Sialomucin CD34 Is the Major L-Selectin Ligand in Human Tonsil High Endothelial Venules

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Abstract. Peripheral node addressin (PNAd) is a complex mixture of glycoproteins with L-selectin ligand activity that functions in lymphocyte homing. We have investigated the contribution of the sialomucin CD34 relative to other components of PNAd in lymphocyte tethering and rolling in in vitro laminar flow assays. PNAd was isolated with MECA-79 mAb–Sepharose from tonsillar stroma, and the CD34 component (PNAd,CD34$^+$) and CD34-negative component (PNAd,CD34$^-$) separated on CD34 mAb–Sepharose. Lymphocytes on the PNAd,CD34$^-$ fraction tether less efficiently, roll faster and are less resistant to shear detachment than on PNAd. The PNAd,CD34$^+$ fraction constitutes about half the total functional activity. These studies show that CD34 is a major functional component of PNAd. Ligand activity in both the PNAd,CD34$^+$ and PNAd,CD34$^-$ fractions is expressed on mucin-like domains, as shown with *O*-sialoglycoprotease. The CD34 component of PNAd has about four times higher tethering efficiency than total tonsillar CD34. CD34 from spleen shows no lymphocyte tethering. Although less efficient than the PNAd,CD34$^+$ fraction from tonsil, CD34 from the KG1a hematopoietic cell line is functionally active as an L-selectin ligand despite lack of reactivity with MECA-79 mAb, which binds to a sulfation-dependent epitope. All four forms of CD34 are active in binding to E-selectin. KG1a CD34 but not spleen CD34 are active as L-selectin ligands, yet both lack MECA-79 reactivity and possess E-selectin ligand activity. This suggests that L-selectin ligands and E-selectin ligands differ in more respects than presence of the MECA-79 epitope.

L-SELECTIN is important in several types of leukocyte interactions with vascular endothelium (32). L-selectin was initially identified on lymphocytes as a receptor for high endothelial venules (HEV)[1] of peripheral lymph nodes and shown to be important in lymphocyte homing or recirculation (14, 41). Subsequently, L-selectin was found also to be expressed on neutrophils, monocytes, and eosinophils, and to be important in rolling interactions of neutrophils in post capillary venules of inflamed tissues and in accumulation of leukocytes at inflammatory sites (33, 49).

Studies on the carbohydrate ligands for L-selectin and the glycoproteins bearing these ligands have focused on molecules expressed in HEV. The rat anti–mouse mAb MECA-79 binds specifically to HEV, inhibits binding of lymphocytes to HEV, and identifies a carbohydrate antigenic determinant closely associated with the L-selectin ligand (23, 47). Affinity purification with MECA-79 mAb or recombinant L-selectin yields a collection of glycoprotein bands that appear nearly identical in SDS-PAGE, and have been termed the peripheral node addressin (PNAd) (6, 22). Sialylation and sulfation of PNAd are required for ligand activity, and specific oligosaccharide structures bearing these modifications that are present in PNAd have been identified, but not yet associated with ligand activity (21). MECA-79 mAb cross-reacts with human PNAd, and as in the mouse is completely specific for HEV, as shown by immunohistochemistry of lymphoid tissues (36). PNAd isolated from human tonsil is a ligand for L-selectin (6) and supports tethering and rolling of lymphocytes and neutrophils in shear flow (31). Affinity chromatography on recombinant L-selectin–Sepharose has been used to isolate murine PNAd, and identify two of its components, GlyCAM-1 (28) and CD34 (4). Both molecules contain extensive mucin-like segments that bear sulfated, sialylated *O*-linked oligosaccharides (22). GlyCAM-1 is a secreted molecule limited in expression to HEV and lactating mammary gland (10, 28). CD34 is a transmembrane glycoprotein expressed on many vascular endothelia including

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1. *Abbreviations used in this paper:* ABTS, 2,2-azido-di(3-ethylbenzthiazoline) sulfonic acid; HEV, high endothelial venules; HSA, human serum albumin; OG, octyl-β-glucoside; PNAd, peripheral node addressin; TSA, 20 nM Tris-HCl, 150 mM NaCl, 0.03% sodium azide, pH 8.00.

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capillaries and some large vessels, is increased in angiogenesis, and is also present on hematopoietic precursor cells (5, 12). Electron microscopy of capillary endothelium has shown that the CD34 molecule is luminal and concentrated on membrane processes at endothelial junctions, a critical zone for leukocyte adhesion, and migration at sites of inflammation (12). Thus, CD34 is a candidate for an L-selectin counter-receptor that presents carbohydrate ligands both on HEV in lymphoid organs and also on less specialized vessels in non-lymphoid tissues. However, the function of CD34 in cell adhesion remains to be characterized. Thus far, only small quantities have been isolated from murine lymph nodes, and functional studies have been limited to demonstrating that soluble, radiolabeled CD34 can be isolated with L-selectin affinity matrices (4, 22). CD34 has not been demonstrated to support lymphocyte or neutrophil adhesion, either in static or in flow assays. Antibodies to CD34 have not been reported to inhibit L-selectin–dependent adhesion. Multiple glycoprotein components are present in PNAd, and the contribution of CD34 relative to other PNAd components in adhesive interactions has been unclear.

In this study, we have examined the role of CD34 in mediating lymphocyte tethering and rolling under in vitro flow conditions, and determined its contribution relative to other components in the PNAd complex. We isolated PNAd from human tonsil, and separated CD34 from the other components. CD34 has previously been shown to be well expressed on HEV in tonsil sections (17), but its function has not been characterized, and it has not previously been shown to be present in human PNAd. Studies on lymphocyte tethering, rolling, and resistance to detachment in shear flow show that CD34 is responsible for a majority of adhesive activity. MECA-79–reactive CD34 from tonsil is more efficient as an L-selectin ligand than total tonsil CD34. Furthermore, we compare CD34 isolated from tonsil, spleen, and KG1a cells. We demonstrate that spleen CD34 is inactive, and KG1a cell CD34 is 10% as active as tonsillar MECA-79$^+$ CD34. KG1a cell CD34 lacks MECA-79 reactivity and may present a carbohydrate that is a model for a lower avidity L-selectin ligand such as found on leukocytes (3) and on postcapillary venules in non-lymphoid tissues (33, 50).

Materials and Methods

Monoclonal Antibodies and Cell Lines

The CD34 mAb 581 (IgG1), 563 (IgG1, kappa), and 547 (IgG2a, kappa) were produced as described (15) and have been characterized (18). Antibodies were purified by protein A affinity chromatography. Monoclonals 581 and 547 were biotinylated using biotin *N*-hydroxysuccinimide (Sigma Chemical Co., St. Louis, MO) active ester as described (19). My-10 mAb to CD34 (IgG1, kappa) (9) was purified using protein A–Sepharose from culture supernatants. The hybridoma was obtained from the American Type Culture Collection (ATCC, Rockville, MD).

