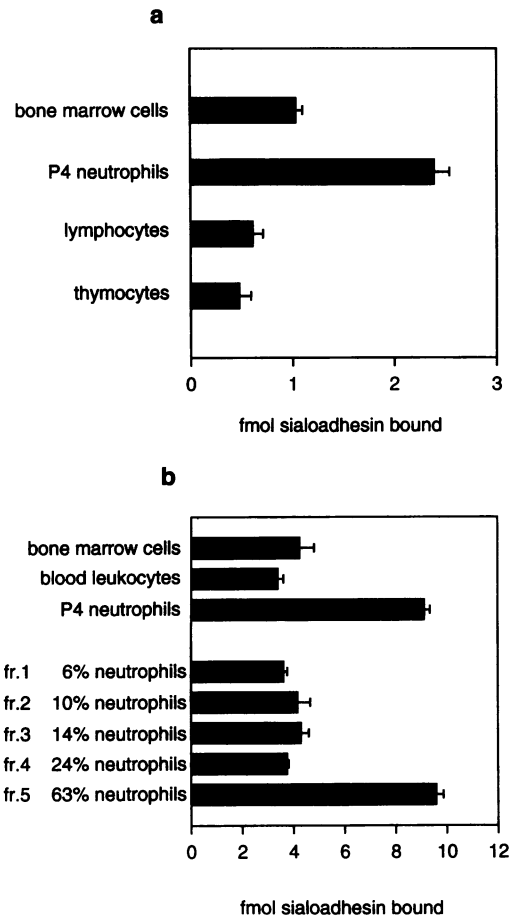

Figure 2. Binding of purified sialoadhesin to different populations of murine cells. 45-fmol (*a*) or 80-fmol (*b*) aliquots of ^{125}I-sialoadhesin at a specific activity of 120 Bq/fmol (*a*) or 75 Bq/fmol (*b*) were incubated with 5×10^4 cells for 60 min at 4°C and washed. Data shown are means±1 SD of triplicate wells, corrected for nonspecific binding determined with 3D6 F(ab')$_2$. Similar results were obtained in two other experiments. In *b*, total blood leukocytes were separated on discontinuous Percoll gradients and different fractions (*fr.*) were compared for binding to ^{125}I-sialoadhesin. The percentage of neutrophils in each fraction is shown; in the other populations, the percentages of neutrophils were as follows: blood leukocytes, 21%; P4 neutrophils, 86%.

be performed in the absence of calcium and magnesium, which excluded the contribution of Mϕ adhesion receptors that require divalent cations for function. Direct evidence that the binding of BM cells to Mϕ was mediated by sialoadhesin, rather than by another co-induced sialic acid–dependent receptor, was demonstrated by the nearly complete inhibition of BM cell binding with Fab or F(ab')$_2$ fragments prepared from mAbs 3D6 and IC2 and a rabbit polyclonal antiserum raised to the purified receptor (Fig. 3 and Table III).

Binding assays to Mϕ were then performed with blood leukocytes, P4 neutrophils, lymphocytes isolated from mesenteric lymph nodes, and thymocytes (Fig. 3). In contrast to binding assays with the purified receptor, binding assays with Mϕ resulted in a variable but significant "background" binding that was not inhibitable by 3D6 (Fig. 3). This was most noticeable for P4 neutrophils, thymocytes, and mesenteric lymphocytes and indicates a role for other divalent cation–independent Mϕ adhesion receptors in binding to these cell types.

