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A Schiff Base with Mildly Oxidized Carbohydrate Ligands Stabilizes L-selectin and not P-selectin or E-selectin Rolling Adhesions in Shear Flow*

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Selectins are a family of lectins, that mediate tethering and rolling of leukocytes on endothelium in vascular shear flow. Mild periodate oxidation of the L-selectin ligand CD34, or L-selectin ligands on leukocytes, enhanced resistance to detachment in shear and decreased rolling velocity equivalent to an 8-fold increase in ligand density, yet had little effect on the rate of tethering. Enhanced interactions were also seen with mildly oxidized sialyl Lewis^a and sialyl Lewis^x glycolipids. Enhancement was completely reversed by borohydride reduction, yielding a strength of interaction equivalent to that with the native ligands. No effect on the strength of P-selectin and E-selectin interactions was seen after mild oxidation of their ligands. Completeness of modification of sialic acid by mild periodate was verified with monoclonal antibody to sialyl Lewis^x-related structures and resistance to neuraminidase. The addition of cyanoborohydride to leukocytes rolling through L-selectin on mildly oxidized but not native CD34 caused arrest of rolling cells and formation of EDTA-resistant bonds to the substrate, suggesting that a Schiff base was reduced. Cyanoborohydride reduction of mildly oxidized cells rolling on P-selectin and E-selectin also caused arrest and formation of EDTA-resistant bonds but with slower kinetics. These data suggest that interactions with a sialic acid aldehyde group on mildly oxidized ligands that include interconversion to a Schiff base can occur with three selectins yet only stabilize binding through the selectin with the fastest k_{off}, L-selectin.

The selectins are a family of three Ca^{2+}-dependent membrane-bound lectins that initiate adhesion of leukocytes to platelets or endothelial cells under the shear forces found in the venular circulation (1–3). L-selectin is expressed on leukocytes (4) and binds certain sulfated glycoproteins from lymph node high endothelial venules (HEVs),[1] initially defined by mAb MECA-79 and collectively known as peripheral node addressin (PNAd) (5). The components of PNAd include the sialomucins GlyCAM-1 (6) and CD34 (7). CD34 is the major L-selectin ligand from human tonsil HEV and mediates leukocyte tethering and rolling in shear flow (8). L-selectin also binds to uncharacterized ligands on neutrophils and hematopoietic progenitor cells (9, 10). P-selectin, expressed by thrombin-activated platelets and endothelial cells, and E-selectin, expressed by cytokine-activated endothelial cells, bind to carbohydrate ligands on myeloid cells and subsets of lymphocytes. P-selectin glycoprotein ligand (11–13) and E-selectin ligand (14) bear selectin ligands on myeloid cells.

Selectins contain highly homologous (60–70% amino acid identity) Ca^{2+}-dependent N-terminal lectin-like domains. The three-dimensional structure is known for E-selectin (15). Site-directed mutagenesis studies have identified a number of residues that are critical to P-selectin- and E-selectin-mediated carbohydrate recognition (15–17). All three selectins bind sialylated, fucosylated lactosaminoglycans, of which the prototype is sialyl Lewis^x (sLe^x; Neu5Acα2–3Galβ1–4(Fucα1–3)GlcNAc) (18, 19). *O*-linked glycans of GlyCAM-1 have been structurally characterized and include a 6′-sulfosialyl Lewis^x core 2 structure attached to a T-antigenic core 1 structure that also bears sialic acid (20, 21). E-selectin ligand is also decorated with sLe^x (14). P-selectin glycoprotein ligand has *O*-linked glycans extended with poly-*N*-acetyl lactosamine, carries sLe^x, and can also bind E-selectin (12). Sialic acid is essential to ligand activity as shown by abolition with neuraminidase treatment of binding by all three selectins (18, 19).

A rapid k_{on} may be important for tethering of leukocytes in shear flow through selectins to the vessel wall, and rapid k_{on} and k_{off} rate constants are thought to be responsible for the transient adhesive interactions that allow rolling of cells on selectins in response to hydrodynamic drag forces. Recent measurements show an unstressed k_{off} of 1 s^{-1} for P-selectin (22), 0.5 s^{-1} for E-selectin (23), and a substantially faster k_{off} of 7 s^{-1} for L-selectin tethers.[2] Rolling through L-selectin is faster than through E-selectin or P-selectin, even when ligand density is adjusted to give identical resistance of rolling adhesions to detachment by shear.[3] These findings suggest that k_{off} is an important determinant of rolling velocity. When the selectin-ligand bond is stressed by the hydrodynamic drag forces acting on the cell, k_{off} increases only modestly (22).[2] Selectins thus have high tensile stability, a factor that may be important for maintenance of adhesion in the vasculature.

Mild periodate treatment of fixed cryostat sections of rat lymph nodes enhanced binding to HEV of lymphocytes and staining by L-selectin-IgG chimera (24). Evidence was obtained for reversible Schiff bases between newly generated aldehyde

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[1] The abbreviations used are: HEV, high endothelial venule; mAb, monoclonal antibody; PNAd, peripheral node addressin; sLe^a and sLe^x, sialyl Le^a and Le^x, respectively; CHO, Chinese hamster ovary; CHO-E cells, recombinant full-length human E-selectin-transfected CHO cells; dyn, dyne(s).

[2] R. Alon, S. Chen, K. D. Puri, R. C. Fuhlbrigge, E. B. Finger, and T. A. Springer, manuscript in preparation.

[3] K. D. Puri, E. B. Finger, and T. A. Springer, manuscript in preparation.

groups of HEV ligands and lysine amino groups in the lectin domain of L-selectin. Mild periodate selectively oxidizes at the exocyclic C-7, C-8, and C-9 positions of sialic acid, and results in cleavage of the C-7–C-8 and C-8–C-9 bonds and a product with an aldehyde group at C-7. Other carbohydrate groups in complex glycans are unaffected by mild periodate (25, 26). The effect of mild periodate treatment on the ligands of P-selectin and E-selectin, or on ligands for L-selectin distinct from those on HEV, remains to be determined. Furthermore, the effect of this modification on tethering and rolling of leukocytes under laminar flow conditions is not known.

In the present study, we have investigated the effect of selective oxidization of sialic acid in selectin ligands on tethering, rolling velocity, and the strength of rolling adhesions under laminar flow conditions. Our results show that interactions with several physiologic ligands through L-selectin, but not through P-selectin and E-selectin, are enhanced after mild periodate treatment. Leukocytes roll more slowly and possess markedly higher resistance to shear detachment on mild periodate-treated PNAd. However, the rate of tethering in shear flow is unaffected. The effect on enhanced interactions of mild periodate treatment was reversed by reduction with borohydride. Furthermore, modification of the terminal sialic acid attached to *N*-acetyl lactosamine in sLe^x or to its isomer lacto-*N*-biose in sLe^a is sufficient to enhance L-selectin interactions. We demonstrate by reduction with cyanoborohydride that reversible Schiff bases are formed not only between L-selectin and ligand sialic acid aldehyde groups but also between E-selectin and P-selectin and their mildly oxidized ligands. A labile Schiff base thus can occur in complexes of all three selectins with mildly oxidized ligands, although perhaps only for a brief fraction of the time that the ligand is bound to the receptor. However, the C-7 aldehyde group of the mildly oxidized sialic acid in the ligand only enhances the kinetics or equilibria for the interaction with the selectin with the fastest k_{off}, L-selectin.

EXPERIMENTAL PROCEDURES

Monoclonal Antibodies and Cell Lines—Monoclonals 581 (IgG1) and 547 (IgG2a, κ) to human CD34 (27, 28) were kindly provided by Dr. Gustav Gaudernack (Oslo, Norway). MECA-79 mAb (rat IgM, κ) (5) was a generous gift of Dr. Eugene Butcher (Stanford, CA). Purified Dreg-56 (IgG1) mAb to L-selectin (29), BB11 (IgG2b) mAb to E-selectin (30), and G1 (IgG) mAb to P-selectin (31) were kind gifts of Drs. T. K. Kishimoto (Boehringer-Ingelheim, Ridgefield, CT), R. Lobb (Biogen, Cambridge, MA), and R. McEver (University of Oklahoma Health Sciences Center, Oklahoma City, OK), respectively, and were used for blocking selectin function. X63 (myeloma, IgG1) was used as a control as a 1:5 dilution of culture supernatant. The human promyelocytic HL-60 and hematopoietic progenitor KG1a cells were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 5 mM glutamine, and 50 μg/ml gentamycin. Recombinant full-length human E-selectin-transfected CHO (CHO-E) cells were grown in α-minimum essential medium (30). Cells were harvested by a 10-min incubation with H/H medium (0.2% human serum albumin, Hanks' balanced salt solution, 10 mM HEPES, pH 7.3) containing 5 mM EDTA at 37 °C, washed and resuspended in the same medium at 2×10^7 cells/ml, and kept at room temperature until use.

Lymphocytes and Neutrophils—Mononuclear cells and neutrophils were isolated from citrate anticoagulated whole blood by dextran sedimentation followed by density separation over Ficoll-Hypaque (32), and lymphocytes (>97%) were purified by depleting monocytes by plastic adhesion (8). Neutrophils and lymphocytes were stored in Hanks' balanced salt solution, Ca^{2+} and Mg^{2+}-free (Life Technologies, Inc.), 10 mM HEPES, pH 7.3, 0.5% human serum albumin. Cells were washed with and suspended in H/H binding medium (Ca^{2+} and Mg^{2+}-free Hanks' balanced salt solution supplemented with 2 mM Ca^{2+}, 0.2% human serum albumin, 10 mM HEPES, pH 7.3) before flow assays.