MECA-79 mAb (rat IgM kappa) (47) was a generous gift of Dr. Eugene Butcher (Stanford, CA). Purified DREG-56 (IgG1) mAb to L-selectin (25) was used at 10 μg/ml. X63 (myeloma, IgG1) was used as a control at 1:5 dilution of culture supernatant. Hematopoietic progenitor KG1a cells (ATCC) and CHO cells stably transfected with full-length human E-selectin (34) (CHO-E) were maintained in RPMI 1640 and α-MEM medium, respectively, containing 10% heat-inactivated FBS, 5 mM glutamine, and 50 μg/ml gentamycin. Cells were harvested by a 10 min incubation with H/H medium containing 5 mM EDTA at 37°C. Cells were washed and resuspended in the same medium at 2×10^7 cells/ml and kept at room temperature until use.

Lymphocytes

Mononuclear cells were isolated from citrate anti-coagulated whole blood by dextran-sedimentation followed by density separation over Ficoll-Hypaque (37). Lymphocytes were prepared by incubating the mononuclear cells on tissue culture dishes for 1 h and collecting the nonadherent cells. Lymphocytes (>97%) were stored in HBSS (Ca^{2+} and Mg^{2+} free; GIBCO BRL, Gaithersburg, MD), 10 mM Hepes, pH 7.3, 0.5% human serum albumin (HSA). Cells were washed with and suspended in H/H binding medium (Ca^{2+}- and Mg^{2+}-free HBSS containing 2 mM $CaCl_2$, 0.2% HSA, and 10 mM Hepes, pH 7.3) before flow assays.

Immunoaffinity Purification and Blotting of Peripheral Node Addressin and CD34

PNAd (MECA-79 antigen) from human tonsil and CD34 from human tonsil, spleen and KG1a cells were isolated using sequential WGA-agarose (Sigma) and immunoaffinity chromatography. In a typical purification, ~10 g fresh human tonsils (Children's Hospital, Boston, MA), after removal of any blood clots, were minced with scissors on a stainless steel screen (200 mesh = 74 μm, Type 316; Tylinter, Mentor, Ohio). Lymphocytes that passed through the screen were eliminated by frequent flushing with RPMI-1640 medium (16). Stromal elements (8 g) remaining on the screen were collected. Tonsil stroma (8 g), human spleen (10 g), or KG1a cells (10-ml pellet) were homogenized and detergent extracts prepared in 250 ml of lysis buffer (2% Triton X-100, 150 mM NaCl, 0.02% NaN_3, 50 mM Tris-HCl, pH 7.5 containing 10 $\mu g\ ml^{-1}$ each of aprotinin, pepstatin, and leupeptin, 1 mM of PMSF, 1 mM benzamidine, and 5 mM iodoacetamide). After 1 h at 4°C the lysate was clarified by centrifugation (8,000 *g* for 30 min) followed by filtration through 0.45-μm filters and the supernatants were passed over WGA-coupled (9 mg/ml, 5 ml) agarose columns. After washing, material was eluted with 0.5 M *N*-acetylglucosamine, 150 mM NaCl, 0.02% NaN_3, 50 mM Tris-HCl, pH 7.5 containing 1% octyl-β-glucoside (OG) (WGA eluate). WGA eluates were passed through a control antibody–Sepharose column and then a column containing either MECA-79 mAb (2.64 mg/ml, 1.5 ml) or 581 mAb (2.5 mg/ml, 2 ml) coupled to Sepharose 4B (Pharmacia Inc., Piscataway, NJ). Columns were washed extensively (1% OG, 150 mM NaCl, 0.2% NaN_3, 50 mM Tris-HCl, pH 8.0) and eluted with 1% OG, 150 mM NaCl, 0.02% NaN_3 in 0.2 M acetic acid for MECA-79 antigen (PNAd) or with 1% OG, 150 mM NaCl, 0.02% NaN_3, 20 mM triethanolamine, pH 11.8, for 581 antigen (CD34). Eluted material was neutralized by collecting fractions in 1/10 vol of 1 M Tris-HCl, pH 8.5 for MECA-79 antigen or pH 7.4 for CD34. Protein concentrations were determined using the bicinchoninic acid assay (Pierce Chem. Co., Rockford, Illinois).

SDS-PAGE and Western blotting followed standard procedures (19, 26). Immobilon-P blots were incubated with MECA-79 mAb (5 μg/ml) or with a control antibody, followed by alkaline phosphatase-conjugated mouse anti–rat IgM (kappa and lambda light chains; Sigma) and developed according to manufacturer's instructions.

For amino acid sequence analysis, purified PNAd was subjected to SDS 7.5% PAGE, and electroblotted on PVDF membranes (Millipore). Bands were visualized with Ponceau-S (Sigma) and the major component at 105 kD was excised and subjected to in situ trypsin digestion. Peptides were extracted from the membrane, separated using reversed phase columns, and sequenced (27).

ELISA for CD34

mAb 547 (10 μg/ml in PBS, 50 μl/well) was added to 96-well flat-bottom microtiter plates (Linbro, Flow Laboratories, McLean, Virginia) for 1 h at room temperature or overnight at 4°C. Wells were washed three times with PBS and blocked with 200 μl of 2% blot grade BSA (Promega Corp., Madison, WI) PBS for 1 h at 37°C. Wells were flicked empty and 50 μl of CD34 standards (twofold serial dilutions, 1,000 to 31.25 ng/ml) and samples for CD34 estimation diluted in 1% BSA-PBS were added in triplicate for 1 h at 37°C. Wells were washed three times with PBS. Biotinylated mAb 581 (50 μl of 2 μg/ml) was added for 30 min at 37°C. HRP-streptavidin (Zymed, South San Francisco, CA) diluted 1:4,000 (50 μl) was added for 30 min at 37°C. Wells were washed three times with PBS, once with 2,2-azido-di(3-ethylbenzthiazoline) sulfonic acid (ABTS) substrate buffer (Zymed), and 50 μl of ABTS substrate buffer was then added. Plates were incubated at room temperature and read after 20 min on a Dynatech Microtiter ELISA reader (410 nm). Mean OD readings were calculated and

the CD34 concentrations were calculated from a standard curve generated from the CD34 titration.

CD34 Depletion from PNAd

581 mAb–Sepharose 4B (125 μl wet bed, 2.5 mg/ml) was packed in a 10-ml polyprep chromatography column (Bio Rad Labs., Richmond, CA). The column was washed with the elution buffer (1% OG, 150 mM NaCl, 0.02% NaN_3, 20 mM triethanolamine, pH 11.8) and then equilibrated with the binding buffer (1% OG, 150 mM NaCl, 0.02% NaN_3, Tris-HCl, pH 7.5). The column was allowed to drain by gravity and drops on the side of the column were removed. PNAd (500 μl, 30 μg/ml) was allowed to pass through the column and the flow-through was collected. The flow-through was passed through again and this process repeated five times. The column was washed with six column volumes of binding buffer. The flow-through and washing were combined to obtain the PNAd,$CD34^-$ fraction. Bound CD34 was eluted with nine column volumes of elution buffer and neutralized with 1/10 volumes of 1 M Tris-HCl, pH 7.5 to obtain the PNAd,$CD34^+$ fraction. In parallel, another batch of PNAd was passed through a control mAb column (mouse IgG1) identically as described for the CD34 mAb column and the same fractions collected. Protein concentrations were determined by bicinchoninic acid and by CD34 ELISA. Recovery of PNAd protein when the depletion was carried out with the control antibody column was >95%. This material is referred to as the PNAd fraction in functional experiments.