Table III. Characteristics of BM Cell Binding to Induced Mϕ Compared with Sheep RBC Binding

Treatment	Sheep RBC	Murine BM cells
	*% Rosettes**	*Cells bound/ 100 Mϕ*
Experiment 1‡		
Control	97±2	525±80
1 mM EDTA	96±1	496±105
5 mM Sialyllactose	53±2	11±4
10 mM Sialyllactose	28±1	12±1
20 mM Sialyllactose	3±1	6±3
Experiment 2§		
Control	30±8	124±22
V. cholerae sialidase	0	22±12
Experiment 3‖		
Control	ND	125±3
Rab anti–sialoadhesin preimmune IgG	ND	116±34
Rab anti–sialoadhesin immune IgG	ND	4±2
Rab anti–sialoadhesin immune F(ab')$_2$	ND	4±3
Rab anti–sialoadhesin immune Fab	ND	8±2

* Binding of Mϕ to sheep RBCs was evaluated as the percentage of Mϕ binding more than four sheep RBCs. Data represent mean±range values from two (experiments 1 and 2) or three (experiment 3) coverslips. At least 200 Mϕ were counted per coverslip. ‡ Mϕ were incubated in EDTA and sialyllactose for 15 min at 37°C prior to addition of cells, and the reagents were maintained throughout the assay. § Cells pretreated in 1 ml of PBS (10^7 BM cells or 0.5% sheep RBCs) were pretreated with 10 mU of *V. cholerae* sialidase for 60 min at 37°C. ‖ Mϕ were preincubated with antibodies for 15 min before addition of cells. Preimmune IgG was added at 50 μg/ml, and all other immune reagents were at 20 μg/ml. ND, not determined; Rab, rabbit.

In three separate experiments, the rank order and relative degree of sialoadhesin-dependent binding for the different cell types was similar to that observed with the soluble receptor. In the experiment shown in Fig. 3, only 35% of the Mϕ bound more than five sheep RBCs. This low level of expression allowed clear differences in binding among the different cell populations to be seen. Thus, sialoadhesin-dependent binding of neutrophils was 3 times higher than BM cells, 7 times higher than circulating leukocytes, and 15 times higher than mesenteric lymphocytes and thymocytes. In general, binding at the single-Mϕ level was heterogeneous and was observed only on the subpopulation of Mϕ that expressed the highest levels of sialoadhesin. This meant that even for low binding cells, like mesenteric lymphocytes and thymocytes, most of the sialoadhesin-positive Mϕ bound more than one cell. In all cases, binding of the different cell populations was not accompanied by phagocytosis.

Comparison of cell binding to sialoadhesin and CD22 using recombinant Fc-adhesins. Through molecular cloning, we have recently shown that sialoadhesin is a type I transmembrane glycoprotein and a new member of the IgSF with 17 extracellular Ig-like domains (3). The amino-terminal four domains share sequence similarity with CD22, a sialic acid–dependent adhesion molecule of B lymphocytes with seven Ig-like domains (21, 22). The availability of cDNA clones encoding murine sialoadhesin and CD22 allowed us to compare the binding prop-

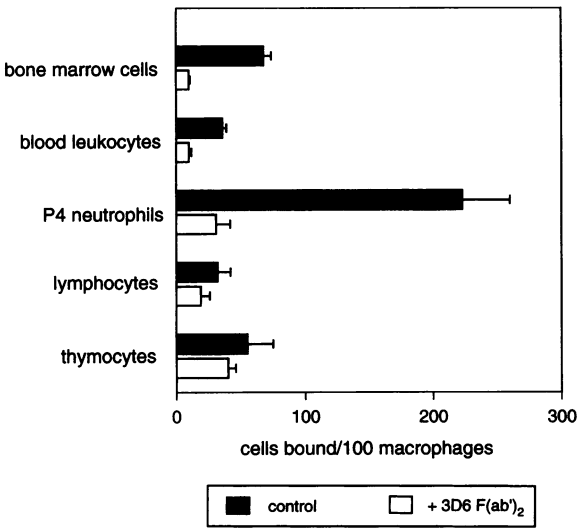

Figure 3. Binding of different murine cell populations to sialoadhesin expressed by Mϕ. Resident peritoneal Mϕ were induced to express sialoadhesin by culture for 5 d in 15% mouse serum. 2×10^6 cells of each type were allowed to bind for 30 min at room temperature in divalent cation–free PBS, and nonbound cells were rinsed off. Non-sialoadhesin-mediated binding was assessed in groups of coverslips by preincubation of Mϕ in 50 μg/ml 3D6 $F(ab')_2$ for 15 min before addition of cells. Data represent means+1 SD of triplicate coverlips. In three other experiments a similar rank order of sialoadhesin-dependent binding was observed for each cell type.

