

Distinct Cell Surface Ligands Mediate T Lymphocyte Attachment and Rolling on P and E Selectin Under Physiological Flow

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Abstract. Memory T lymphocytes extravasate at sites of inflammation, but the mechanisms employed by these cells to initiate contact and tethering with endothelium are incompletely understood. An important part of leukocyte extravasation is the initiation of rolling adhesions on endothelial selectins; such events have been studied in monocytes and neutrophils but not lymphocytes. In this study, the potential of T lymphocytes to adhere and roll on endothelial selectins in vitro was investigated. We demonstrate that T cells can form tethers and rolling adhesions on P selectin and E selectin under physiologic flow conditions. Tethering and rolling on P selectin was independent of cell-surface cutaneous lymphocyte antigen (CLA) expression, which correlated strictly with the capacity of T cells to form rolling adhesions under flow on E

selectin. T cell tethering to P selectin was abolished by selective removal of cell surface sialomucins by a *P. haemolytica* O-glycoprotease, while cutaneous lymphocyte antigen expression was unaffected. A sialomucin molecule identical or closely related to P selectin glycoprotein ligand-1 (PSGL-1), the major P selectin ligand on neutrophils and HL-60 cells, appears to be a major T cell ligand for P selectin. P selectin glycoprotein ligand-1 does not appear to support T cell rolling on E selectin. In turn, E selectin ligands do not appear to be associated with sialomucins. These data demonstrate the presence of structurally distinct ligands for P or E selectins on T cells, provide evidence that both ligands can be coexpressed on a single T cell, and mediate tethering and rolling on the respective selectins in a mutually exclusive fashion.

THE mechanism used by circulating leukocytes to initiate contact with the luminal aspect of inflamed endothelium lining vessel walls requires transient interactions (tethers) mediated by selectins (Lawrence and Springer, 1991; Ley et al., 1991; Mayadas et al., 1993; von Andrian et al., 1993). It has been proposed that tethered leukocytes, unlike cells flowing freely in vessels, can be selectively activated at the level of the endothelial cell membrane by chemokines or related molecules; these events appear to be essential for activation of integrin adhesiveness and trans-endothelial migration (Butcher, 1991; Bargatze and Butcher, 1993; Taub et al., 1993; Springer, 1994). The latter process depends on interactions between leukocyte integrins and endothelial ligands (e.g., VLA-4/VCAM, LFA-1/ICAM). The available in vitro data indicate that these integrins cannot efficiently initiate attachment of a freely flowing leukocyte to an endothelial cell lining the vessel wall (Butcher, 1991; Springer, 1994).

Several immunologically mediated human diseases are characterized by the pathophysiologic extravasation of memory T cells into peripheral tissue (Zhang, 1992; Kupper, 1994; Picker, 1994), yet there is no compelling evidence that the paradigm developed to explain neutrophil trans-endothelial migration can also apply to T cell extravasation into peripheral tissues. However, binding of memory T cell subsets to E selectin in static binding assays has been reported (Picker et al., 1991, 1993; Shimizu et al., 1992), and more recently, evidence that subsets of T lymphocytes bind to P selectin has been presented (Damle et al., 1992; Moore and Thompson, 1992; Rossiter et al., 1994). There is also evidence that chemokines (e.g., MIP-1 β , RANTES, MCP-1) and other T cell-triggering molecules can be trapped on the luminal aspect of the vessel wall by proteoglycans, thus setting the stage for antigen independent memory T cell activation in situ (Tanaka et al., 1993). Analogous multi-step mechanisms are postulated for T cell homing to peripheral lymph nodes and emigration through high endothelial venules (Shimizu et al., 1992), though in this case primary rolling interactions are mediated by lymphocyte surface L selectin and carbohydrate ligands expressed on the specialized endothelial walls (Imai et al., 1991; Lasky, 1992).