Laminar Flow Assays

Different PNAd fractions and immunopurified CD34 from tonsil, spleen, and KG1a cells were diluted at the indicated concentrations in TSA, pH 7.5, and a final concentration of 0.1% OG, and a 50-μl drop was adsorbed to plastic slides overnight at 4°C. Nonspecific sites were blocked by incubating the plate with HBSS supplemented with 2% HSA for 30 min at room temperature. Substrate or HSA adsorbed slides were assembled in a parallel plate laminar flow chamber (260-μm gap thickness) and mounted on the stage of an inverted phase-contrast microscope (Diaphot-TMD; Nikon Inc., Garden City, NY), as previously described (29). Cells were resuspended at a concentration of 5×10^5 ml^{-1} in the H/H binding medium for flow assays (30). Tethering, resistance to detachment, and rolling velocity were measured twice on different 10× microscopic fields for each substrate containing an immobilized CD34 preparation or PNAd fraction. Cells were detached with 5 mM EDTA in H/H medium in between observation on different fields. Three or four independent substrates on different days were prepared for each preparation or fraction, at three different concentrations each. Data is reported as the average and SD of the three or four experiments.

Tethering in flow was defined as the number of nonadherent cells that tethered within the field of view and remained rollingly adherent for at least 5 s. The number of cells that tethered in one minute was measured at different wall shear stresses.

For detachment assays, cells were infused into the chamber at 0.84 dyn/cm^2, allowed to adhere until equilibration between rolling and nonadherent cells was reached (~3 min) and then the wall shear stress was increased every 10 s to a maximum of 36 dyn/cm^2 to generate a detachment force. The number of cells remaining bound was calculated as a percentage of the number of cells rolling on the substrate at 0.84 dyn/cm^2.

Rolling velocities were calculated as previously described (29) for 15 to 20 of the cells observed during detachment assays. Cell displacement was measured over 3–5-s intervals. Velocities were measured only for cells that remained adherent throughout the 10-s period during which a given shear was applied.

Inhibitors

To test CD34 mAbs, a CD34 substrate to which tethering had already been measured was incubated with a mixture of 10 μg/ml each of 547, 563, 581, and My-10 mAb for 20 min. Tethering was in the presence of these mAb in the binding medium each at a concentration of 5 μg/ml. For inhibition studies with L-selectin or control (IgG) mAbs, cells (10^7/ml) were preincubated with 10 μg/ml purified antibody for 10 min at room temperature in binding medium. The cell suspension was diluted 20-fold in binding medium and immediately perfused into the flow chamber. Inhibition studies with anionic polysaccharides were carried out at final concentrations of 17.5 μg/ml fucoidan, 20 μg/ml heparin, and 30 μg/ml of keratan sulfate from bovine cornea, all from Sigma. The antibody or the anionic polysaccharides were continuously present in the binding media.

Enzyme Digestion

A substrate to which tethering had already been measured was incubated with *Vibrio cholera* neuraminidase (5 mU/ml) (Oxford Glycosystems, Rosedale, NY) for 30 min in 50 mM sodium acetate, 4 mM $CaCl_2$, 0.1% BSA, pH 5.5 or with *O*-sialoglycoprotease (0.2 mg/ml) (Cedarlane, Ontario, Canada) in the binding medium for 1 h at room temperature by injecting the enzyme through a side port of the flow chamber. The chamber was then washed with binding medium and tethering was measured to the same field. To study the effect of other glycosidases, substrates were incubated with *N*-glycanase (2 U/ml) (Genzyme, Cambridge, MA) in 50 mM NaH_2PO_4, pH 7.5, or endo-β-galactosidase (1 U/ml) (Oxford Glycosystems) in 50 mM sodium acetate, pH 5.8, and for each glycosidase, 20 μM leupeptin, 30 μM antipain, 1 mM benzamidine, and 0.02% NaN_3 for 6 h at 37°C. After the treatment the slides were washed with PBS and blocked again with 2% HSA for 20 min at room temperature before the experiment. Several fields of view of substrates treated with glycosidase or sham treated with buffer alone were monitored.

Site Density Determination

581 mAb to CD34 was iodinated (7.5 μCi/μg specific activity) using IODO-BEADS (Pierce) following the instructions of the manufacturer and site densities of CD34 at the concentrations used for flow experiments, were determined by saturation binding (11). Protein samples (10 μl) were coated at 4°C for 12 h, using removawell microtiter plates (Immulon 3; Dynatech Laboratories Inc., Chantilly, VA), and other adsorption sites on plastic were blocked with PBS containing 2% HSA. Saturating concentrations of ^{125}I-labeled mAb (2 μg/ml) in 50 μl binding medium (PBS/NaN_3, 0.1% HSA) were added to the wells and incubated for 1 h at 22°C. The wells were washed five times with binding medium, removed from the plate, and counted in a γ counter. Specific binding was defined as counts min^{-1} bound to substrate-coated wells minus counts min^{-1} bound to HSA-coated wells. Site densities were calculated assuming binding of one IgG molecule per antigen molecule at saturation. All assays were performed in triplicate. The values of sites per μm^2 for PNAd,$CD34^+$ fraction adsorbed at 440, 220, and 147 ng/ml for the flow assay were 287 ± 35, 195 ± 27, and 96 ± 12, respectively. Site densities/μm^2 for CD34 from KG1a, spleen and tonsil adsorbed at 440 ng/ml were 300 ± 37, 302 ± 40, and 295 ± 30, respectively. PNAd adsorbed at 1,460 ng/ml had 279 sites/μm^2 of CD34.

Results

A Prominent 105-kD Component of Human Tonsil PNAd Is the Sialomucin CD34

WGA affinity chromatography (28) was used to enrich L-selectin ligands in tonsil stroma detergent lysates. Western blots of the tonsil stromal lysate and the flow-through from the WGA column showed that all species reactive with MECA-79 mAb were bound to the column (data not shown). Sandwich ELISA for CD34 also showed that it was quantitatively bound to the WGA column (data not shown). The eluate from the WGA–Sepharose column was applied to a MECA-79 mAb–Sepharose column to isolate PNAd. Blotting of the isolated PNAd with MECA-79 mAb showed a prominent polypeptide migrating at 105 kD with additional bands at 60, 165, and 205 kD (Fig. 1 *A*). The same bands with the 105-kD band again prominent were visualized by silver staining purified PNAd (Fig. 1 *B*) and after ^{125}I-labeling (data not shown) (6). Isolation and sequencing of tryptic peptides from the 105-kD band revealed several with identity with the sequence predicted from the human CD34 cDNA, including Leu-Gly-Glu-Asp-Pro-Tyr-Tyr-Thr-Glu-Asn-Gly, corresponding to residues 293–303 (44).