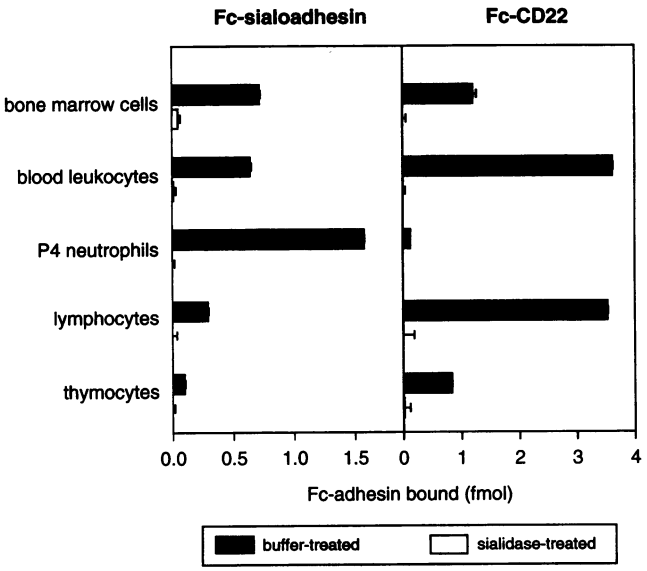

Figure 4. Fluid-phase binding assays of complexed, ^{125}I-Fc-sialoadhesin, and ^{125}I-Fc-CD22 to different cells. ^{125}I-Fc-adhesins containing the first three (CD22) or four (sialoadhesin) Ig-like domains, at 2.5 nM and a specific activity of 50 Bq/fmol, were complexed with an equimolar concentration of goat anti–rabbit IgG (Fc specific), and 20-fmol aliquots were incubated with 1.5×10^5 glutardialdehyde-fixed cells for 60 min at room temperature and washed. The sialic acid dependence of binding was determined using sialidase-treated cells under identical conditions. Data shown are means+1 SD of triplicate wells.

erties of both receptors. These experiments were done with recombinant forms of CD22 and sialoadhesin containing the first three or four extracellular domains, respectively, fused to the Fc portion of human IgG1 (19). In other studies, these truncated Fc-adhesins have been shown to exhibit full sialic acid–dependent binding activity to human RBCs when compared with the respective full-length forms (11).

In initial experiments, ^{125}I-labeled Fc-adhesins exhibited no detectable binding activity to cells in fluid-phase binding assays. To increase the avidity of the normally dimeric Fc-adhesins, fluid-phase binding assays were performed using radiolabeled Fc-adhesins that had been precomplexed with an equimolar concentration of anti–human IgG antiserum. This resulted in stable, specific binding to cells that was temperature independent since, similar binding of complexed Fc-sialoadhesin and Fc-CD22 was observed at 4°C and room temperature. For all cell types examined (Fig. 4), binding of both Fc-sialoadhesin and Fc-CD22 was sialic acid dependent, but the patterns of cell recognition were distinct. Similarly to the native protein, Fc-sialoadhesin bound most strongly to P4 neutrophils, with intermediate binding to BM cells and blood leukocytes, whereas lymphocytes and thymocytes bound poorly. In striking contrast, Fc-CD22 bound most strongly to lymphocytes and blood leukocytes, whereas binding to P4 neutrophils was weak, with intermediate binding to BM cells and thymocytes.