Preparation of Substrates—PNAd was purified with MECA-79 mAb from tonsil stromal lysates. The CD34 component of PNAd was isolated with mAb-Sepharose, its concentration was determined by capture enzyme-linked immunosorbent assay, it was immobilized on plastic, and its site density was determined as described (8). Recombinant full-length human E-selectin was purified from the detergent lysate of CHO cells transfected with E-selectin cDNA (a generous gift of Dr. R. Lobb, Biogen, Cambridge, MA), by immunoaffinity chromatography using E-selectin mAb, BB11 coupled to Sepharose 4B (30). P-selectin purified from human platelets (33) was a kind gift of Dr. R. McEver (University of Oklahoma Health Sciences Center, Oklahoma City, OK). Liposomes containing selectins were prepared by dialysis from solutions in octylglucoside as described previously (34). Synthetic sialyl $Lewis^a$ (NeuAcα2,3Galβ(Fucα4)3GlcNAcβ3Galβ4Glcβ1-ceramide) and sialyl $Lewis^x$ (NeuAcα2,3Galβ(Fucα3),4GlcNAcβ3Galβ4Glcβ1-ceramide) glycohexaosylceramides (kind gifts of Dr. T. Ogawa, Institute of Physical and Chemical Research, Riken, Japan) (35, 36) were immobilized at about 12,000 molecules/μm^2 as described (37). The human L-selectin-IgG chimera (38) was a kind gift of Dr. L. A. Lasky (Genentech, San Francisco, CA). The human P-selectin-IgG chimera (11) was kindly provided by Dr. Dale Cumming (Genetics Institute, Cambridge, MA). Selectin chimeras were diluted in 20 mM Tris-HCl, 150 mM NaCl, 0.03% sodium azide, pH 8.0 (TSA), and a 50-μl drop was adsorbed to plastic slides overnight at 4 °C. Nonspecific sites were blocked by incubating slides with Hanks' balanced salt solution supplemented with 2% human serum albumin for 30 min at room temperature.

Laminar Flow Assay—A glass slide supporting a lipid bilayer containing a selectin, or a polystyrene slide on which CD34, glycolipid, or selectin-IgG chimera was adsorbed, was assembled in a parallel plate laminar flow chamber and mounted on the stage of an inverted phase-contrast microscope as described previously (34). Cells resuspended at 5×10^5 cells/ml in H/H binding medium were perfused through the flow chamber at the desired shear stress. Tethering in flow, accumulation of cells, resistance to detachment by shear forces, and rolling velocity were measured twice on different 10 × microscopic fields for each immobilized substrate. Cells were detached with 5 mM EDTA in H/H medium between observations on different fields or between tethering and accumulation assays at different shear stresses. For each ligand concentration, cell interactions were measured twice before and after substrate modification and averaged. Binding was measured on one to two substrates each day on two to three different days; data reported are the average and S.D. of results on four to five substrates unless stated otherwise.

Tethering in flow was measured as the number of cells free in flow that tethered in 1 min within the field of view and remained rollingly adherent for at least 5 s. Tethers that were initiated after a nonadherent cell touched an adherent cell were not counted. Cell accumulation was measured as the total number of cells present within the field of view after 3 or 5 min as cells/unit area. Cells were allowed to accumulate on the substrates at different wall shear stresses, and the number of accumulated cells/unit area was determined after 3 or 5 min. For detachment assays, cells were infused into the chamber at 0.84 dyn/cm^2 and allowed to adhere until a sufficient number of cells accumulated (about 2 min). Nonadherent cells were cleared by perfusion with H/H binding medium at 0.84 dyn/cm^2 for 30 s. Then the wall shear stress was increased every 3 s for glycolipids or 10 s for other substrates to a maximum of 36 dyn/cm^2 to generate a detachment force. A shorter interval was used on glycolipids because the cells rolled faster than on other substrates, and it was difficult to distinguish detachment from cells rolling out of the field of view. 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 described previously (34) for 15–20 of the cells observed during detachment assays.

Inhibition with L-selectin mAb or control myeloma IgG and with fucoidan and EDTA was determined as described previously (8). Inhibition with E-selectin mAb or P-selectin mAb was carried out by incubating selectin bilayers to which tethering had already been measured with 10 μg/ml mAb for 20 min and repeating the measurements. All mAbs and inhibitors remained present during the adhesion assay.

Substrate Treatments—Periodate or sham treatments of CD34 or glycolipid substrates to which control tethering measurements had already been made were carried out in the dark at the indicated concentrations of sodium metaperiodate (Sigma). Periodate solution in phosphate-buffered saline (pH 7.2) or in phosphate-buffered saline alone was infused through the flow chamber for 30 min at 4 °C. Reduction of the periodate-oxidized substrate was carried out for 30 min by infusing 100 mM sodium borohydride (Sigma) in phosphate-buffered saline, pH 7.2, at room temperature. The flow chamber was equilibrated with the binding medium, and binding was measured to the same field as examined for control measurements.

Periodate- or sham-treated substrates were treated with *Vibrio chol-*

era neuraminidase (5 milliunits/ml) (Oxford Glycosystems, Rosedale, NY) for 30 min in 50 mM sodium acetate, 4 mM $CaCl_2$, 0.1% bovine serum albumin, pH 5.5, 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.

Cell Treatments—Periodate or sham treatment of one volume of cells at 10^7/ml was carried out by incubation with 20 volumes of H/H medium (pH 7.2) containing 5 mM meta-periodate or medium alone, respectively, for 20 min at 4 °C. Cells were then washed twice, resuspended in binding medium, and immediately perfused into the flow chamber.

Periodate- and sham-treated cells were treated for 30 min with or without *V. cholera* neuraminidase (5 milliunits/ml) in the binding medium at room temperature. Cells were washed three times and resuspended in the binding medium.

Flow Cytometry—Flow cytometry was on a Becton-Dickinson FACSCAN. Cells (10^5) were stained with the mAbs CSLEX-1 (IgM), HECA-452 (IgM), FH6 (IgM), or X63 myeloma or CD63 mAb Mo11 (Fifth International Workshop on Leukocyte Differentiation Antigens) as controls. Fluorescein isothiocyanate-conjugated goat anti-mouse (IgG + IgM) was used as secondary antibody.

Cyanoborohydride Treatment—Lymphocytes were perfused for 3 min and allowed to accumulate on substrates containing periodate or sham-treated CD34 at a wall shear stress of 0.84 dyn/cm^2. Alternatively, periodate- or sham-treated neutrophils were perfused on E-selectin or P-selectin under the same conditions. Perfusion at 0.84 dyn/cm^2 was then continued with 5 mM sodium cyanoborohydride (Sigma) in phosphate-buffered saline (pH 7.2) containing 2 mM Ca^{2+} for 5–10 min at room temperature and then with Hanks'/HEPES containing 5 mM EDTA to detach noncovalently linked cells.

RESULTS

L-selectin, and Not E-selectin and P-selectin, Shows Enhanced Binding to Ligands Subjected to Mild Periodate Oxidation—The CD34 component of PNAd immobilized on the lower wall of a flow chamber was subjected to mild (5 mM) periodate treatment, under conditions that are selective for sialic acid and result in cleavage of C-8 and C-9 and oxidation of C-7 of sialic acid to an aldehyde (25, 26). Lymphocytes were perfused through the chamber, and adhesive interactions to the same microscopic fields were compared before and after the periodate treatment. Accumulation of rolling lymphocytes on CD34 was enhanced more than 2-fold by mild periodate treatment (Fig. 1*A*). This enhancement after 3 min of continuous flow of lymphocytes over mildly oxidized CD34 substrates was observed at all shear stresses tested. However, the rate of tethering of lymphocytes free in flow to the substrate, *i.e.* the rate of formation of initial rolling attachments to the substrate, was enhanced little, by less than 1.2-fold (Fig. 1*B*). This suggests that mild periodate treatment had little effect on the rate of association and that its effect was to stabilize rolling adhesions by decreasing the rate of spontaneous detachment from the substrate. Certain leukocytes including neutrophils (9) and KG1a cells (10) express L-selectin ligands on their surface. These ligands mediate tethering and rolling on substrates bearing immobilized L-selectin.[4] Treatment of KG1a cells (data not shown) and neutrophils (Fig. 1*C*) with mild periodate enhanced accumulation in shear flow on L-selectin.

Borohydride reduces the C-7 aldehyde group in mildly oxidized sialic acid to a C-7 hydroxyl. Borohydride treatment reversed the effect of mild oxidation, reducing lymphocyte accumulation on CD34 to control levels (Fig. 1*A*). The aldehyde moiety at C-7 of sialic acid therefore appears to be required for enhanced L-selectin-dependent binding to mildly oxidized CD34, whereas removal of C-8 and C-9 appears to have no effect. In contrast, strong periodate treatment, which cleaves between endocyclic carbons of sugar moieties containing vicinal hydroxyls and is expected to leave more aldehyde groups per glycan than mild periodate treatment, completely abolished lymphocyte binding (Fig. 1*A*). These data suggest that aldehyde groups generated on sialic acid side chains of CD34 are specifically involved in the enhanced interaction.