CD34 in PNAd was separated from the other components in this complex glycoprotein mixture to allow their relative contributions to L-selectin ligand activity to be in-

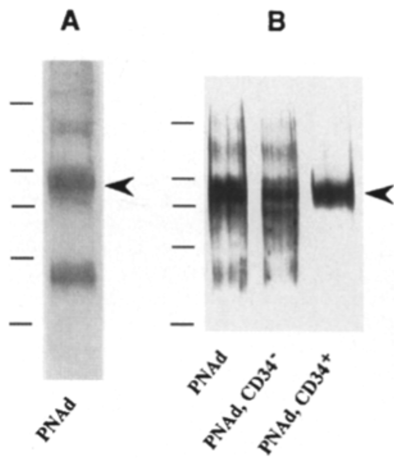

Figure 1. Purified PNAd and fractionation with CD34 mAb–Sepharose. (*A*) Immunoaffinity-purified PNAd (~1 μg) was electophoresed under reducing conditions in SDS 8% PAGE, and electophoretically transferred onto Immobilon-P membranes (Millipore) that were probed with MECA-79 mAb (5 μg/ml) and alkaline phosphatase-conjugated mouse anti-rat mAb. (*B*) Equal proportions of purified PNAd, PNAd depleted of CD34 (*PNAd,CD34⁻*), and CD34 purified from PNAd (*PNAd,CD34⁺*) were subjected to SDS 7.5% PAGE under reducing conditions and silver staining. Arrowheads denote the band corresponding to CD34; lines to the left of *A* and *B* denote the position of markers: 200, 116, 97.4, 66, and 45 kD.

vestigated in shear flow. PNAd was subjected to affinity chromatography on Sepharose conjugated to the CD34 mAb 581. This mAb reacts well with CD34 expressed in tonsil HEV, in contrast with some mAb that show diminished reactivity with this CD34 glycoform, and recognizes an epitope that is resistant to neuraminidase and *O*-sialoglycoprotease digestion (17, 18). The band at 105 kD (Fig. 1 *B*, *arrow*) was significantly depleted in the PNAd,CD34⁻ fraction, and was the only band seen in the PNAd,CD34⁺ eluate fraction. A capture ELISA was used to quantitate CD34 in the different fractions. Protein concentrations of the fractions were determined with bicinchoninic acid assay, and the concentration of the CD34 fraction was used to standardize the ELISA (Table I). CD34 was 30% of PNAd glycoprotein. This was reduced to 4% in the PNAd,CD34⁻ fraction (Table I); thus the extent of CD34 depletion was 88%. Comparisons of the amount of protein in the depleted and eluted fractions to the starting material showed quantitative recoveries of the glycoproteins. This was also apparent from the silver stained gel in which an equal proportion of the fractions was loaded (Fig. 1 *B*). Some material migrating near to CD34 remained in the PNAd,CD34⁻ fraction, which may correspond to a distinct glycoprotein.

Table I. Protein Preparations

Sample	Protein conc.	Volume	Total protein	CD34 conc.	% CD34 protein
	μg/ml	*μl*	*μg*	*μg/ml*	
PNAd	29.2	500	14.6	8.8*	30
PNAd,CD34⁻	8.1	1250	10.1	0.33*	4.0
PNAd,CD34⁺	3.52	1250	4.4	3.52‡	100

*CD34 ELISA.
‡Protein assay.

Tethering in Flow on PNAd Components

To determine the contribution of CD34 in PNAd to tethering under laminar flow conditions, lymphocytes were perfused through a parallel wall flow chamber in which several concentrations of PNAd,CD34-depleted PNAd (PNAd,CD34⁻) or CD34 purified from PNAd (PNAd, CD34⁺) were immobilized. Under continuous flow conditions freshly purified lymphocytes tethered to and rolled on PNAd and PNAd fractions, but not on HSA-coated control substrates. Tethering required Ca^{2+} and was completely inhibited by mAb to L-selectin (Fig. 2). Comparisons of equal proportions of PNAd (1,460 ng/ml), PNAd, CD34⁻ (1,020 ng/ml), and PNAd,CD34⁺ (440 ng/ml) fractions, diluted to correspond to the amount of each component in PNAd as a whole, showed that the PNAd,CD34⁺ fraction had slightly more tethering activity than the PNAd,CD34⁻ fraction and that each had close to 50% of the activity of the PNAd fraction (Fig. 2).

The density of immobilized CD34 was similar in the PNAd (279 sites/μm^2) and PNAd,CD34⁺ (287 sites/μm^2)

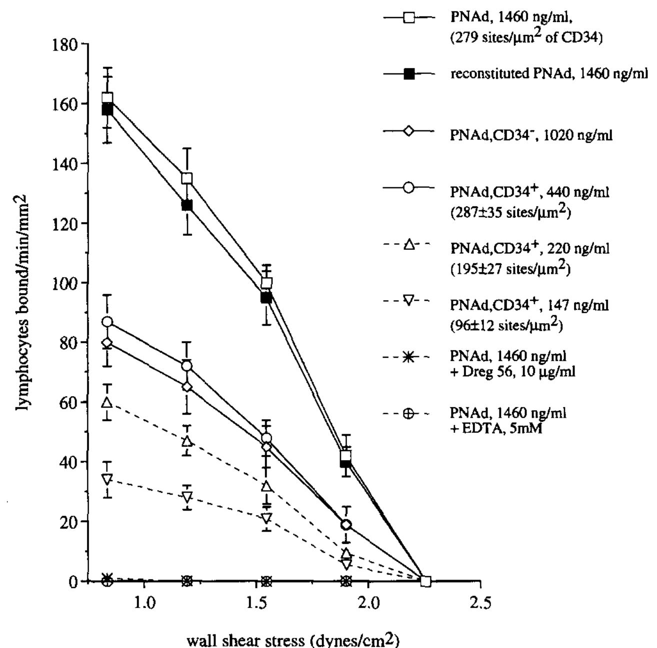

Figure 2. Lymphocyte tethering in shear flow to PNAd fractions. Lymphocytes (5 × 10^5 cells/ml) were infused at varying wall shear stresses through the parallel wall flow chamber. PNAd fractions were immobilized at the indicated concentrations and yielded the indicated CD34 site densities. Fractions are PNAd; CD34 purified from PNAd (*PNAd,CD34⁺*); PNAd depleted of CD34 (*PNAd,CD34⁻*); and the recombined PNAd,CD34⁺ and PNAd,CD34⁻ fractions (*reconstituted PNAd*). CD34 (440 ng/ml) and PNAd,CD34⁻ (1,020 ng/ml) correspond to the amounts of these fractions present in PNAd (1,460 ng/ml). The number of tethers formed per min at a given shear stress was quantitated. The data points represent the mean ± range of the number of tethered lymphocytes in two randomly selected 10× fields and are representative of three to four different independent experiments. The presence of mAb DREG-56 to L-selectin or EDTA inhibited tethering to all the substrates.

fractions, showing that other components in PNAd had little effect on immobilization of CD34. Tethering efficiency was related to the protein concentration used for immobilization, and to site density, as shown with three concentrations of the PNAd,CD34$^+$ preparation. To confirm that functional activity had not been lost during fractionation on CD34 mAb–Sepharose, we also mixed together equal proportions of PNAd,CD34$^-$and PNAd,CD34$^+$ fractions (reconstituted PNAd) to compare to the starting PNAd. Full activity was recovered in the reconstituted PNAd (Fig. 2). Experiments with each fraction diluted two- and threefold further showed proportionally less tethering, and the same overall results, with the PNAd,CD34$^+$ fraction containing slightly more than half the total PNAd activity.