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Recently, specific carbohydrate ligands for the endothelial selectins, E and P selectin, have been identified on neutrophils and myelomonocytic cell lines (Moore et al., 1992; Levinovitz et al., 1993; Norgard et al., 1993). Leukocyte binding to P selectin is mediated by a newly described sialomucin glycoprotein, termed P selectin glycoprotein ligand-1 (PSGL-1)¹ (Sako et al., 1993), while E selectin binding appears to be mediated by multiple glycoconjugate ligands sharing lactosamine motifs related to the sialyl Lewis x/a (SLe^{x/a}) antigen (Lowe et al., 1990; Picker et al., 1991; Polley et al., 1991; Rosen, 1993). We have shown previously (Rossiter et al., 1994) that T cell clones derived from peripheral blood and skin of atopic dermatitis patients differ in their levels of CLA (Berg et al., 1991), a SLe^x containing ligand for E selectin defined by mAbs HECA-452 and CSLEX-1 (Fukushima et al., 1984; Duijvestijn et al., 1988; Picker et al., 1990; Munro et al., 1992; van Reijssen et al., 1992). Binding of T cell clones to P selectin in this study did not correlate with either E selectin binding or CLA expression, raising the intriguing possibility that T cells express distinct ligands for E and P selectins (Rossiter et al., 1994).

In the present study, we report that T lymphocytes can indeed form rolling adhesions on purified endothelial selectins under conditions simulating physiological flow. Through the study of both normal peripheral blood T cells and specific CD4⁺ T cell clones, we conclude that distinct ligands appear to mediate T cell interactions with P and E selectins. We used a novel *O*-glycoprotease derived from *Pasteurella haemolytica* that is highly specific for *O*-sialo mucin-like glycoproteins (Abdullah et al., 1992; Sutherland et al., 1992) to characterize their contribution to rolling adhesions on P and E selectins. We have identified PSGL-1 (or a closely related molecule) as the major ligand for P selectin on T cells, and found it does not contribute to T cell rolling adhesions on E selectin. The physiological significance of distinct ligands on T lymphocytes for P and E selectin is discussed.

Materials and Methods

Antibodies

HECA-452 (Rat IgM), a gift of Dr. L. Picker (University of Texas/Southwestern, Dallas, TX) was produced and FITC labeled as previously described (Duijvestijn et al., 1988; Picker et al., 1990). FITC-conjugated Rat IgM was used to control for nonspecific binding. The anti-human P selectin murine mAbs used in this study as purified Igs were gifts of Dr. R. McEver (University of Oklahoma, Oklahoma City, OK); mAb G1 was used for blocking P selectin function; mAb S12, a non-function-blocking mAb, was used for site density determinations of P selectin in the planar membranes (Geng et al., 1990). MAb BB11(IgG2b), a function-blocking anti-E selectin mAb (Lobb et al., 1991) was obtained from Dr. R. Lobb (Biogen, Cambridge, MA). Rb 3026, a well-characterized polyclonal antibody to the fucosylated extracellular portion of PSGL-1 (Sako et al., 1993), was a gift of Dr. G. Larsen (Genetics Institute, Cambridge, MA). FITC- and phycoerythrin (PE)-conjugated secondary antibodies were obtained (Southern Biotechnology, Birmingham, AL), and used according to manufacturer's instructions.

Flow Cytometry

FACS analysis: flow cytometry was performed on a Becton-Dickinson FACSCAN; 10⁵ cells were analyzed per test, using FITC-labeled HECA

452. FITC-conjugated Rat IgM was used as a control. Cells were also stained with anti-PSGL-1 polyclonal antiserum (Rb 3026) (1:100 dilution), or control rabbit serum, washed, and labeled with a secondary FITC for single color or PE conjugated goat anti-rabbit antibody for two color analysis.