These studies indicate that sialoadhesin binds preferentially to cells of the granulocytic lineage, whereas CD22 shows preference for lymphocytes. To obtain direct evidence for this, we performed cell adhesion assays on plastic wells that had been coated with Fc-adhesins and then characterized the cells that were specifically bound. In this system, binding of cells depended on the coating concentration, with optimal binding being observed at around 10 nM Fc-adhesin (Fig. 5 *a*). No binding was observed with wells coated with Fc-NCAM as a negative control (Fig. 5 *a*). Binding of cells to Fc-sialoadhesin was sialic acid dependent (data not shown) and inhibited to background levels in the presence of a mixture of $F(ab')_2$ fragments from SER-4 and 3D6 (Fig. 5 *b*). When the various hemopoietic cell populations were compared, the rank order of binding to Fc-sialoadhesin was the same as that observed in all other assays (Fig. 5 *b*).

For the single-cell analysis, we used BM cells and blood leukocytes since these are complex mixtures of cells that allowed us to determine whether particular subpopulations of cells were selected by Fc-sialoadhesin and Fc-CD22. As shown in Table IV, Fc-sialoadhesin selectively bound to cells of the granulocytic lineage. Thus, blood leukocytes contain ~ 20% neutrophils, 70% lymphocytes, and 5% monocytes, yet up to 97% of the cells bound were neutrophils, as defined by the NIMP-R14 mAb (16) and Giemsa staining. In contrast, CD22 showed a striking preference for lymphocytes. This was most clearly seen with the BM cell suspensions in which > 70% of the cells bound to Fc-CD22 were developing B lymphocytes as defined by the B220 mAb TIB 146, compared with only 15% B lymphocytes in the total BM cell suspension (Table IV).

Binding of BM cells to COS cells transfected with sialoadhesin cDNA. The single-cell analysis described above was extended to COS cells that had been transfected transiently with a cDNA encoding full-length sialoadhesin. BM cells bound only to the transfected cells, and this was sialic acid dependent and mAb inhibitable (data not shown). Staining of the bound cells with Giemsa (Fig. 6 *a*) or by immunoperoxidase labeling with the neutrophil marker NIMP-R14 (Fig. 6 *b*) showed that the

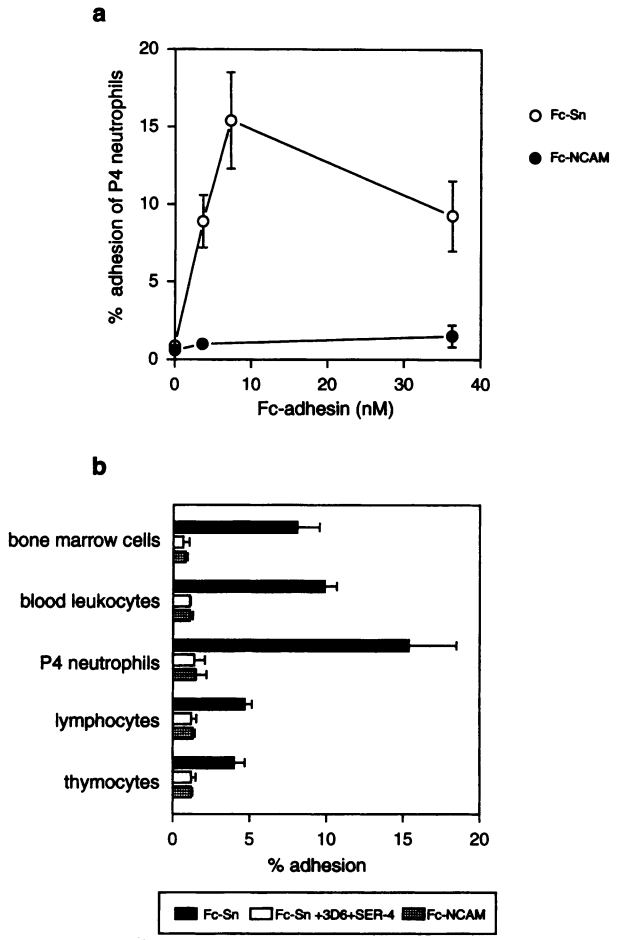

Figure 5. Solid-phase binding assays of Fc-sialoadhesin to different ^{51}Cr-labeled cells. In *a*, adhesion assays were performed with P4 neutrophils to plastic wells coated sequentially with goat anti-human IgG followed by the indicated concentration of Fc-sialoadhesin containing the first four Ig-like domains. In *b*, various cell types were added to plastic wells coated as previously described, with Fc-sialoadhesin at 10 nM. Specificity of binding in each case was determined in the presence of 3D6 and SER-4 F(ab')$_2$ fragments, as well as by plating cells on wells coated with Fc-NCAM. Data shown are means±1 SD (*a*) or means+1 SD (*b*) of triplicate wells. Similar results were obtained in three independent experiments.