Lymphocyte Rolling on PNAd Components

Having established that CD34 is a major L-selectin tethering ligand in human tonsil PNAd, we monitored the contribution of CD34 to lymphocyte rolling. The rolling velocity of lymphocytes decreased as the site density of immobilized CD34 was increased, suggesting more L-selectin bonds with the substrate (Fig. 3). Rolling velocity increased as shear stress was increased. The rolling velocities for lymphocytes, measured at a range of shear stress values, were higher on the PNAd,CD34$^+$ (440 ng/ml) and PNAd,CD34$^-$ (1,020 ng/ml) fractions than on PNAd (1,460 ng/ml), showing that both fractions stabilize rolling interactions. Lymphocytes rolled slightly slower on the PNAd, CD34$^+$ (440 ng/ml) fraction than on the PNAd,CD34$^-$ (1,020 ng/ml) fraction. Mixing of these fractions to yield the reconstituted PNAd resulted in a lower velocity identical to that seen on PNAd.

Strength of Lymphocyte Adhesion to PNAd Components

To examine further the function of the CD34$^-$ and CD34$^+$ components of PNAd, we assayed the strength of lymphocyte rolling adhesion as measured by resistance to detachment by increasing wall shear stress (8, 29). Resistance to detachment by shear was dependent on CD34 site density, suggesting that the density of CD34 on the plate was related to the number of receptor-ligand bonds that could form rolling interactions (Fig. 4). Lymphocytes on PNAd were more resistant to shear detachment than on the PNAd,CD34$^+$ and PNAd,CD34$^-$ fractions. Resistance to detachment on the PNAd,CD34$^+$ fraction was somewhat higher than on the corresponding concentration of the PNAd,CD34$^-$ fraction, showing a significant participation of CD34 compared to the other components in PNAd for L-selectin mediated interactions. At two lower concentrations, rolling adhesions formed on the PNAd,CD34$^+$ fraction were also more resistant to detachment than on corresponding concentrations of the PNAd,CD34$^-$ fraction (not shown). Reconstituted PNAd exhibited similar detachment profiles as PNAd suggesting additive contributions of the fractions (Fig. 4).

CD34 from Different Tissues

We isolated total tonsillar CD34; CD34 from spleen, where lymphocyte homing is L-selectin independent; and CD34 from hematopoietic progenitor KG1a cells, for comparisons to one another and to the CD34 component of PNAd. Immunoaffinity-purified CD34 from human tonsil migrates as a 100–115-kD broad band under reducing (Fig. 5 *A*) and nonreducing conditions (not shown). Similar amounts of CD34 from spleen and KG1a migrated as relatively sharper bands with slightly lower molecular mass indicating less heterogeneity in extent of their glycosylation (Fig. 5 *A*). Western blots showed that CD34 from tonsil but not from spleen or KG1a cells is reactive with MECA-79 mAb (Fig. 5 *B*). MECA-79 reactivity occurred in a relatively higher molecular mass subset of the total CD34 from tonsil.

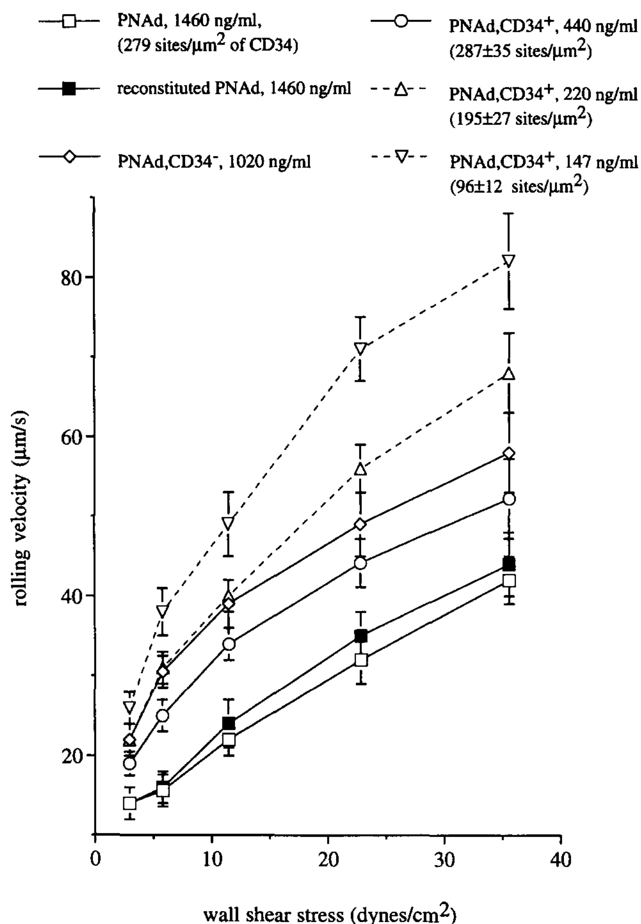

Figure 3. Lymphocyte rolling on PNAd fractions. Lymphocytes were tethered at a wall shear stress of 0.84 dyn/cm^2 at the indicated concentrations and site densities of PNAd; CD34 depleted PNAd (*PNAd,CD34$^-$*); CD34 purified from PNAd (*PNAd, CD34$^+$*); and reconstituted PNAd. Wall shear stress was then increased every 10 s to a maximum of 36 dyn/cm^2 and rolling velocities was measured as described in experimental procedures. PNAd,CD34$^+$ (440 ng/ml) and PNAd,CD34$^-$ (1,020 ng/ml) correspond to the amounts present in PNAd (1,460 ng/ml). The data points represent the mean rolling velocity ± the standard error of the mean and are representative of three to four different independent experiments.

Lymphocytes were perfused at a representative wall shear stress of 0.84 dyn/cm^2 through a parallel plate flow chamber to compare tethering to the different CD34 preparations immobilized at 300 sites/μm^2 (Fig. 6). Total CD34