Isolation of Peripheral Blood T Cells and Generation of T Cell Clones

CFTS 4:3.1 and CFT 4:1.7 were derived from a patient known to develop atopic dermatitis after exposure to the house dust mite *Dermatophagoides pteronyssinus*. CFTS 4:3.1 was obtained from a biopsy of an epicutaneous patch test to *Dermatophagoides pteronyssinus* 24 h after challenge as previously described (van Reijssen, 1992). Clone CFT 4:1.7 was obtained from peripheral blood and was derived in an identical fashion; these cells are not antigen specific for Der.p. 1. Both clones are CD4⁺ and express the α/β TCR. Clone C4B5 was the generous gift of Dr. R. Modlin (UCLA, Los Angeles, CA) and was derived from a patient with leprosy. It is CD4⁺, produces a TH0 profile of cytokines, and is antigen specific for M. leprae. Clones were maintained as previously described (Rossiter, 1994).

Peripheral blood T lymphocytes were isolated by Ficoll Hypaque density gradient centrifugation, followed by depletion of monocytes, B lymphocytes, and NK cells using mAbs to selective surface markers followed by magnetic bead-mediated negative selection, as described (Carr et al., 1994). CD3 immunoreactivity was greater than 90% as determined by flow cytometry.

Cell Adhesion Assays

⁵¹Cr-labeled lymphocytes were preincubated at 4°C with various dilutions of PSGL-1-specific antiserum or normal rabbit serum (NRb) in binding medium (HBSS/Hepes/10 mM Ca²⁺/10 mM Mg²⁺/2% BSA). The cell suspension was added to multiwell plates precoated with anti-human IgG Fc Ab followed by P-selectin Rg, E-selectin Rg, or control CD4-Rg chimera absorption (Zettlmeissl et al., 1990), as previously described (Rossiter et al., 1994). The plates were gently rotated at room temperature for 30 min in order to maintain shear conditions. Unbound cells were separated from bound cells by inverting the plates, as described previously (Rossiter et al., 1994).

Preparation of Selectin Containing Planar Bilayers

Recombinant full length human E selectin was purified from a CHO cell line transfected with E selectin cDNA, (a generous gift of Dr. R. Lobb), by immunoaffinity chromatography using anti-E selectin mAb BB11 coupled to Sepharose. P selectin, purified from human platelets, was a gift of Dr. R. McEver. Liposomes containing the reconstituted selectins, were prepared as previously described (Lawrence and Springer, 1991) by the method of octyl-glucoside (OG; Sigma Chem. Co., St. Louis, MO) dialysis.

Determination of Selectin Site Densities

Liposomes were reconstituted with different quantities of a purified selectin and planar membranes were formed as described (Lawrence and Springer, 1991). Radiolabeled mAbs S12 or BB11 were used at 20 μ g/ml for site density determination of P or E selectin-containing membranes, respectively. The purified antibodies were iodinated to a known specific activity and site densities of each planar bilayer were determined by saturation binding as previously described (Dustin and Springer, 1989). Site densities were determined in triplicate.

Cell Treatments

To remove terminal cell-surface sialic acids, cells were incubated with 0.1 U/ml *Vibrio Cholera* neuraminidase (Calbiochem, San Diego, CA) for 30 min at 25°C in HBSS supplemented with 10 mM Hepes and 2 mM Ca²⁺ (H/H Ca²⁺ medium). To assess the role of *O*-sialo mucin-like ligands for selectins in leukocyte rolling adhesions to each selectin, lymphocytes or HL-60 cells (10⁷/ml) were incubated for 40 min at 37°C in binding medium: H/H Ca²⁺ medium supplemented with 2 mg/ml HSA (Calbiochem, San Diego, CA) in the presence of 50 μ g/ml of *P. haemolytica* *O*-glycoprotease (a generous gift of Dr. A. Mellors, University of Guelph, Ontario). Control cells were incubated at 37°C in this medium in absence of enzymes. Reactions were terminated by washing the cells twice with H/H medium + 5 mM EDTA. Cells were kept at 4°C up to 2 h in binding medium.