CD34 can support E-selectin-dependent tethering and rolling of E-selectin transfected CHO-E cells (8). However, mild periodate modification of CD34 did not affect CHO-E cell accumulation in shear flow (Fig. 1*A*). To extend these studies to ligands for P-selectin and E-selectin on myeloid cells, we examined mild oxidation of neutrophils, the promyelocytic cell line HL-60, and hematopoietic progenitor KG1a cells, for an effect on interaction with immobilized P-selectin and E-selectin under laminar flow conditions. Both P-selectin and E-selectin-mediated accumulation of HL-60 cells were unaffected by mild periodate treatment (Fig. 1*C*). Similar results were obtained with neutrophils and KG1a cells (data not shown).

Inhibition studies were performed to confirm that the interactions with mild periodate-treated substrates were specific. Dreg-56 mAb to human L-selectin (29), fucoidan, and chelation of Ca^{2+} with EDTA abrogated the binding of lymphocytes to both sham- and periodate-treated CD34 (Fig. 1*A*), whereas a class-matched control antibody had no effect (not shown). Similarly, both sham- and periodate-treated neutrophil and KG1a cell interactions with immobilized L-selectin were abrogated with Dreg-56 mAb (not shown). E-selectin and P-selectin-mediated interactions with their ligands were abolished by EDTA and by pretreatment with mAb BB11 to E-selectin and mAb G1 to P-selectin, respectively (Fig. 1*C*).

Complete Modification of Sialic Acid in Selectin Ligands by Mild Periodate—To confirm the completeness of oxidation of sialic acid and the specificity for sialic acid of mild periodate, selectin ligands were treated before and after mild oxidation with *V. cholera* neuraminidase, which requires the presence of C-8 and C-9 on sialic acid for cleavage (39). Neuraminidase treatment of CD34 or neuraminidase treatment of CD34 prior to periodate modification completely abolished both the basal and the enhanced L-selectin-dependent interactions (Fig. 1*A*). By contrast, neuraminidase treatment of modified CD34 had no effect (Fig. 1*A*). These results show that most if not all of the sialic acid side chains of CD34 were modified by mild periodate treatment. Furthermore, neuraminidase treatment had little effect on the activity of mildly oxidized ligands of E-selectin and P-selectin (Fig. 1*C*), providing a positive control for the completeness of modification of these ligands.

To further confirm oxidation of sialic acid by periodate treatment, several mAbs directed against sialyl Lewis^x-related structures were tested on sham- and periodate-treated neutrophils, HL-60, and KG1a cells. Neuraminidase treatment of sham-treated cells completely abrogated binding by all mAbs (Fig. 2). Furthermore, the epitopes of CSLEX-1 and HECA-452 mAb, but not of the FH6 mAb, were sensitive to mild periodate oxidation. Moreover, mild periodate treatment rendered the FH6 epitope completely resistant to neuraminidase, confirming that sialic acid side chains were indeed quantitatively modified.

Terminal Sialic Acid on an N-Acetyl Lactosamine Chain Can Participate in the Enhanced Interactions with L-selectin—Recently, the structures of several *O*-linked glycans of GlyCAM-1, an HEV-specific L-selectin counter-receptor, have been reported, and they contain terminal sialic acids on both T-antigen (Galβ1, 3GalNAc) and *N*-acetyl lactosamine (Galβ1, 4GlcNAc) moieties of the glycans (21). Sialyl Lewis^x and sialyl Lewis^a have terminal sialic acid on *N*-acetyl lactosamine and its isomer lacto-*N*-biose (Galβ1,3GlcNAc), respectively. To investigate the effect of modification of terminal sialic acid on *N*-acetyl lactosamine chains, we examined SKW3 and CHO-E cell binding to sialyl Lewis^x and sialyl Lewis^a glycohexaosylceramides. These glycolipids can mediate both L-selectin- and E-selectin-

[4] R. C. Fuhlbrigge, R. Alon, K. D. Puri, J. B. Lowe, and T. A. Springer, manuscript in preparation.

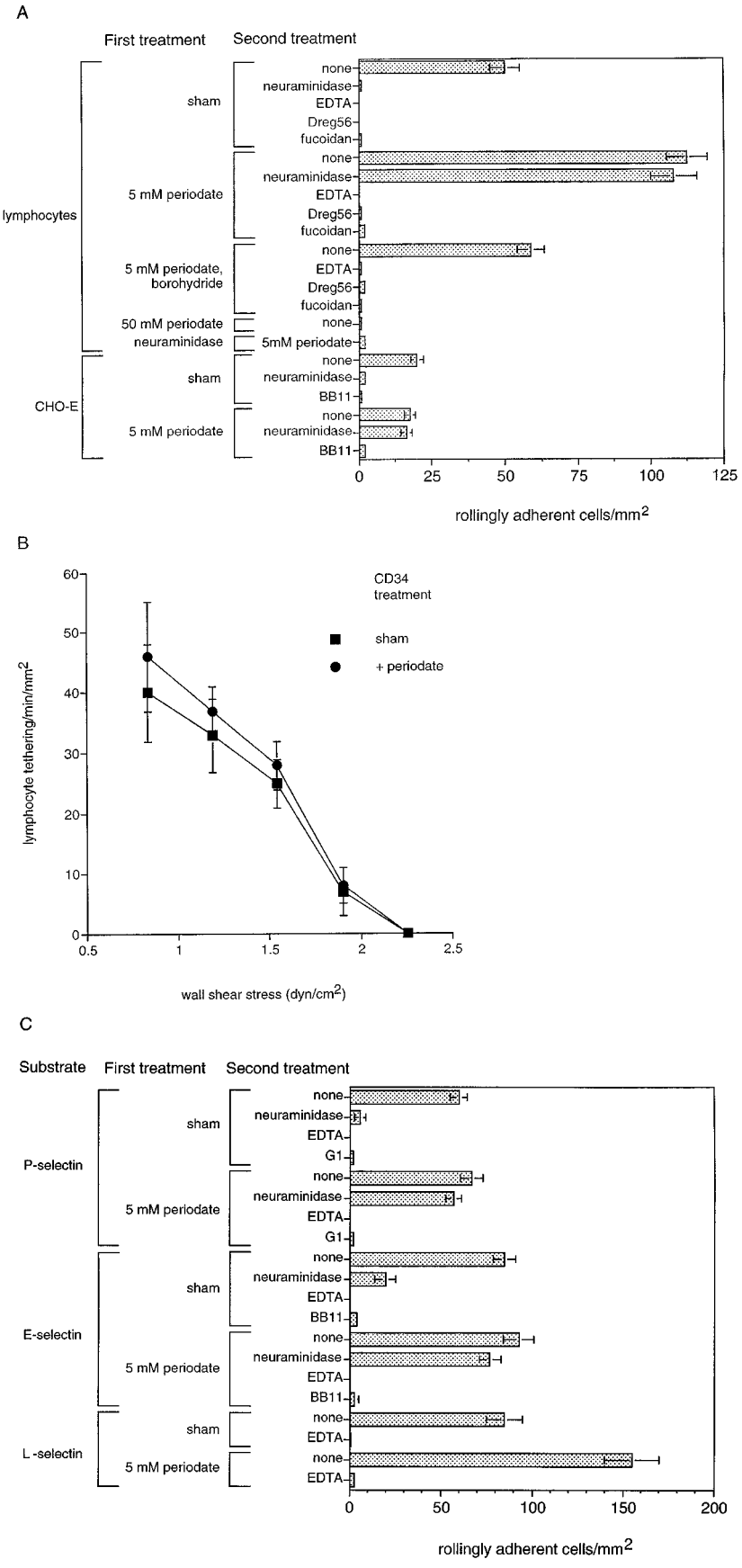

FIG. 1. **Effect of periodate treatment on selectin-mediated cell binding in shear flow.** *A*, lymphocytes or CHO-E cells (5×10^5 cells/ml) were infused at a wall shear stress of 0.84 dyn/cm^2 on polystyrene slides incubated with CD34 at 185 ng/ml to yield 150 CD34 sites/μm^2, and assembled in a parallel plate flow chamber. The number of cells that accumulated in 3 min of continuous flow within the field of view was calculated and expressed per mm^2. Periodate, borohydride, and neuraminidase treatments of immobilized CD34 and treatments with DREG-56 mAb to L-selectin, BB-11 mAb to E-selectin, and fucoidan were as indicated in the *figure* and described under "Experimental Procedures" and were carried out on substrates to which binding in absence of inhibition had previously been measured. The data represent the mean ± range of two randomly selected 10 × fields and are representative of two independent experiments. *B*, lymphocytes (5×10^5 cells/ml) were infused at varying wall shear stresses through the parallel wall flow chamber containing sham- or mild periodate-treated CD34 at 150 sites/μm^2. The number of tethers formed per minute at a given shear stress was quantitated. The data points represent the mean ± range of the number of tethered lymphocytes in a 10 × field and are representative of two independent experiments performed in duplicate. *C*, human promyelocytic HL-60 cells (5×10^5/ml) were infused at 0.84 dyn/cm^2 through the parallel plate flow chamber containing P-selectin (110 sites/μm^2) or E-selectin (175 sites/μm^2) in supported lipid bilayers, or neutrophils were infused at the same conditions over a substrate containing immobilized L-selectin-IgG chimera, and the number of cells that accumulated after 3 min of continuous flow was quantitated. Cells were treated with periodate and neuraminidase as described under "Experimental Procedures," and cell accumulation on the same microscopic field was determined. Treatments with the G1 mAb to P-selectin and the BB11 mAb to E-selectin were as described under "Experimental Procedures." The data represent the mean ± range of two randomly selected 10 × fields and are representative of two independent experiments.

dependent tethering and rolling of cells (37). Mild periodate-modified sLe^a- and sLe^x-hexaosylceramide substrates showed 2.2- and 2.8-fold higher L-selectin-dependent accumulation of SKW3 cells (Fig. 3*A*); however, CHO-E cell binding was unaffected (Fig. 3*B*).