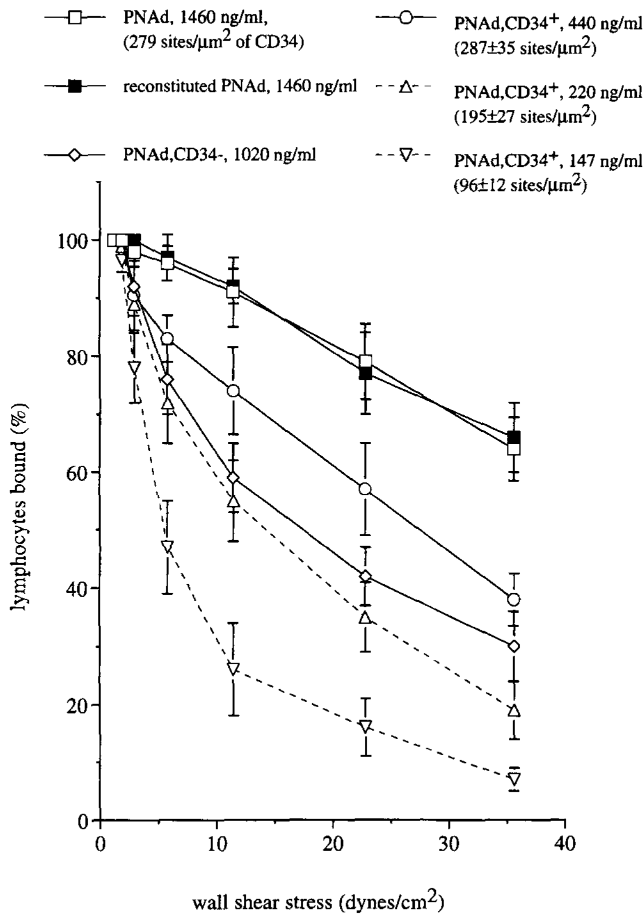

Figure 4. Resistance to detachment by shear of lymphocytes on PNAd fractions. Lymphocytes were allowed to tether at 0.84 dyn/cm^2 for 2 to 3 min at the indicated concentrations and site densities of PNAd; CD34 depleted PNAd (*PNAd,CD34*$^-$); CD34 purified from PNAd (*PNAd,CD34*$^+$); and reconstituted PNAd. The shear stress was then increased every 10 s to a maximum of 36 dyn/cm^2 and the percentage of cells remaining bound at each shear was determined. CD34 (440 ng/ml) and PNAd,CD34$^-$ (1,020 ng/ml) correspond to the amounts of these fractions present in PNAd (1,460 ng/ml). The data points represent the mean ± range of the number of lymphocytes that remained bound in two randomly selected 10× fields and are representative of three to four different independent experiments.

from tonsil supported tethering at an efficiency 3.6-fold lower than the PNAd,CD34$^+$ fraction; i.e., the MECA-79–reactive subset of tonsillar CD34. CD34 from KG1a cells supported tethering at an efficiency 8.5-fold lower than the PNAd,CD34$^+$ fraction and 2.4-fold lower than total tonsillar CD34. Though less efficient, tethering to KG1a CD34 was specific as shown by complete inhibition by DREG-56 mAb to L-selectin, neuraminidase treatment (Fig. 6), and EDTA (not shown). Furthermore, KG1a CD34 supported rolling interactions similar to those on CD34 from tonsil. Rolling velocities at 2.96 dyn/cm^2 on the PNAd,CD34$^+$ fraction, total tonsillar CD34, and KG1a CD34 were 19, 35, and 42 μm/s, respectively. Thus, KG1a CD34 supported tethering and rolling interactions through L-selectin despite lack of the MECA-79 epitope. By contrast, CD34 from spleen supported no tethering or rolling interactions.

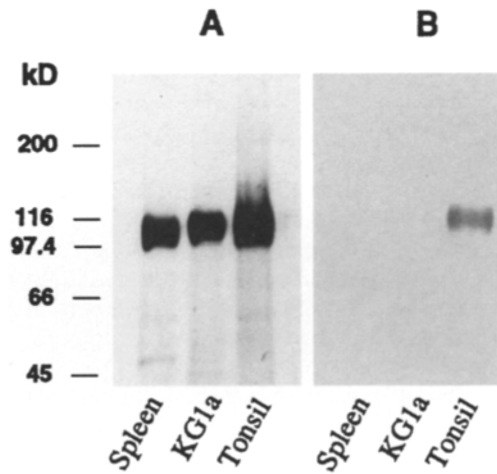

Figure 5. SDS-PAGE and Western blot analysis of immunopurified CD34. CD34 was purified from detergent extracts of tonsil, spleen, and KG1a cells using CD34 antibodies conjugated to Sepharose, as described in experimental procedures. (*A*) Similar amounts of CD34 (~1 μg) each from spleen, KG1a, and tonsil were subjected to SDS 7.5% PAGE under reducing conditions followed by silver staining. (*B*) About 0.25 μg of each CD34 preparation electrophoresed in a similar gel was electophoretically transferred onto Immobilon-P membrane and probed with MECA-79 mAb (5 μg/ml). The bound antibody was detected by color developed using an alkaline phosphatase-conjugated mouse anti–rat mAb. MECA-79 reacts with CD34 from tonsil but not from spleen and KG1a cells.

E-selectin transfectants were perfused through the parallel plate flow chamber to compare E-selectin ligand activity of the CD34 preparations. The PNAd,CD34$^+$ fraction and total CD34 from tonsil, KG1a cells, and spleen all supported tethering and rolling of E-selectin transfected CHO cells. The adhesion was completely inhibited by BB11 mAb to E-selectin (Fig. 6) and required Ca^{2+} as shown by complete inhibition with EDTA (data not shown). Unlike L-selectin, E-selectin did not show a preference for the HEV glycoform of CD34 and bound to CD34 from all sources.

Characteristics of the Interactions of L-selectin with the PNAd,CD34$^-$ and PNAd,CD34$^+$ Fractions

Inhibitors and enzymatic treatments were characterized for effect on interactions with the CD34$^+$ and CD34$^-$ fractions of PNAd. Certain anionic mono- and polysaccharides block L-selectin–dependent binding (46, 51, 52). Fucoidan and heparin both inhibited L-selectin mediated tethering of lymphocytes to PNAd,CD34$^+$ (Fig. 7). However, the control polysaccharide keratan sulfate was completely inactive at a concentration as high as 30 μg/ml. A cocktail of 4 mAb to CD34, 547, 563, 581, and My-10, had no effect on lymphocyte tethering. MECA-79 mAb blocked lymphocyte adhesion to both the PNAd,CD34$^+$ and PNAd,CD34$^-$ fractions by more than 85%, as previously reported for total PNAd (6).

L-selectin ligands in HEV are sialylated and neuraminidase treatment abrogates binding (42, 43). Treatment with neuraminidases from *Vibrio cholera* (Fig. 7) or Newcastle disease virus (data not shown) completely prevented lym-