1. *Abbreviation used in this paper:* CLA, cutaneous lymphocyte antigen; Der.p.1, *Dermatophagoides pteronyssinus* antigen; NRb, normal rabbit serum; OG, octyl-glucoside; PE, phycoerythrin; PSGL-1, P-selectin glycoprotein ligand-1; SLe^x, Sialyl Lewis x.

Laminar Flow Assays

A glass slide containing a planar bilayer was assembled in a parallel flow chamber (260- μ m gap thickness) and mounted on the stage of an inverted phase-contrast microscope (Diaphot-TMD; Nikon Inc., Garden City, NY), as previously described (Lawrence and Springer, 1991). Cells were resuspended at a concentration of 1×10^6 /ml in binding medium and attachment during flow was assayed. The number of adherent cells per field of view (0.43 ± 0.01 mm²) which attached during initial periods of continuous flow was quantitated by a visual count of the fields videotaped while scanning the lower plate of the flow chamber. Cell attachment events were expressed in rate of attachment. The wall shear stress was calculated assuming a viscosity of assay buffer equal to the viscosity of water at room temperature (1.0 centipoise, 24°C). T clones or HL-60 failed to attach to the selectin containing membranes at shear stresses higher than 1.8 dynes/cm². PMN attached to identical selectin containing bilayers at shears as high as 3.6 dynes/cm².

For detachment assays, cells were infused into the chamber at a shear of 0.73 dynes/cm², allowed to adhere until equilibration was reached and the shear force was then increased every 20 s to a maximum of 36 dynes/cm². A given batch of cells, pretreated with the different enzymes, was compared for attachment rate to a given field of the selectin-containing membrane.

Analysis of Cell Rolling

All cells were bound during shear flow (0.73 dyne/cm²) for 1–2 min and the shear force was increased every 20 s. Images were recorded on a time-lapse video cassette recorder and analyzed as previously described (Lawrence and Springer, 1991) except that cell displacements were measured over 5–10-s intervals. Rolling was assessed only for cells which remained adherent for at least 20 s at the shear applied. Rolling of a given group of cells, differently pretreated with the various reagents, was measured in identical fields of view in order to directly assess the effects of these treatments. In each experiment, under the highest shear applied, at least 20% of the cells originally attached remained adherent and rolling.

Results

Normal Peripheral Blood T Lymphocytes Tether to P and E Selectin

We wished to determine the relative percentages of normal peripheral blood T cells that tethered to P and E selectin, respectively, under shear stress. At saturable selectin densities coated on the substrate, ~15% of T cells bound to P selectin, and a similar percentage of T cells bound to E selectin (Fig. 1). Tethering under these conditions was always followed by rolling of all cells on both selectins (not shown). Subsequent experiments to determine whether these ligands were distinct, and whether they were present on the same or different T cells, yielded equivocal results. While these data and our prior results (Rossiter et al., 1994) suggested that different ligands might mediate these interactions on peripheral blood T cells, such an analysis of a heterogeneous population of T cells could not shed further light on the expression and function of each type of putative selectin ligand on individual T cells. Subsequent analyses focused on homogeneous populations of cloned human T cells.

CLA is not Required for Tethering and Rolling of T Cells on P Selectin Under Physiological Flow

We characterized the properties of three representative T cell clones with regards to attachment and rolling under physiological flow conditions on artificial membranes containing P or E selectin (Lawrence and Springer, 1991). HL-60, a promyelocytic cell line that exhibits well-characterized binding to both E and P selectins, served as a positive control. All three T clones are CD4+, express $\alpha\beta$ T cell receptors, and are CD45RO positive and RA negative (Rossiter et