A Schiff Base Can Participate in the Interaction of L-selectin

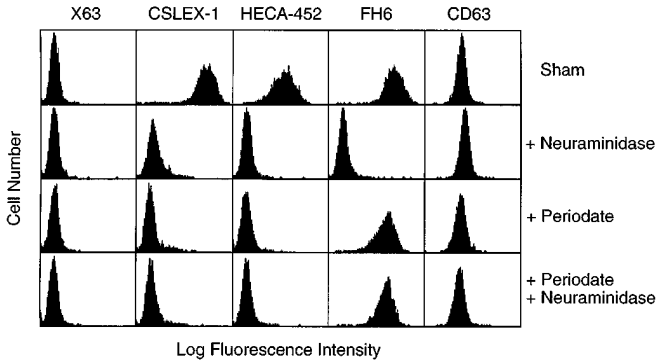

FIG. 2. **Effect of mild periodate and neuraminidase on carbohydrate antigens related to sialyl Lewis^x.** Periodate and neuraminidase treatments of HL-60 cells were carried out as described under "Experimental Procedures." Cells were stained with mAbs that bind to sialyl Lewis^x or related structures, CSLEX-1, HECA-452, and FH6, and with mAb to CD63 and the myeloma X63 as binding and nonbinding controls, respectively. Cells were stained with fluorescein isothiocyanate goat anti-mouse Ig and subjected to immunofluorescent flow cytometry.

with Modified CD34—An aldehyde group of mildly oxidized sialic acids could interact with a lysine ϵ-amino group of L-selectin through a hydrogen bond, and alternatively through several types of partially covalent and covalent structures, which would likely include a Schiff base. To attempt to reduce a reversible Schiff base to an irreversible secondary amine, lymphocytes were allowed to accumulate on mildly oxidized or sham-treated CD34 in the flow chamber at 0.84 dyn/cm^2, and then perfusion was continued in the presence of 5 mM sodium cyanoborohydride. Subsequently, 5 mM EDTA was added to the perfusate. Cyanoborohydride selectively reduces Schiff bases and not aldehydes. The kinetics of reduction by cyanoborohydride treatment were followed by measuring the rolling velocities of 20–25 cells every 30 s. Cyanoborohydride had no effect on the rolling velocity of cells rolling on sham-treated CD34, and subsequent addition of EDTA detached all the rolling cells. However, cyanoborohydride treatment caused about 40% of cells rolling on the periodate-treated substrate to stop within 30 s, and almost all of the rollingly adherent cells had stopped by 3 min of exposure (Fig. 4*A*). Subsequent treatment with EDTA failed to detach these cells, indicating the formation of irreversible covalent bonds (Fig. 4*D*). Similar experiments were carried out on neutrophils subjected to mild periodate oxidation that had accumulated on and were rolling on E-selectin or P-selectin. Addition to the perfusate of cyanoborohydride caused rolling periodate-treated but not sham-treated neutrophils to come to a stop, with kinetics somewhat slower than seen with L-selectin (Fig. 4, *B* and *C*). By 10 min, almost all of the cells had stopped rolling and were irreversibly linked to the substrate as shown by resistance to detachment by EDTA (Fig. 4*D*). These results suggest that all three selectins are capable of forming Schiff bases with their corresponding mild periodate-treated ligands.

Effect of Modification on the Strength of Rolling Interactions—The effect of periodate treatment on the strength of selectin-mediated rolling adhesion was measured by resistance to detachment by increasing wall shear stress (34, 40). L-selectin-mediated lymphocyte rolling interactions on modified CD34 were markedly more resistant to shear detachment than on sham-treated CD34 (Fig. 5*A*). Less than 10% of lymphocytes on CD34 (150 sites/μm^2) remained bound and rolling at the highest shear of 36 dyn/cm^2, whereas on modified CD34 more than 88% of lymphocytes remained rollingly adherent. The similarity in detachment profiles on mock-treated CD34 at 290 sites/μm^2 and on mildly oxidized CD34 at 35 sites/μm^2 showed

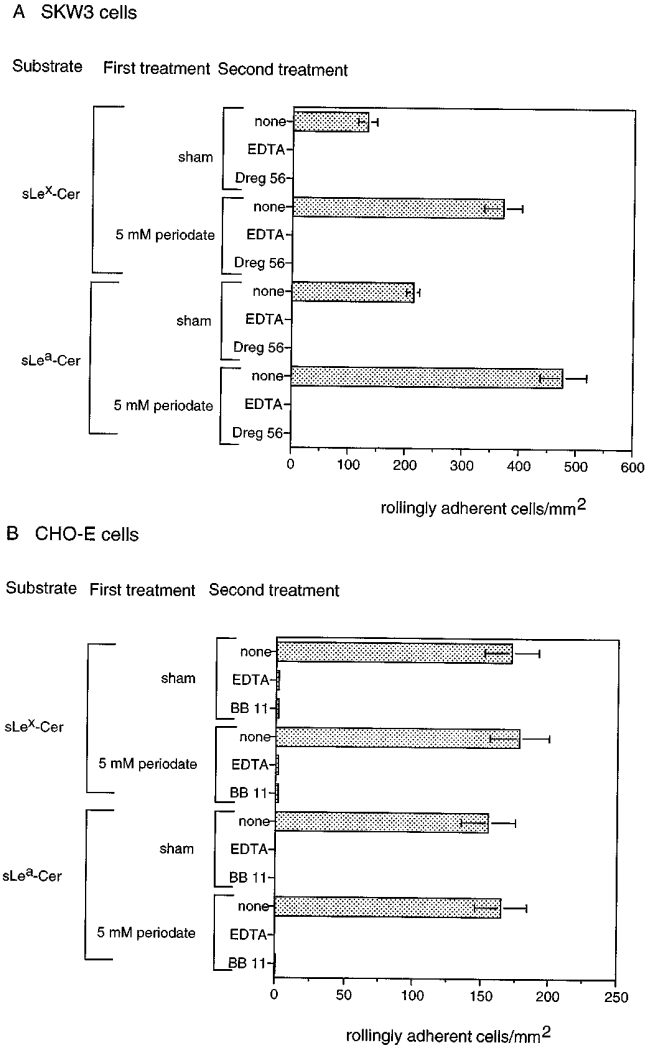

FIG. 3. **Effect of mild periodate treatment on selectin-mediated accumulation of cells on glycolipids.** *A*, L-selectin-dependent binding of SKW3 cells. *B*, E-selectin-dependent binding of CHO-E cells. Cells (5×10^5/ml) were infused at 0.84 dyn/cm^2 on polystyrene slides containing immobilized glycolipids assembled in a parallel plate flow chamber. The number of cells that accumulated after 3 min of continuous flow was calculated and expressed per mm^2. Periodate treatment of immobilized glycolipids was carried out as described under "Experimental Procedures." DREG-56 mAb to L-selectin, BB-11 mAb to E-selectin, and 5 mM EDTA were used as described under "Experimental Procedures." The data represent the mean ± S.D.

that mild periodate oxidation strengthened adhesion equivalently to an 8-fold increase in CD34 density. The strength of rolling interactions of neutrophils and KG1a cells on L-selectin were also increased after mild periodate modification of the cell surface L-selectin ligand (Fig. 5, *C* and *D*). However, the strength of neutrophil, KG1a, and HL-60 rolling interactions on P-selectin (Fig. 5, *C*, *D*, and *E*) and on E-selectin (Fig. 5*F* and data not shown) were not significantly different after mild periodate treatment of the cells. Similarly, periodate treatment of sLe^x and sLe^a glycolipids strengthened L-selectin- but not E-selectin-mediated rolling interactions (Fig. 5*G* and data not shown).

Neuraminidase treatment of sham- but not periodate-treated CD34 abrogated lymphocyte resistance to detachment (Fig. 5*B*). Similar results were obtained with P-selectin and E-selectin ligands (Fig. 5, *E* and *F*). Borohydride reduction after modification reversed the increased strength of lymphocyte rolling interactions on CD34, further confirming the specific involvement of aldehyde groups (Fig. 5*B*).