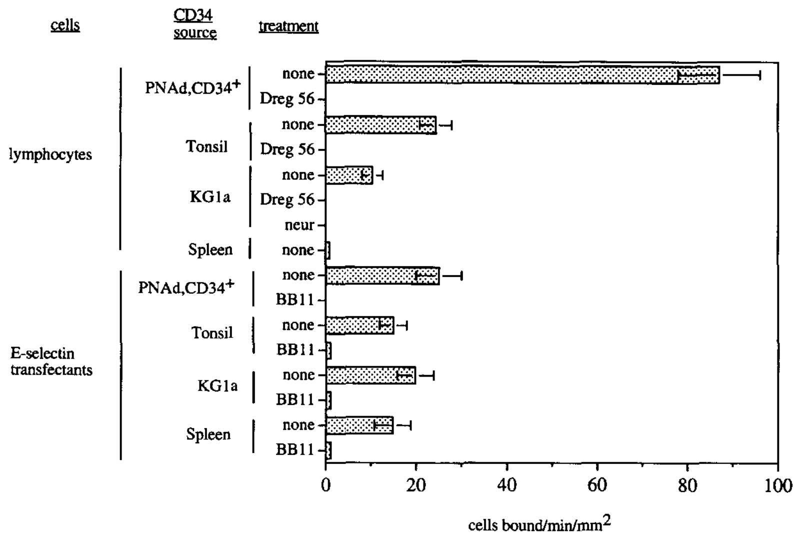

Figure 6. Lymphocyte and E-selectin transfectant (CHO-E) cell tethering in hydrodynamic flow to CD34 from different sources. Lymphocytes or CHO-E cells (5×10^5 cells/ml) were infused at a wall shear stress of 0.84 dyn/cm^2 on polystyrene slides assembled in a parallel plate flow-chamber. CD34 purified from PNAd, total CD34 from human tonsil, spleen, and KG1a cells were adsorbed at 440 ng/ml and yielded 287 ± 35, 300 ± 37, 302 ± 40, and 295 ± 30 sites of CD34/μm^2, respectively. The number of cells that tethered within the field of view per min per mm^2 was calculated. DREG-56 mAb to L-selectin and BB-11 mAb to E-selectin at 10 μg/ml completely inhibited tethering of lymphocytes, or CHO-E cells, respectively. *Vibro cholera* neuraminidase digestion of CD34 from KG1a cells was performed as described in Materials and Methods. The data represent the mean ± SD of two randomly selected 10× fields and are representative of two independent experiments.

phocyte tethering to both the PNAd,CD34$^+$ and PNAd, CD34$^-$ fractions.

O-Sialoglycoprotease is an enzyme produced by the bacteria *Pasteurella hemolytica* (1) that selectively degrades *O*-sialomucins and has been shown to abolish binding of neutrophils to P-selectin (39, 48). Treatment of the immobilized PNAd,CD34$^+$ and PNAd,CD34$^-$ fractions with *O*-sialoglycoprotease inhibited lymphocyte tethering by 95 and 92%, respectively (Fig. 7). The few lymphocytes that tethered after *O*-sialoglycoprotease treatment rolled only a few cell diameters after tethering and completely detached when the shear stress was raised to 1.5 dyn/cm^2 (data not shown). These results show that mucin-like domains are important for interactions of both the CD34$^+$ and CD34$^-$ fractions of PNAd with L-selectin. The cDNA sequence of human CD34 predicts nine potential N-glycosylation sites (20). *N*-glycanase treatment of the substrates did not affect lymphocyte tethering; however, it was difficult to determine whether the N-linked glycans had been removed because there was little effect on electrophoretic mobility (~7 kD) of the PNAd,CD34$^+$ fraction in SDS-PAGE (data not shown). Endo-β-galactosidase hydrolyzes internal β1-4 linkages between galactose and *N*-acetylglucosamine (Galβ1-4GlcNAc) in extended unbranched poly–*N*-acetyllactosamine chains and removes most of the poly–*N*-acetyllactosamine chains of the P-selectin glycoprotein ligand from human neutrophils (38). This enzyme neither altered the SDS-PAGE mobility of PNAd,CD34$^+$ (data not shown) nor affected lymphocyte tethering to the CD34$^+$ or CD34$^-$ fractions of PNAd (Fig. 7). The enzyme

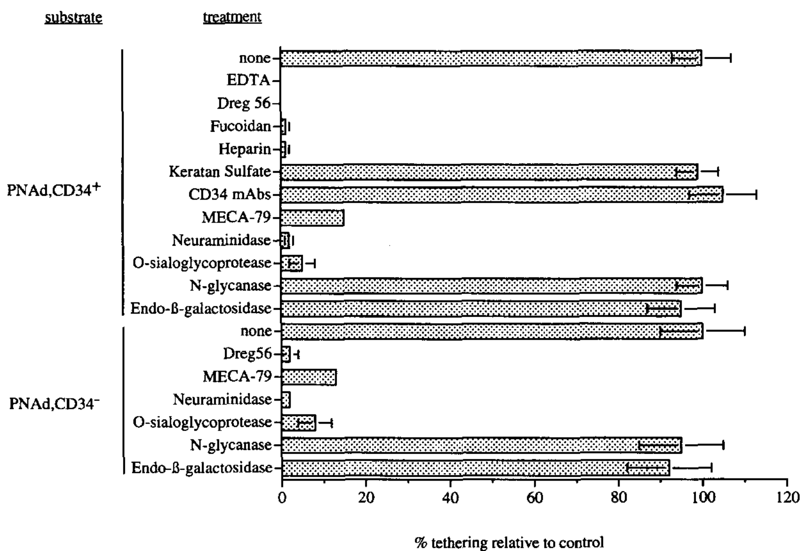

Figure 7. Characteristics of the interaction of lymphocyte 3 with PNAd fractions under flow conditions. CD34 purified from PNAd (PNAd,CD34$^+$, 440 ng/ml, 287 ± 35 sites/μm^2) and CD34-depleted PNAd (PNAd,CD34$^-$, 520 ng/ml) were adsorbed onto plastic Petri dishes and assembled into the flow apparatus. Lymphocytes (5×10^5 /ml) were perfused through the flow chamber at a wall shear stress of 0.84 dyn/cm^2 and the number of tethers in the field of view formed per minute per mm^2 was calculated. Lymphocytes were preincubated with anionic polysaccharides for 5 min in the binding medium and infused without dilution into the flow chamber. Concentrations of fucoidan, heparin, and keratan sulfate were 17.5, 20, and 30 μg/ml, respectively. Immobilized CD34 was incubated with a mixture of CD34 mAbs (10 μg/ml each of 547, 563, 581, and My-10) for 20 min and flow assay was performed in the presence of these antibodies (5 μg/ml) in the binding medium. Immobilized PNAd fractions were treated with 20 μg/ml MECA-79 mAb for 30 min and tethering was determined in the continued presence of mAb. Immobilized PNAd fractions were treated with indicated glycosidases as described in Materials and Methods. The data represent the mean ± SD of two to three independent experiments normalized to control binding (88 ± 6 and 58 ± 6 cells per min per mm^2 for PNAd,CD34$^+$ and PNAd,CD34$^-$ substrates, respectively). Several fields of immobilized substrate were analyzed for sham or *N*-glycanase and endo-β-galactosidase treatments. The number of lymphocytes was kept constant in all the above comparisons.

resistance suggests either the absence of poly-*N*-acetyllactosamine chains or modifications by substitutions such as sulfate or fucose that lead to resistance.

Discussion

The present study demonstrates that the sialomucin CD34 can support L-selectin–dependent lymphocyte adhesive interactions in shear flow. Previous results have shown that peripheral lymph node addressin (PNAd), a complex mixture of glycoproteins from human tonsil recognized by the MECA-79 mAb, mediates lymphocyte binding through L-selectin (7, 31). Studies in the murine system have identified two components of PNAd, GlyCAM-1, and CD34 (4, 28), but the relative contributions of these two mucin-like molecules, and other components of PNAd, to cell adhesive interactions through L-selectin have been unclear.

We have determined the contribution of CD34 relative to other components in the PNAd complex in mediating lymphocyte tethering, rolling, and resistance to detachment in shear. Furthermore, we have demonstrated that CD34 is a prominent component of human PNAd; indeed, it appears to correspond to a previously uncharacterized 105-kD glycoprotein noted to be a major component of PNAd (6). We have identified this major component as CD34 by immunodepletion, and by sequencing of tryptic peptides.