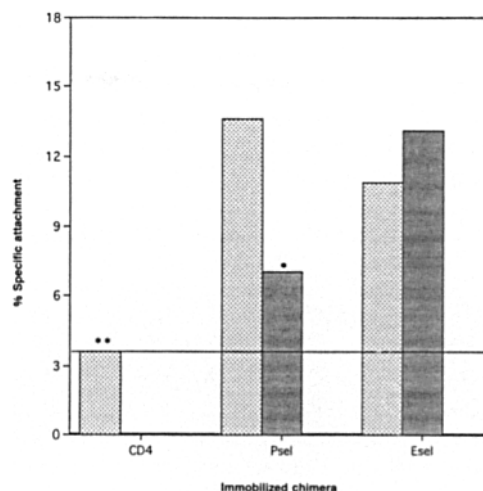


Figure 1. Attachment of peripheral blood T lymphocytes to P and E selectin under shear conditions. Purified ⁵¹Cr labeled T cells (5×10^4 /well) in binding medium (HBSS/Hepes/10 mM Ca²⁺/10 mM Mg²⁺/2% BSA) were allowed to attach to either P or E selectin chimeras, immobilized in multiwell plates. A CD4 chimera served as a control. Plates were gently rotated to maintain shear conditions under which selectin-mediated tethering predominates. In the presence of EDTA or EGTA all cell binding was abolished. E selectin function-blocking mAb (BB11) reduced T cell binding to background levels (not shown). Results are the means of triplicates. A representative of three experiments is shown. * $p < 0.05$ and ** $p < 0.001$ with respect to cell attachment to P selectin. □, No antibody; ■, a P selectin blocking antibody.

al., 1994; R. Modlin, unpublished data). These clones were selected because they expressed high, low, or absent levels of CLA, as defined by HECA-452 reactivity. This monoclonal antibody is an IgM that defines a sialylated carbohydrate antigen associated with multiple leukocyte glycoconjugates terminating with SLe^x-related carbohydrates (Berg et al., 1991). Clone C4B5 is a TH0 clone that lacks detectable CLA, while clone CFTS 4:3.1 expresses high levels of CLA (Fig. 2). Clone CFT 4:1.7 expresses low but detectable levels of CLA (Fig. 2).

Despite lacking any carbohydrate structures recognized by HECA-452, C4B5 lymphocytes attached and rolled on P selectin under flow. In fact, they did so more readily than HL-60 cells, which express high HECA-452 levels (Fig. 3 a). This attachment could be fully blocked by the anti-P selectin function-blocking antibody mAb G1 (data not shown). In contrast, C4B5 cells did not attach to E selectin under the flow conditions of the assay, while HL-60 cells did so readily (Fig. 3 a). We conclude that the T clone C4B5 expresses a functional P selectin ligand(s) that is not recognized by HECA-452 antibody and is thus distinct from CLA.

P Selectin Ligands on T Cells Are O-glycoprotease-sensitive Sialomucins

A novel endopeptidase that selectively degrades O-sialomucins (Abdullah et al., 1992) was recently shown to selectively abolish binding of HL-60 and PMN to P selectin (Steininger et al., 1992). Treatment of C4B5 cells or HL-60 cells with O-glycoprotease completely abolished their tethering to P selectin, but did not influence HL-60 attachment to E selectin (Fig. 3 a). Similar elimination of C4B5 attachments to P selectin was observed after neuraminidase treat-