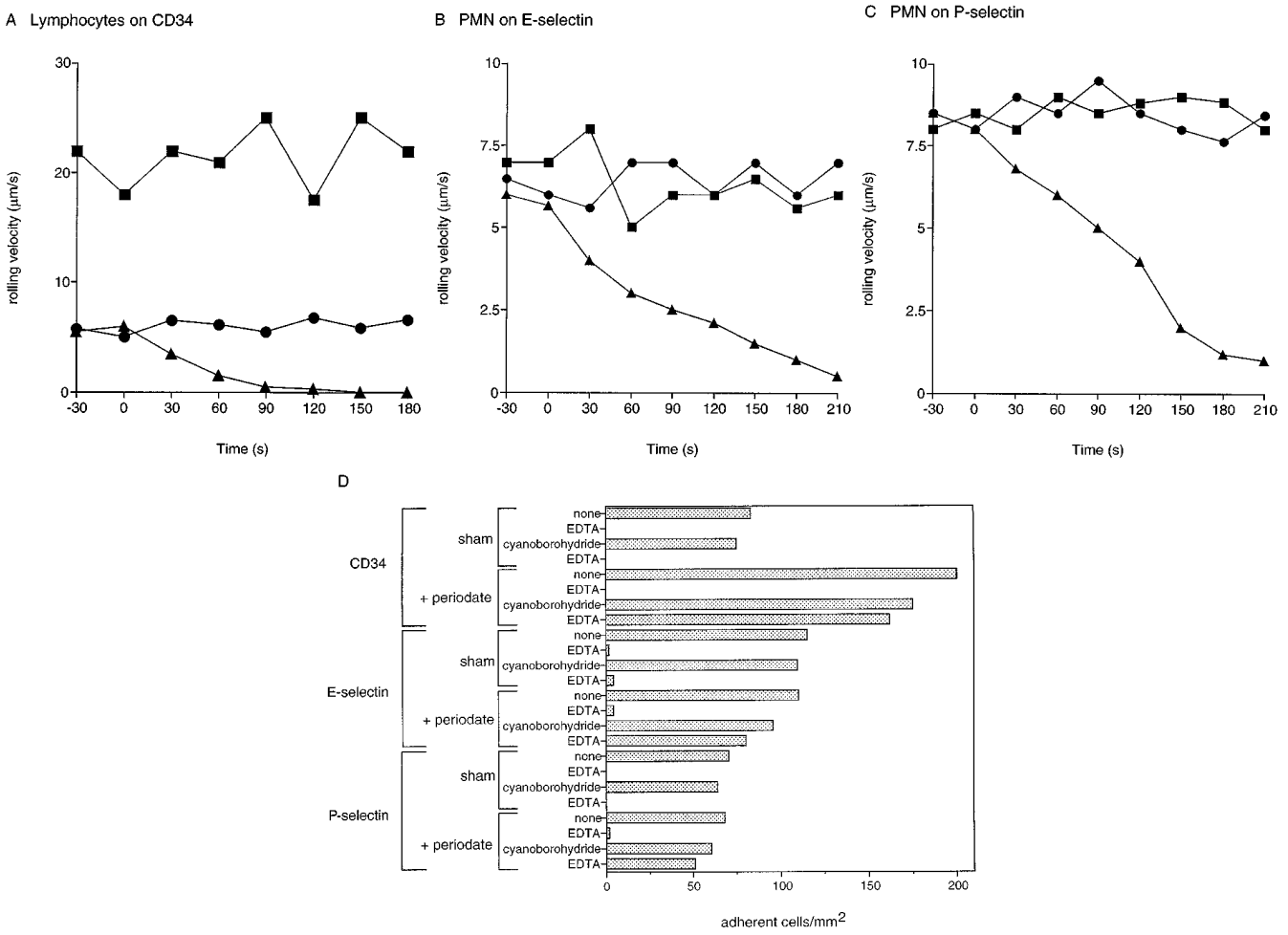

FIG. 4. **Reduction of the Schiff base formed between selectins and mild periodate-treated ligands.** *A*, rollingly adherent lymphocytes on mild periodate-treated or sham-treated CD34 at 150 sites/μm^2 were treated by perfusion with 5 mM sodium cyanoborohydride at 0.84 dyn/cm^2, and rolling velocities of 20–25 randomly selected cells were determined as described under "Experimental Procedures." ■, sham + cyanoborohydride; ●, periodate; ▲, periodate + cyanoborohydride. *B*, rolling adherent sham- or mild periodate-treated neutrophils on E-selectin at 0.84 dyn/cm^2 were subjected to perfusion with 5 mM cyanoborohydride at $t = 0$, and rolling velocities were determined for 10–15 cells. *C*, as in *B*, except on P-selectin. Data points in *A*, *B*, and *C* are mean rolling velocity (stationary cells are included with velocity = 0) and are representative of two to three independent experiments. *D*, rollingly adherent lymphocytes on sham- or mild periodate-treated CD34 or sham- or mild periodate-treated neutrophils rollingly adherent on P-selectin and E-selectin were treated by perfusion with 5 mM cyanoborohydride at 0.84 dyn/cm^2 for 10 min and then with EDTA. Bound cells were enumerated before and after perfusion with medium containing 5 mM EDTA.

Rolling Velocity—The rolling velocity of lymphocytes decreased as the site density of immobilized CD34 was increased, suggesting more L-selectin bonds with the substrate, and it increased as shear stress was increased (Fig. 6*A*). Lymphocytes on modified CD34 rolled markedly slower than on sham-treated CD34. Rolling velocities measured at a range of shear stresses on modified CD34 were about 3–7-fold lower than on sham-treated CD34. Rolling velocities on sham-treated CD34 at 290 sites/μm^2 and on mildly oxidized CD34 at 35 sites/μm^2 were almost identical, mirroring identical shear resistance on these substrates. Rolling velocities of neutrophil and KG1a cells on L-selectin were also significantly slower after modification of the cell surface ligands (not shown). L-selectin- but not E-selectin-bearing cells roll slower on periodate-treated sLe^x and sLe^a glycolipids (Fig. 6*B* and data not shown). Rolling velocities on P-selectin and E-selectin of neutrophils, HL-60 cells, and KG1a cells were similar whether or not the cells were treated with periodate (Fig. 6, *C* and *D*). Neuraminidase treatment of sham- but not periodate-treated substrates affected selectin-mediated rolling velocity (Fig. 6, *C* and *D*, and data not shown). Furthermore, lymphocytes roll with approximately the same velocities on sham-treated CD34 and on mildly oxidized, borohydride-treated CD34 (Fig. 6*A*).

DISCUSSION

In this study, we have determined the effect of mild periodate oxidation of the carbohydrate ligands of L-selectin, E-selectin, and P-selectin on adhesive interactions in hydrodynamic shear flow. A previous study showed enhanced lymphocyte binding under static conditions to mild periodate-treated fixed cryostat sections of lymph node HEVs and enhanced binding of L-selectin IgG to mildly oxidized PNAd (24). We have quantitated the effect of mild periodate oxidation on several measures of L-selectin-dependent interactions in shear flow. We have extended observations to L-selectin ligands on leukocytes and to the simple L-selectin ligands sLe^a and sLe^x. Furthermore, we demonstrate that interactions through L-selectin but not P-selectin or E-selectin are enhanced by mild periodate treatment and that Schiff bases are formed with all three selectins.

To determine the effect of mild periodate treatment on dynamic selectin-mediated interactions, we monitored four different parameters, stable tethers, cell accumulation, rolling velocity, and resistance to shear detachment. Stable tethers are initial interactions between a cell in flow and the substrate that result in rolling interactions that last for at least 3 s. We