majority of cells bound were immature cells of the granulocytic lineage.

Discussion

Here we demonstrate that the Mϕ-restricted receptor, sialoadhesin, can function as a selective cellular interaction molecule, with strong preference for cells of the granulocytic lineage. This was observed in a wide range of binding assays using both native and recombinant forms of the receptor, expressed either in the plasma membrane or as purified molecules. In rank order, sialoadhesin bound neutrophils > BM cells = blood leukocytes > lymphocytes > thymocytes.

The oligosaccharide determinants recognized by sialoadhesin, NeuAcα2,3Galβ1,3GalNAc and NeuAcα2,3Galβ1,3(4)-GlcNAc, are commonly found on mammalian cells in both glycoproteins and glycolipids. It is therefore important to understand how sialoadhesin can mediate differential binding to diverse cell types. Similarly to selectin ligands (23), a number of possible mechanisms could influence presentation of the carbohydrate determinants and thereby contribute to selective recognition. These include the nature of the carrier molecules, the effect of ligand clustering, and modification of the sialic acid. Recent studies with murine sialoadhesin (10) and human CD22 (24) have shown that 9-O-acetylation of sialic acid masks recognition. This is likely to be of particular relevance to the cellular interactions mediated by sialoadhesin since murine RBCs contain large amounts of this form of sialic acid (25). Mϕ in the BM are in close proximity to both developing RBCs and myeloid cells, but sialoadhesin is concentrated only at the contact sites with myeloid cells (12). It is possible that the presence of 9-O-acetylated sialic acid on RBCs prevents high avidity binding to sialoadhesin. This could also explain why sialoadhesin showed a clear preference for developing myeloid cells over erythroid cells in single-cell analyses of BM cell suspensions.

If the expression of high avidity ligands for sialoadhesin increases in parallel with granulocyte maturation, this raises the interesting question of how differentiating granulocytes are able to dissociate from sialoadhesin expressed on Mϕ in the BM and enter the blood stream. De-adhesion could be achieved by proteolytic cleavage and shedding of the receptor, as demonstrated for L-selectin (26). Alternatively, de-adhesion may be promoted by the enzymatic removal of sialic acid from the granulocyte surface by extracellular sialidases or by intracellular modification of the sialic acid by O-acetylation or hydroxylation to forms that are not recognized by sialoadhesin (10). Another possibility is that enzymatic modification of either the receptor or the ligand is not required and that detachment results from the enhanced motility of terminally differentiating myeloid cells.

Compared with granulocytes, lymphocyte populations bound very weakly to sialoadhesin in a wide range of binding assays. Previously, a frozen tissue section overlay assay was used to demonstrate that lymphocytes can bind to sialoadhesin expressed by splenic and lymph node Mϕ (13). Since the level of binding was dependent on the lymphocyte subset and stage of maturation, this suggests that if sialoadhesin does play a role in lymphocyte interactions, it is likely to be restricted to a discrete subset of these cells. The relatively weak binding of sialoadhesin to lymphocytes is even more apparent if its binding characteristics are compared side by side with CD22, as in the present study. Using recombinant forms of both receptors, CD22 and sialoadhesin exhibited opposite cellular specificities with respect to lymphocytes and granulocytes. At an evolutionary level, this could indicate that the carbohydrate binding specificity of sialoadhesin has become tailored toward interactions with cells of the granulocytic lineage, whereas that of CD22 has become tailored toward interactions with T and B lymphocytes.