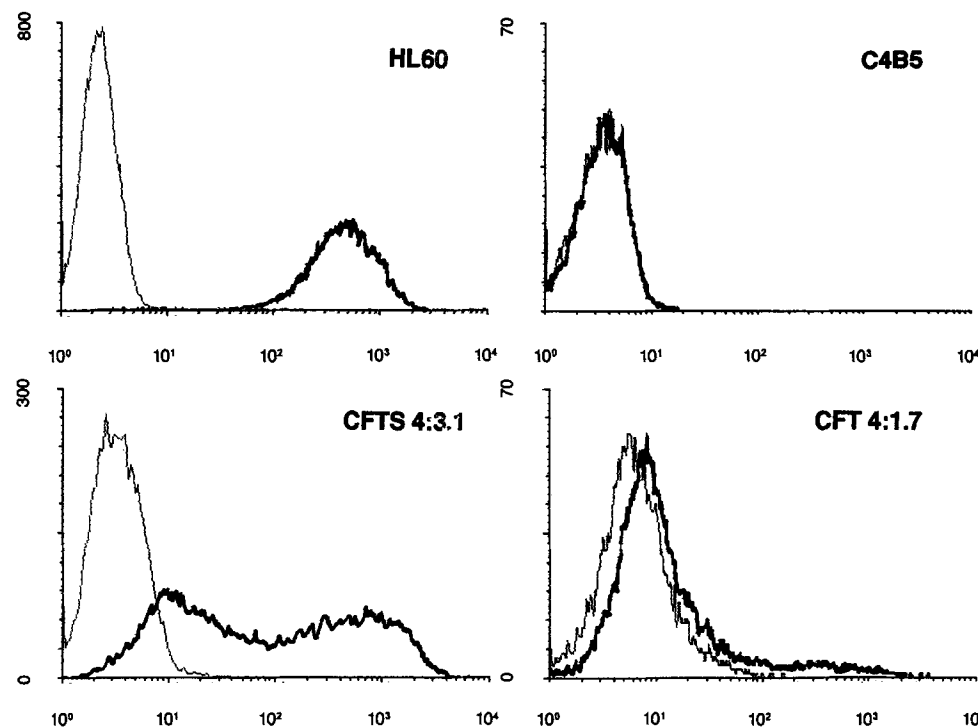


Figure 2. HECA-452 FACS analysis of HL-60 and T lymphocyte clones. Cells were stained with FITC-labeled HECA 452 or control IgM.

ment. The P selectin ligand on HL-60 cells was partially susceptible to neuraminidase treatment at 24°C (Fig. 3 *a*), and neuraminidase treatment at 37°C completely abolished the P selectin ligand (not shown). Collectively, the effects of these different enzyme treatments suggest that the predominant P selectin ligand on the C4B5 T cell clone is a sialomucin, just as in neutrophils and HL-60 cells (Steininger et al., 1992). This P selectin ligand does not appear to support the clone tethering and rolling on E selectin. It is notable that adherent C4B5 cells exhibited an extremely high resistance to increasing shear stresses applied on cells tethered to P selectin. The majority of C4B5 cells continued to roll on this selectin at shear stresses equivalent to the highest measured in post capillary venules (35 dynes/cm²) (Fig. 3 *b*). In contrast, the few T cells or HL-60 which tethered to P selectin after *O*-glycoprotease treatment, had transient rolling and readily detached from the selectin at low shear stresses, indicating near complete removal of P selectin ligands.

Binding of T Cells to E Selectin Is Not Mediated by *O*-glycoprotease Sensitive Sialomucins

The T cell clone CFTS 4:3.1, which expresses high levels of cell surface CLA (Fig. 2), attached and rolled on E selectin-containing membrane in a manner comparable to HL-60 cells (Fig. 4, *a* and *b*). These rolling adhesions were completely abolished by neuraminidase treatment (Fig. 4 *b*). CFTS 4:3.1 T cells had considerable resistance to increasing shear on E selectin, with a significant proportion of cells exhibiting persistent rolling at shear stresses as high as 15 dynes/cm² (Fig. 4 *b*). *O*-glycoprotease treatment of CFTS 4:3.1 had a negligible effect on E-selectin-mediated adhesions (Fig. 4 *b*). CFTS 4:3.1 T cells also express ligand(s) for P selectin. In contrast to interactions with E selectin, both attachment under flow (Fig. 4 *a*) and rolling on P selectin (Fig. 4 *c*) were eliminated by *O*-glycoprotease treatment,

indicating that the P selectin ligand on these cells is a sialomucin. *O*-glycoprotease-treated 4:3.1 cells appear to retain completely their functional E selectin ligands, despite having lost virtually all cell surface sialomucins to this endopeptidase (including their P selectin ligand[s]). Identical results were obtained with HL-60 cells (Fig. 4, *a* and *b*); after loss of their entire P selectin binding capacity, these cells could still attach (Fig. 4 *a*) and roll (Fig. 4 *b*) normally on E selectin. Taken together, these data suggest that CLA, or other sialylated E selectin ligands on T cells resistant to *O*-glycoprotease treatment, do not support rolling attachments to P selectin. This is consistent with previous studies on myeloid cell lines (Larsen et al., 1992).