CD34 accounted for close to 30% of the protein in PNAd. CD34 was isolated from PNAd in high purity as shown by SDS-PAGE, and this PNAd,CD34$^+$ fraction was compared to the CD34-depleted PNAd or PNAd,CD34$^-$ fraction. ELISA showed at least 88% depletion of CD34 from this fraction. Tethering efficiency, a direct measure of the ability of a ligand to mediate interactions with flowing cells, was reduced somewhat more than 50% after CD34 depletion. Furthermore, the CD34 component of PNAd contained somewhat more than 50% of the tethering activity. Comparing tethering efficiency to protein content, the PNAd,CD34$^+$ fraction is 2.3-fold more active than the PNAd,CD34$^-$ fraction. The caveat is that we do not know the efficiency of immobilization of the PNAd, CD34$^-$ fraction; however, it clearly is immobilized as shown by its activity, and we suspect that immobilization efficiency will be similar to the PNAd,CD34$^+$ fraction. Moreover, both the studies with the depleted fraction, and the comparison of the PNAd,CD34$^+$ fraction to PNAd and to the reconstituted PNAd fraction, suggest that CD34 is a major functional component of PNAd. CD34 contains an NH_2-terminal region of 145 amino acid residues containing 35% serine and threonine, which is removed by *O*-sialoglycoprotease. This mucin-like domain is followed by a globular domain of 66 amino acids and a COOH-terminal transmembrane domain that remain on the cell surface after *O*-sialoglycoprotease or mild protease digestion (18, 44). Based on its size in SDS-PAGE, CD34 appears to be greater than 60% carbohydrate by weight. Since carbohydrate determinants on selectin ligands are important for selectin recognition (23, 24), we expect that CD34 has a high content of critical carbohydrates efficiently displayed in the mucin-like domains that contribute to the avid interaction with L-selectin. Critical carbohydrate modifications such as sulfation may also contribute to the more efficient binding by CD34; it incorporates a higher amount of sulfation as shown in immunoprecipitates with MECA-79 of tonsil stromal lysates metabolically labeled with $[^{35}S]SO_4$ (not shown). Furthermore, Western blots with MECA-79 mAb emphasize the CD34 105-kD band more than silver-stained gels (Fig. 1, *A* and *B*). Although displayed on distinct proteins, the carbohydrate ligands in both the PNAd,CD34$^-$ and PNAd,CD34$^+$ fractions are associated with mucin-like domains, as shown by sensitivity to *O*-sialoglycoprotease.

We used three independent measures of selectin function: tethering, rolling velocity, and resistance to detachment in shear. Tethering of a flowing cell to a substrate involves formation of the first receptor-ligand bond between the cell and the substrate, and must be followed by some rolling for the tether to persist long enough to be counted. Rolling velocity is a function of the kinetics of bond formation and dissociation and the average number of receptor-ligand bonds between the cell and the substrate at any one time. Resistance to detachment in shear may depend more on the average number of receptor-ligand bonds, and how they respond to applied force. Using all three measures, the CD34 component of PNAd contributed more than half the total activity, and was 10–20% more active than the CD34-depleted component of PNAd. Thus, there was no evidence for a selective function for CD34 in any of these assays. Reconstitution experiments also showed that the functions of the PNAd,CD34$^-$ and PNAd,CD34$^+$ fractions were additive rather than synergistic.

CD34 is widely expressed in the vasculature in both human and mouse. It is present on many large and small vessels including postcapillary venules and capillaries in a wide variety of tissues, as well as on HEV. This has led to the hypothesis of the possible role of CD34 in leukocyte trafficking to both lymphoid and nonlymphoid sites (5, 12). CD34 is also expressed on hematopoietic stem cells, where its function is unknown.

We compared CD34 isolated from tonsil, spleen, and KG1a hematopoietic cells. The CD34 component of tonsillar PNAd was more active than total tonsillar CD34. This may reflect CD34 expression at other sites in tonsil besides HEV, as well as the possibility that not all CD34 expressed in HEV may react with MECA-79 mAb. CD34 in spleen is present in sparse vascular structures between the red and white pulp (5); lymphocyte migration from the blood to spleen is known not to require L-selectin (2). Consistent with this, spleen CD34 did not support L-selectin functions including tethering and rolling, as found previously for CD34 transfectants (40). Interestingly, KG1a CD34 possessed substantial tethering and rolling activity as an L-selectin ligand. KG1a CD34 was 42 and 12% as active as total tonsillar CD34 and PNAd,CD34$^+$ fractions, respectively. KG1a cells and KG1a CD34 do not react with MECA-79 mAb; this dissociates L-selectin ligand activity from MECA-79 reactivity. This is in agreement with recent observations that neutrophils have an L-selectin ligand that contributes to neutrophil aggregation (45) and can support rolling interactions of one layer of neutrophils on another (3). It is of further interest that all forms of CD34 were active as E-selectin ligands. Previous immunohistochemical and precipitation studies have shown that

both L-selectin and E-selectin can recognize ligands on HEV (35) and bind to purified PNAd (7). Furthermore, both L-selectin and E-selectin can bind to sialyl Lewis^x and related structures, and this has been proposed as a minimal recognition motif that requires additional modifications (such as sulfation) for optimal recognition (7, 13, 24). The major capping group of GlyCAM-1 has been defined as 6′-sulfo sLe^x (21), and the MECA-79 epitope also requires sulfation (22). The finding that the MECA-79$^+$ PNAd,CD34$^+$ fraction is more active than the MECA-79$^-$ KG1a CD34 is consistent with the importance of sulfation in a high-affinity L-selectin ligand. However, our observation that KG1a and spleen CD34 are active as E-selectin ligands, yet are active and inactive as L-selectin ligands, respectively, implies that a modification that may be distinct from sulfation, or at least from the MECA-79 epitope, is also important in modifying a sLe^x minimal recognition motif into an optimal L-selectin ligand. We have unpublished evidence that other molecules besides CD34 on KG1a cells bear L-selectin ligand activity, consistent with another report (40). It remains to be determined whether the L-selectin ligand on postcapillary venules in nonlymphoid tissues important in neutrophil rolling (33, 49) is expressed on CD34, and whether the carbohydrate expressed on KG1a CD34 will be similar in structure to L-selectin ligands expressed on these MECA-79$^-$ venules and on neutrophils (3).

In summary, our data provide the first demonstration that sialomucin CD34 mediates cell adhesion and supports lymphocyte tethering and rolling under in vitro flow conditions. We show that CD34 is the predominant L-selectin ligand in the mixture of peripheral node addressins from human tonsil. Furthermore, HEV specific glycosylation is required for high affinity binding to L-selectin. A modification distinct from the MECA-79 epitope can also modulate L-selectin ligand and differentiate it from E-selectin ligand activity. Further studies, such as comparison of the differential glycosylation responsible for the L-selectin binding activity of CD34 from tonsil but not from spleen will further our understanding of modulation of L-selectin ligand activities.

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