With respect to mature granulocytes, an important question is whether both resting (circulating) and activated cells bind similarly to sialoadhesin. Using a wide range of binding assays, we failed to see a clear difference between neutrophils obtained from the blood and inflammatory P4 neutrophils. However, we cannot formally exclude the possibility that the circulating neutrophils used in our study were activated during their isolation, although it should be pointed out that the single-cell analyses were performed with blood leukocytes subjected to minimal ex vivo manipulation. In contrast, the P4 neutrophils are likely to consist mostly of activated cells since they had been recruited to an inflammatory site prior to collection.

Table IV. Single-Cell Analysis of Cells Specifically Adherent to Fc-Sialoadhesin and Fc-CD22

	Percent cells labeled (range)*				
mAb Cells recognized	5C6 Myeloid	NIMP-R14 Neutrophils	TIB 146 B lymphocytes	GK1.5 CD4+ T cells	TIB 165 CD8+ T cells
Cell population					
Bone marrow, total	42–49	32–43	16	ND	ND
Bone marrow, adherent to Fc-sialoadhesin‡	83–85	80–90	5–10	ND	ND
Bone marrow, adherent to Fc-CD22§	4–5	2	71–80	0–1	1–2
Blood leukocytes, total	12–22	10–14	40	ND	ND
Blood leukocytes, adherent to Fc-sialoadhesin	77–97	88–96	19–26	ND	ND
Blood leukocytes, adherent to Fc-CD22	0–2	1–2	64–78	14–18	7–9

* Cells were labeled by immunoperoxidase staining with the indicated mAbs. Data show the range of values determined from between one and three independent experiments. For the adherent cells, at least 200 cells were counted in triplicate for each experiment; for the total cell populations, a single preparation was scored for each experiment. ‡ Fc-sialoadhesin contained the first four amino-terminal Ig-like domains. § Fc-CD22 contained the first three amino-terminal Ig-like domains. ND, not determined.

In vivo, the adhesion of granulocytes to Mϕ via sialoadhesin could result in a number of possible events relating to their function and turnover. For example, it has been demonstrated that the respiratory burst activity of granulocytes can be suppressed by sialic acid–binding molecules like influenza A hemagglutinin (27) or P-selectin (28). This could be important for preventing undesired tissue damage caused by activated granulocytes. A second consequence of granulocyte binding to Mϕ via sialoadhesin could be to promote engagement of "phagocytic" receptors of the Mϕ, leading to uptake of the bound cells.

The fate of circulating neutrophils under steady-state conditions is poorly understood, but a proportion of the senescent cells are probably cleared in the circulation by Mϕ of the reticu-