CFT 4:1.7, a T cell clone derived from peripheral blood, expresses low but detectable levels of CLA (Fig. 2). Attachment rates of this clone to P selectin were comparable to those observed with HL-60 (Figs. 3 *a* and 5 *a*), and as always, attachment was followed by rolling (Fig. 5 *b*). All interactions of this clone with P selectin were completely abolished by *O*-glycoprotease treatment (Fig. 5, *a* and *b*). FACS analysis indicated that virtually complete removal of P selectin-binding activity by *O*-glycoprotease treatment did not result in any decrease in the cell surface expression of CLA or other HECA-452 reactive molecules, even on cells where the baseline expression of such epitopes was low (not shown).

Another parameter that assesses the average number of tethers being formed by a rolling cell is resistance to detachment of forces continuously applied on it (Lawrence and Springer, 1993). We compared the three T clones, all preattached under low flow conditions to P selectin, for their relative shear resistance on this selectin. The CLA^{neg} C4B5 clone had the highest shear resistance on P selectin, whereas the CLA^{high} T clone 4:3.1 had the lowest value (Fig. 6). Based on this assay, and its highest cell attachment rates to

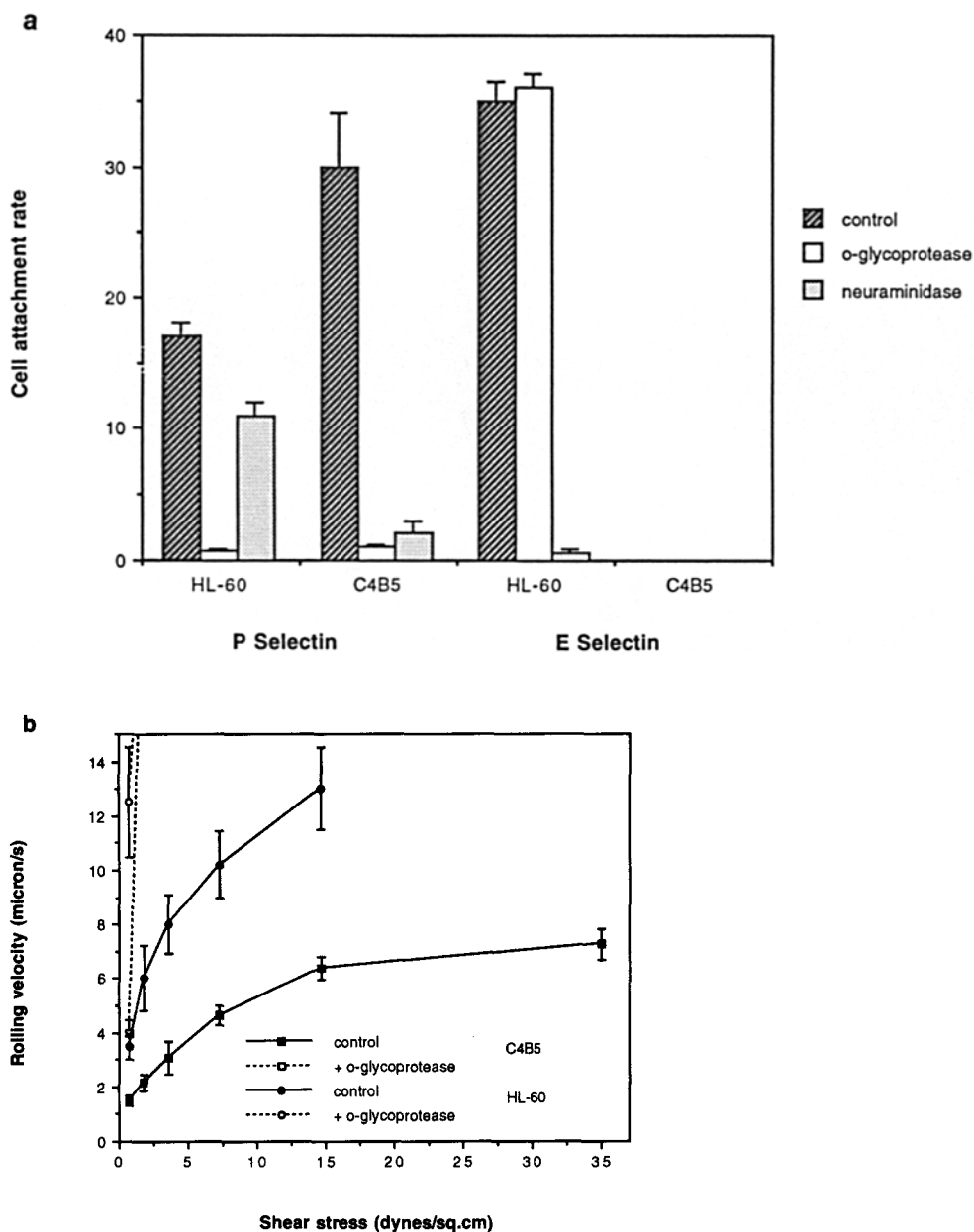


Figure 3. Attachment of C4B5 and HL-60 cells under flow conditions to purified P and E selectins reconstituted in lipid bilayers. (a) Effect of enzymatic pretreatments of the flowing cells. Cells (10^7 /ml) were preincubated with control binding medium alone or with O-glycoprotease (at 50 μ g/ml; 20) or 0.1 U/ml *Vibrio cholera* neuraminidase (Calbiochem, San Diego, CA) at 37 or 25°C, respectively, for 30 min. Reactions were terminated by washing the cells twice with H/H medium + 5 mM EDTA. Cells (1×10^6 /ml) were infused in binding