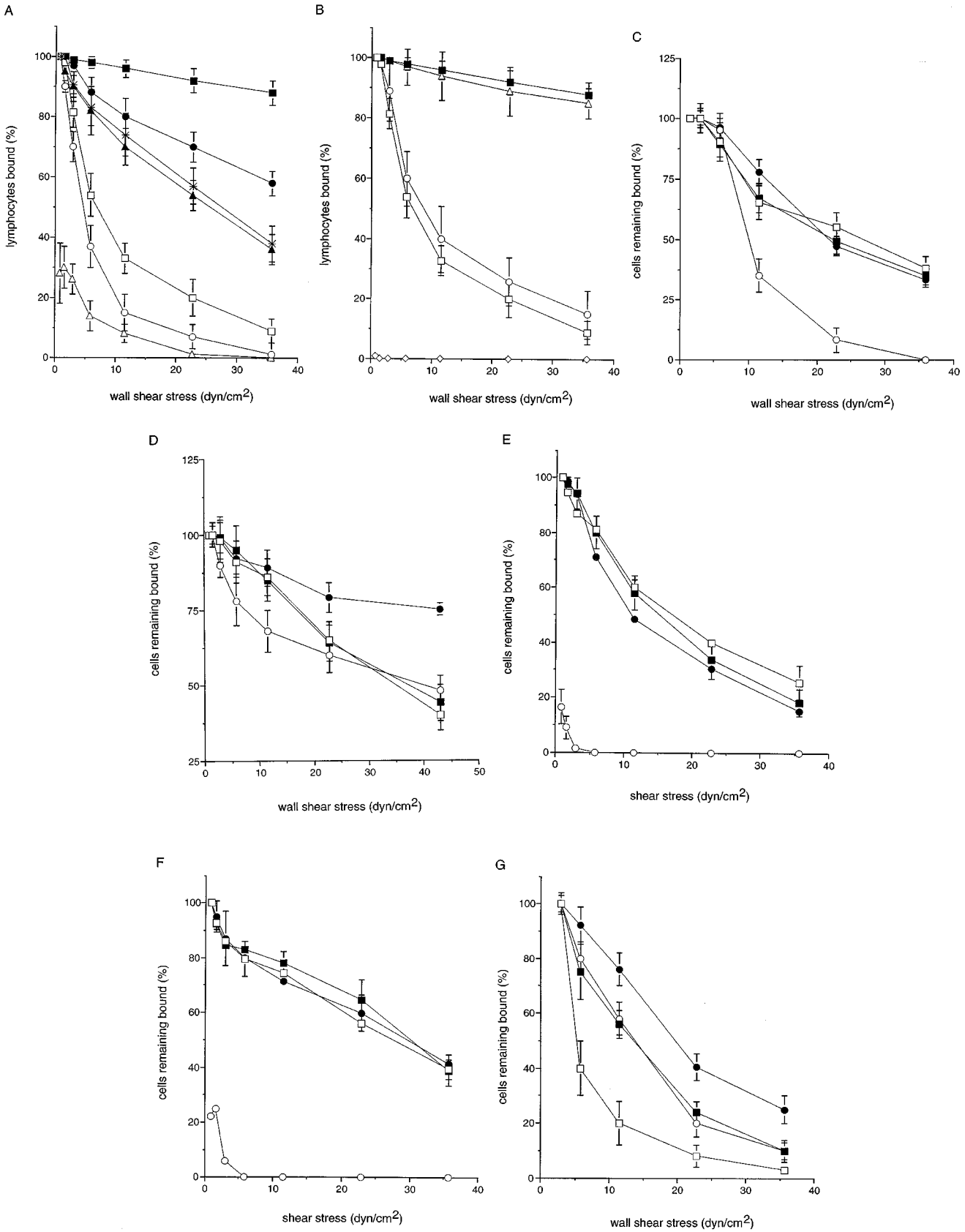

FIG. 5. **Effect of mild periodate treatment on selectin-mediated resistance to cell detachment by shear.** *A*, lymphocytes were allowed to tether at 0.84 dyn/cm^2 at the following site densities of sham- or periodate-treated CD34: □, 150 sites/μm^2 (sham); ■, 150 sites/μm^2 (+periodate); ○, 60 sites/μm^2 (sham); ●, 60 sites/μm^2 (+periodate); △, 35 sites/μm^2 (sham); ▲, 35 sites/μm^2 (+periodate). *, 290 sites/μm^2 (no treatment). Wall 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. *B*, periodate, borohydride, and neuraminidase treatments of immobilized CD34 at 150 sites/μm^2 were carried out in the order described under "Experimental Procedures" prior to the detachment assay. 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 two to three different independent experiments. □, sham; ■,

hypothesize that the rate of the initial formation of the tether is related to the on rate of the reaction between the selectin and ligand; the requirement for subsequent rolling would also be influenced by the off rate. The finding that mild oxidation had little effect on formation of stable tethers on CD34 suggests that k_{on} either was not increased or was not rate-limiting in the assay system. Cell accumulation may reflect both the kinetic constants and the equilibrium constant; its enhancement for L-selectin by mild periodate oxidation of CD34 suggests an increase in $K_{eq} = k_{on}/k_{off}$. Rolling velocity is hypothesized to reflect the average number of receptor-ligand bonds between the cell and the substrate, governed by k_{on}/k_{off}, and the rate at which bonds break (k_{off}). Receptor-ligand dissociation during rolling is thought to allow the cell to move forward in response to hydrodynamic drag until it is held by other bonds. Additionally, how k_{off} responds to tensile force on the bond will influence rolling velocity (22). Resistance to detachment by hydrodynamic shear force is hypothesized to be influenced by the same parameters as rolling velocity. The hydrodynamic drag force experienced by a cell near a wall in shear flow is proportional to and can be calculated from the wall shear stress (41). It is interesting that rolling velocity and resistance to detachment by shear were the parameters most dramatically influenced by mild periodate oxidation; rolling velocity was decreased 3–4-fold, and resistance to detachment was increased approximately 10-fold higher in wall shear stress. Changes in both parameters were equivalent in effect to an 8-fold increase in CD34 density on the substrate. Based on these results, we suspect that periodate oxidation either diminished the L-selectin:ligand k_{off} with no effect on k_{on} or diminished k_{off} much more than k_{on}. It will be important to experimentally test this prediction.

Selective enhancement of L-selectin interactions by mild periodate oxidation of PNAd was generalized to other L-selectin ligands. L-selectin ligands on HEV, *i.e.* PNAd and the CD34 fraction of PNAd studied here, bear the sulfation-dependent MECA-79 carbohydrate epitope (42, 43); however, ligands for L-selectin are present on human neutrophils and human hematopoietic progenitor KG1a cells (8–10) that lack this epitope. Therefore, we also studied interactions of sham- and mild periodate-treated neutrophils and KG1a cells with L-selectin adsorbed to a substrate, which mediates rolling of these cells.[4] Consistent with results with HEV-specific ligands, L-selectin-mediated interactions of both neutrophils and KG1a cells were enhanced after mild periodate treatment. Both cell types accumulated better, rolled slower, and were more resistant to shear detachment after mild periodate treatment. Our studies were further extended to sLe^x and sLe^a. *O*-linked glycans of an HEV-specific L-selectin counter-receptor have been shown to have two terminal sialic acids, one each on the T-antigen and *N*-acetyl lactosamine carbohydrate structures (21). Therefore, we tested the effect of mild periodate treatment on sLe^x and sLe^a-containing glycolipids, which have sialic acid only on the counterpart of the *N*-acetyl lactosamine moiety of the HEV ligands. Mild periodate modification of both of these glycolipids enhanced L-selectin-mediated binding of SKW3 T cells, as reflected in slower rolling velocity and higher resistance to shear detachment. These results support the participation of the *N*-acetyl lactosamine sialic acid in the enhanced interactions with HEV ligands of L-selectin, although we cannot rule out an additional interaction with the terminal sialic acid on the T-antigen structure. Furthermore, neuraminidase treatment of immobilized CD34, KG1a cells, and neutrophils completely abolished ligand activity. These results show that enhanced interaction after mild periodate treatment is a general phenomenon for L-selectin ligands and may reflect modification of the terminal sialic acid on the *N*-acetyl lactosamine structure.

Control studies with EDTA, mAb specific for E-selectin, P-selectin, and L-selectin and with fucoidan, which blocks L-selectin but not E-selectin interactions, showed that interactions with mildly oxidized substrates were specific. CD34 treated with neuraminidase prior to periodate modification was inactive, confirming the selective involvement of modified sialic acids in enhanced interactions with L-selectin. On the other hand, periodate modification rendered sialic acid insensitive to neuraminidase and protected ligand activity for E-selectin, P-selectin, and L-selectin. Results with the mAbs CSLEX and HECA-452 show that their epitopes include C-8 and C-9 of sialic acid, unlike all three selectins and the epitope of the FH6 mAb. These results and results on inhibition by mild periodate oxidation of digestion by neuraminidase showed that essentially all sialic acid side chains required for E-selectin, P-selectin, and L-selectin ligand activity, and for the CSLEX-1, HECA-452, and FH6 epitopes, were modified by mild periodate.

Mild periodate oxidation of carbohydrate ligands did not enhance interactions with E-selectin or P-selectin. The sialomucin CD34 is also a ligand for E-selectin and mediates tethering and rolling interactions of CHO-E cells that express E-selectin. However, E-selectin-mediated interactions were not affected by mild periodate treatment of CD34 as shown by lack of effect on rolling velocities and detachment profiles. Interactions of CHO-E cells with sLe^a and sLe^x glycolipids were also unaffected by mild periodate oxidation. Moreover, mild periodate oxidation of HL-60 and KG1a cells had no effect on rolling interactions with P-selectin and E-selectin, in contrast to enhancement of interactions with L-selectin. Subsequent reduction with borohydride also had no effect on interaction with E-selectin or P-selectin, in agreement with findings that truncation of the sialic acid exocyclic side chain does not destroy recognition by E-selectin (44).

Our results suggest that an aldehyde group on mildly oxidized ligands is responsible for the enhanced interaction with L-selectin. Sialic acid is a nine-carbon sugar containing an

+periodate; ○, +periodate + borohydride; △, +periodate + neuraminidase; ◇, sham + neuraminidase. *C*, L-selectin-IgG chimera and P-selectin-IgG chimera were immobilized on plastic substrates at 1.5 and 2 μg/ml, respectively. The P-selectin density was about 150 sites/μm^2. Periodate or sham-treated neutrophils were allowed to tether at 0.84 dyn/cm^2, and detachment was measured by increasing the wall shear stress every 10 s to a maximum of 36 dyn/cm^2. The data represent mean ± S.D. of two to three independent experiments. □, sham-treated, P-selectin; ■, periodate-treated, P-selectin; ○, sham-treated, L-selectin; ●, periodate-treated, L-selectin. *D*, human hematopoietic progenitor KG1a cells after sham and periodate treatments were infused at 0.84 dyn/cm^2 on substrates containing L-selectin-IgG or P-selectin-IgG chimeras immobilized at 2.5 μg/ml, and after sufficient accumulation of cells, the wall shear stress was increased every 20 s to a maximum of 42.7 dyn/cm^2 and the percentage of cells remaining bound at each shear was determined. The density of P-selectin was 180 sites/μm^2. The data represent the mean ± S.D. □, sham-treated, P-selectin; ■, periodate-treated, P-selectin; ○, sham-treated, L-selectin; ●, periodate-treated, L-selectin. *E* and *F*, human promyelocytic HL-60 cells after sham, periodate, and neuraminidase treatments were allowed to accumulate on supported lipid bilayers containing P-selectin (110 sites/μm^2) (*E*) or E-selectin (175 sites/μm^2) (*F*) at 0.84 dyn/cm^2, and the detachment assay was performed by increasing wall shear stress every 10 s to a maximum of 36 dyn/cm^2. Data represent mean ± S.D. of three independent experiments. □, sham; ■, +periodate; ●, +periodate + neuraminidase; ○, +neuraminidase. *G*, SKW3 T cells were allowed to tether at 0.84 dyn/cm^2 on sham- or periodate-treated glycolipid substrates. Wall shear stress was then increased every 3 s to a maximum of 36 dyn/cm^2, and the percentage of cells remaining bound at each shear was determined. Data represent mean ± S.D. □, sLe^x (sham); ■, sLe^x, (+periodate); ○, sLe^a (sham); ●, sLe^a (+periodate).