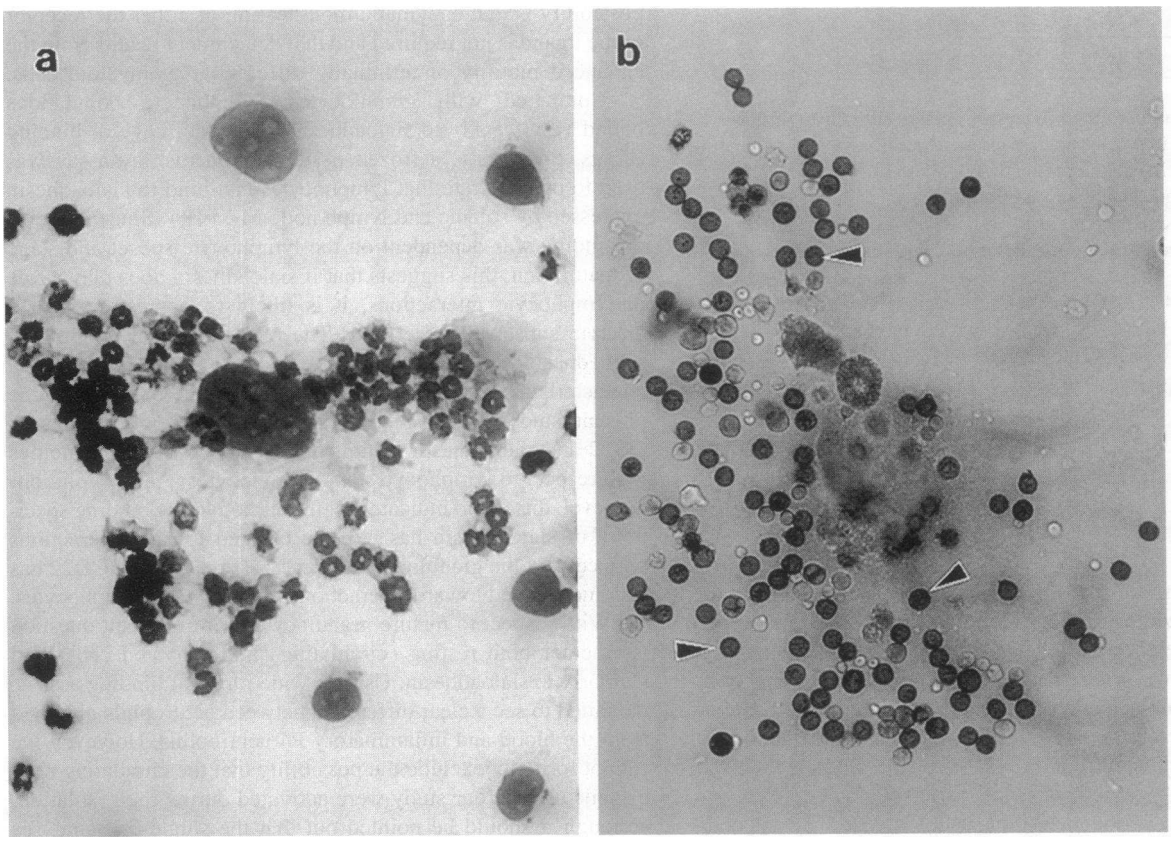

Figure 6. Selective binding of myeloid cells to COS cells expressing sialoadhesin. COS-1 cells were transfected with a cDNA encoding full-length sialoadhesin. 3 d after transfection, cells were overlaid with BM cell suspensions for 60 min at 37°C. Washed, glutardialdehyde-fixed cells were stained with Giemsa (*a*) or by immunoperoxidase labeling with the neutrophil-specific mAb NIMP-R14 (*b*). Arrowheads in *b* show examples of positively labeled cells.

loendothelial system while others randomly enter tissues, where they are removed by stromal Mϕ. In comparison, the majority of neutrophils recruited to sites of inflammation are thought to be cleared in situ (29). Studies in vivo have demonstrated that sialoadhesin can be induced on Mϕ recruited to a site of inflammation, thereby raising the possibility that the receptor expressed by recruited Mϕ could interact with neutrophils within an inflammatory lesion (30). Neutrophils that enter the afferent lymphatics of draining lymph nodes (31) would encounter a dense network of sialoadhesin-positive stromal Mϕ within the subcapsular sinus (14). Initial binding events mediated by sialoadhesin could promote ligation of phagocytic receptors with corresponding ligands on the neutrophil and result in a more efficient uptake process. Recent studies have shown that at least two Mϕ receptors, CD36 and the vitronectin receptor, are important for mediating the uptake of apoptotic neutrophils in vitro (32, 33). Cooperativity between sialoadhesin and other receptors in clearance of neutrophils would be analogous to the coordinated interaction of endothelial cell and neutrophil adhesion receptors during neutrophil margination and extravasation in acute inflammation (reviewed by Butcher [34]).

In conclusion, our study demonstrates that cell type–specific glycosylation of hemopoietic cells results in selective interactions of developing and mature granulocytes with sialoadhesin expressed by Mϕ. Importantly, the highly restricted expression pattern of sialoadhesin would limit these cellular interactions to Mϕ populations within hemopoietic and secondary lymphoid tissues and possibly at sites of inflammation.

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