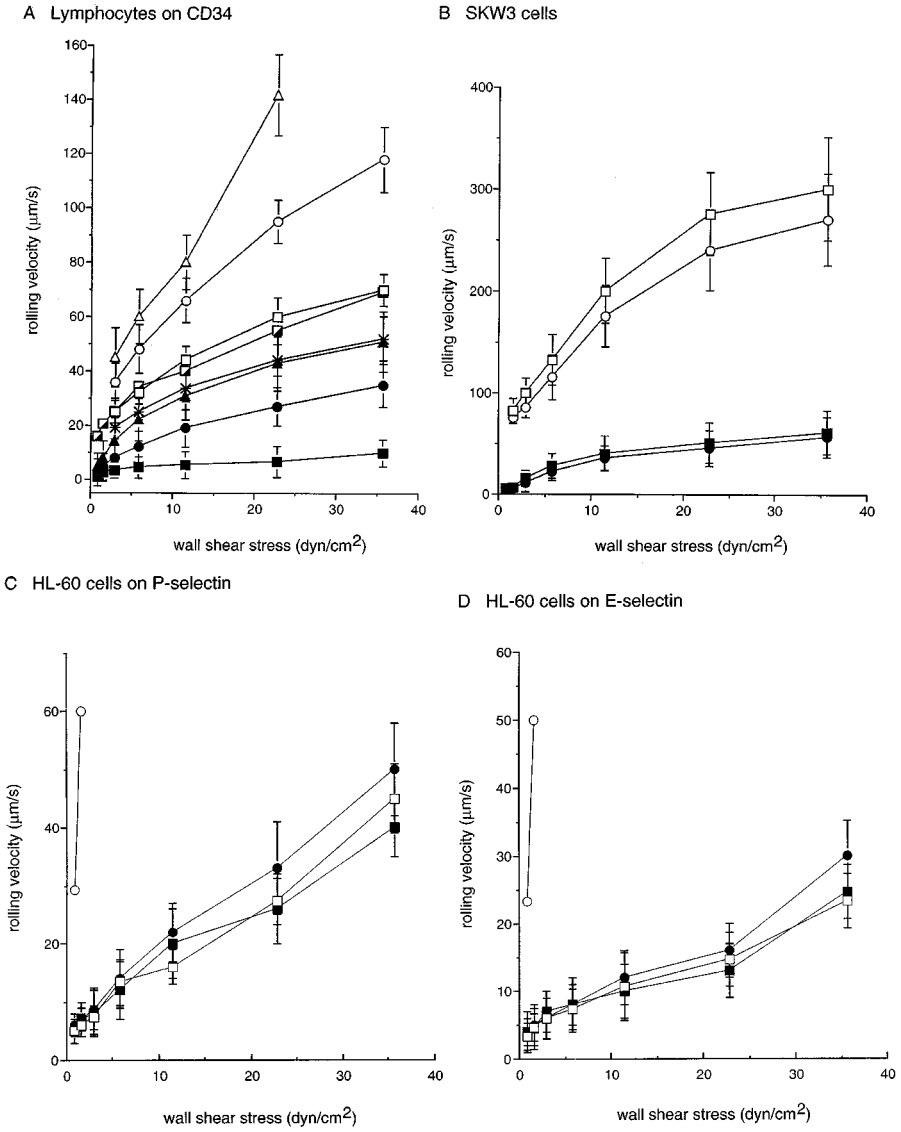

FIG. 6. **Effect of mild periodate treatment on rolling velocity.** *A*, lymphocytes were allowed to tether at 0.84 dyn/cm^2 for 2–3 min at the following site densities of sham- and periodate-treated CD34: □, 150 sites/μm^2 (sham); ■, 150 sites/μm^2 (+periodate); ◪, 150 sites/μm^2 (+periodate + borohydride); ○, 60 sites/μm^2 (sham); ●, 60 sites/μm^2 (+periodate); △, 35 sites/μm^2 (sham); ▲, 35 sites/μm^2 (+periodate). *, 290 sites/μm^2 (no treatment). Wall shear stress was then increased every 10 s to a maximum of 36 dyn/cm^2, and rolling velocities were measured for 15–20 cells. The data points represent the mean rolling velocity ± S.E. and are representative of two to three different independent experiments. *B*, SKW3 T cells were tethered to sham and periodate-treated glycolipid substrates and rolling velocities of 25–30 cells were determined during detachment assays as described in the legend of Fig. 5. Data represent mean ± S.D. of rolling velocities of cells measured in two independent experiments. □, sLe^x (sham); ■, sLe^x, (+periodate); ○, sLe^a (sham); ●, sLe^a (+periodate). *C* and *D*, sham-, periodate-, and neuraminidase-treated promyelocytic HL-60 cells were allowed to accumulate on supported lipid bilayers containing P-selectin (110 sites/μm^2) (*C*) or E-selectin (175 sites/μm^2) (*D*) at 0.84 dyn/cm^2 and subjected to increased shear in detachment assays as described in Fig. 5. Rolling velocities of 15–20 cells were determined in each experiment, and the data represent mean ± S.D. of rolling velocities of cells in two to three independent experiments. □, sham; ■, +periodate; ●, +periodate + neuraminidase; ○, +neuraminidase.

exocyclic chain; mild periodate oxidation under the conditions used here quantitatively generates the seven-carbon aldehyde form (26). Borohydride reduction subsequent to the mild periodate oxidation reduces the 7-aldehyde to a primary alcohol. Borohydride reduction reversed the effect of mild oxidation of CD34, showing that the enhanced interaction with L-selectin is not due to side-chain truncation but required the 7-aldehyde group. Furthermore, equivalent binding to the native and truncated, reduced structures suggests that the three selectins do not interact with the C-8, C-9 diol moiety of sialic acid. These results extend a previous study that found an unexplained disruption by borohydride of complexes of L-selectin chimera with both native and mildly oxidized ligand (24). Our data and those of Norgard *et al.* (24) suggest that the C-7 aldehyde on sialic acid specifically interacts with L-selectin. This interaction is very likely with the ϵ-amino group of a specific lysine residue. The interaction may consist of several different types of bonds that rapidly interconvert. It may include a hydrogen bond of a lysine ϵ-amino hydrogen with the C-7 aldehyde oxygen, which would be predicted to be stronger than a hydrogen bond with the C-7 hydroxyl oxygen. Interconversion could occur to a partially covalent bond, a single bond, and a double bond or Schiff base between the lysine ϵ-N and the C-7 carbon. Predominance as a Schiff base is unlikely, because this requires stabilization by resonance with other double bonds or with aromatic groups. We could demonstrate a Schiff base (24) by reduction with cyanoborohydride, as shown by formation of an EDTA-resistant bond between leukocytes rolling on mildly oxidized CD34 but not on sham-treated CD34. Furthermore, the cells stopped rolling on the mildly oxidized substrate after cyanoborohydride reduction. These findings suggest that covalent bond(s) were formed between L-selectin and CD34.

Oxidation of E-selectin and P-selectin ligands had no effect on measures of interactions in shear flow that appear to reflect both the kinetics and equilibria of selectin binding; nonetheless, Schiff base formation occurred, as shown by reduction with cyanoborohydride. Cyanoborohydride caused cells rolling on E-selectin and P-selectin to arrest and to form an EDTA-resistant bond to the substrate. The kinetics of reduction of the Schiff base were somewhat slower for E-selectin and P-selectin than for L-selectin, as determined by the kinetics of the arrest of the rolling cells. The data show that interconversion to a Schiff base structure can occur in all three selectin-ligand complexes, although interconversion might be less frequent for E-selectin and P-selectin based on the kinetics of reduction. There may be no effect of mild oxidation on E-selectin and P-selectin rolling behavior because gain of interaction with the C-7 aldehyde moiety is compensated for by loss of another interaction, whereas with L-selectin there is a gain with no compensating loss. Another way of looking at this is that the

stabilizing interaction that includes the Schiff base may only be noted kinetically with L-selectin because of its considerably faster k_{off} with native ligands.

The highly homologous lectin domains of selectins may use a common recognition site for sugars and may bind in the same manner the sLe^x motif that is common to all three ligands, with other contacts that provide specificity for distinctive elements in the ligand structures. The N-terminal lectin domains of the three selectins have 10–14 lysine residues. Those at positions 32, 55, 67, 96, 111, and 113 are conserved in all three selectins. Although no carbohydrate ligand has yet been cocrystallized with a selectin, studies on a cocrystal of the homologous mannose binding protein (45) and docking of sLe^x to E-selectin suggest that lysines at positions 111 and 113 are closest to the sialic acid of sLe^x (15). Mutation at Lys^{113} completely abolishes E-selectin and P-selectin ligand-binding function, and mutation at Lys^{111} severely decreases but does not abolish function (15–17). One possible model is that Lys^{113} forms an ionic hydrogen bond to the sialic acid carboxylate in sLe^x in all three selectins and Lys^{111} forms a hydrogen bond to this carboxylate or to the nearby anomeric oxygen or C-7 hydroxyl oxygen, which perhaps is more favorable in E-selectin and P-selectin than L-selectin. In this model, Lys^{111} is available in all three selectins for formation of a hydrogen bond to the C-7 aldehyde oxygen of the mildly oxidized sialic acid, and for interchangeable formation of a Schiff base. Specific assignments of the lysine(s) that form(s) Schiff base(s) in E-selectin, P-selectin, and L-selectin would extend knowledge of how these molecules bind their carbohydrate ligands.

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REFERENCES

1. Lasky, L. A. (1992) *Science* **258,** 964–969
2. Bevilacqua, M. P., and Nelson, R. M. (1993) *J. Clin. Invest.* **91,** 379–387
3. McEver, R. P. (1994) *Curr. Opin. Immunol.* **6,** 75–84
4. Lewinsohn, D. M., Bargatze, R. F., and Butcher, E. C. (1987) *J. Immunol.* **138,** 4313–4321
5. Streeter, P. R., Rouse, B. T. N., and Butcher, E. C. (1988) *J. Cell Biol.* **107,** 1853–1862
6. Lasky, L. A., Singer, M. S., Dowbenko, D., Imai, Y., Henzel, W. J., Grimley, C., Fennie, C., Gillett, N., Watson, S. R., and Rosen, S. D. (1992) *Cell* **69,** 927–938
7. Baumhueter, S., Singer, M. S., Henzel, W., Hemmerich, S., Renz, M., Rosen, S. D., and Lasky, L. A. (1993) *Science* **262,** 436–438
8. Puri, K. D., Finger, E. B., Gaudernack, G., and Springer, T. A. (1995) *J. Cell Biol.* **131,** 261–270
9. Bargatze, R. F., Kurk, S., Butcher, E. C., and Jutila, M. A. (1994) *J. Exp. Med.* **180,** 1785–1792
10. Oxley, S. M., and Sackstein, R. (1994) *Blood* **84,** 3299–3306
11. Sako, D., Chang, X. -J., Barone, K. M., Vachino, G., White, H. M., Shaw, G., Veldman, G. M., Bean, K. M., Ahern, T. J., Furie, B., Cumming, D. A., and Larsen, G. R. (1993) *Cell* **75,** 1179–1186
12. Moore, K. L., Eaton, S. F., Lyons, D. E., Lichenstein, H. S., Cummings, R. D., and McEver, R. P. (1994) *J. Biol. Chem.* **269,** 23318–23327
13. Ma, L., Raycroft, L., Asa, D., Anderson, D. C., and Geng, J-G. (1994) *J. Biol. Chem.* **269,** 27739–27746
14. Steegmaier, M., Levinovitz, A., Isenmann, S., Borges, E., Lenter, M., Kocher, H. P., Kleuser, B., and Vestweber, D. (1995) *Nature* **373,** 615–620
15. Graves, B. J., Crowther, R. L., Chandran, C., Rumberger, J. M., Li, S., Huang, K. -S., Presky, D. H., Famillетti, P. C., Wolitzky, B. A., and Burns, D. K. (1994) *Nature* **367,** 532–538
16. Erbe, D. V., Wolitzky, B. A., Presta, L. G., Norton, C. R., Ramos, R. J., Burns, D. K., Rumberger, J. M., Rao, B. N. N., Foxall, C., Brandley, B. K., and Lasky, L. A. (1992) *J. Cell Biol.* **119,** 215–227
17. Erbe, D. V., Watson, S. R., Presta, L. G., Wolitzky, B. A., Foxall, C., Brandley, B. K., and Lasky, L. A. (1993) *J. Cell Biol.* **120,** 1227–1235
18. Rosen, S. D. (1993) *Semin. Immunol.* **5,** 237–247
19. Varki, A. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 7390–7397
20. Hemmerich, S., and Rosen, S. D. (1994) *Biochemistry* **33,** 4830–4835
21. Hemmerich, S., Leffler, H., and Rosen, S. D. (1995) *J. Biol. Chem.* **270,** 12035–12047
22. Alon, R., Hammer, D. A., and Springer, T. A. (1995) *Nature* **374,** 539–542
23. Kaplanski, G., Farnarier, C., Tissot, O., Pierres, A., Benoliel, A-M., Alessi, M-C., Kaplanski, S., and Bongrand, P. (1993) *Biophys. J.* **64,** 1922–1933
24. Norgard, K. E., Han, H., Powell, L., Kriegler, M., Varki, A., and Varki, N. M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 1068–1072
25. Van Lenten, L., and Ashwell, G. (1971) *J. Biol. Chem.* **246,** 1889–1894
26. Murray, M. C., Bhavanandan, V. P., Davidson, E. A., and Reinhold, V. (1989) *Carbohydr. Res.* **186,** 255–265
27. Gaudernack, G., and Egeland, T. (1995) in *Leukocyte Typing V: White Cell Differentiation Antigens* (Schlossman, S., Boumsell, L., Gilks, W., Harlan, J., Kishimoto, T., Morimoto, C., Ritz, J., Shaw, S., Silverstein, R., Springer, T., Tedder, T., and Todd, R., eds) pp. 861–864, Oxford University Press, New York
28. Greaves, M. F., Titley, I., Colman, S. M., Buhring, H-J., Campos, L., Castoldi, G. L., Garrido, F., Gaudernack, G., Girard, J-P., Ingles-Esteve, J., Invernizzi, R., Knapp, W., Lansdorp, P. M., Lanza, F., Merle-Beral, H., Parravicini, C., Razak, K., Ruiz-Cabello, F., Springer, T. A., Van Der Schoot, C. E., and Sutherland, D. R. (1995) in *Leukocyte Typing V: White Cell Differentiation Antigens* (Schlossman, S., Boumsell, L., Gilks, W., Harlan, J., Kishimoto, T., Morimoto, C., Ritz, J., Shaw, S., Silverstein, R., Springer, T., Tedder, T., and Todd, R., eds) pp. 840–846, Oxford University Press, New York
29. Kishimoto, T. K., Jutila, M. A., and Butcher, E. C. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 2244–2248
30. Lobb, R. R., Chi-Rosso, G., Leone, D. R., Rosa, M. D., Bixler, S., Newman, B. M., Luhowskyj, S., Benjamin, C. D., Dougas, I. R., Goelz, S. E., Hession, C., and Chow, E. P. (1991) *J. Immunol.* **147,** 124–129
31. Geng, J-G., Bevilacqua, M. P., Moore, K. L., McIntyre, T. M., Prescott, S. M., Kim, J. M., Bliss, G. A., Zimmerman, G. A., and McEver, R. P. (1990) *Nature* **343,** 757–760
32. Miller, L. J., Bainton, D. F., Borregaard, N., and Springer, T. A. (1987) *J. Clin. Invest.* **80,** 535–544
33. Moore, K. L., Varki, A., and McEver, R. P. (1991) *J. Cell Biol.* **112,** 491–499
34. Lawrence, M. B., and Springer, T. A. (1991) *Cell* **65,** 859–873
35. Ilda, M., Endo, A., Fujita, S., Numata, M., Matsuzaki, Y., Sugimoto, M., Nunomura, S., and Ogawa, T. (1995) *Carbohydr. Res.* **270,** C15–C19
36. Ilda, M., Endo, A., Fujita, S., Numata, M., Suzuki, K., Nunomura, S., and Ogawa, T. (1995) *Glycoconjugate J.*, in press
37. Alon, R., Feizi, T., Yuen, C-T., Fuhlbrigge, R. C., and Springer, T. A. (1995) *J. Immunol.* **154,** 5356–5366
38. Watson, S., Imai, Y., Fennie, C., Geoffroy, J. S., Rosen, S. D., and Lasky, L. A. (1990) *J. Cell Biol.* **110,** 2221–2229
39. Veh, R. W., Corfield, A. P., Sander, M., and Schauer, R. (1977) *Biochim. Biophys. Acta* **486,** 145–160
40. Chan, P-Y., Lawrence, M. B., Dustin, M. L., Ferguson, L. M., Golan, D. E., and Springer, T. A. (1991) *J. Cell Biol.* **115,** 245–255
41. Goldman, A. J., Cox, R. G., and Brenner, H. (1967) *Chem. Eng. Sci.* **22,** 653–660
42. Berg, E. L., Robinson, M. K., Warnock, R. A., and Butcher, E. C. (1991) *J. Cell Biol.* **114,** 343–349
43. Hemmerich, S., Butcher, E. C., and Rosen, S. D. (1994) *J. Exp. Med.* **180,** 2219–2226
44. Tyrrell, D., James, P., Rao, N., Foxall, C., Abbas, S., Dasgupta, F., Nashed, M., Hasegawa, A., Kiso, M., Asa, D., Kidd, J., and Brandley, B. K. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 10372–10376
45. Weis, W. I., Drickamer, K., and Hendrickson, W. A. (1992) *Nature* **360,** 127–134