medium (HBSS/Hepes/2 mM Ca^{2+} supplemented with 2 mg/ml HSA) at 0.73 dyne/cm² wall shear stress through a parallel plate flow chamber (Lawrence and Springer, 1991, 1993). Rates of cell attachment to the artificial planar bilayer containing P or E selectin at 650 and 500 sites/ μm^2 , respectively, were calculated from the numbers of cells adhered to a given field of view (0.43 ± 0.01 mm²) per minute. All cells started to roll upon adherence to the selectin-containing membrane. Bars show the standard error of the mean. Results are representative of two independent experiments. At 37°C, neuraminidase completely blocked attachment of both HL-60 and C4B5 cells to P selectin. (b) Effect of O-glycoprotease pretreatment on C4B5 and HL-60 rolling on P selectin. Cells, attached at

flow to the P selectin membranes as described in *a* were exposed to increasing shear stresses and their rolling velocities at each stress were determined. Each point represents the mean $\pm$ SEM of 20–30 rolling cells. Velocity values of cells observed to roll at the given shear stress in a transient manner (mean velocities greater than 50 $\mu\text{m/s}$) and eventually detach from the membrane are not shown but are indicated by connecting dashed lines. ▨, Control; □, O-glycoprotease; ■, neuraminidase.

P selectin (Fig. 3 *a*), C4B5 appears to express the highest P selectin ligand level.

These observations suggest that the P selectin ligands on all three T cells are sialomucin structures that do not react with an antibody specific for common SLe^x related structures. In contrast, rolling adhesions of T cells on E selectin are closely associated with the presence of SLe^x bearing glycoproteins (e.g., CLA). Since C4B5, the T cell clone negative for HECA-452 reactivity (Fig. 2), attached very poorly to E selectin under flow, it would appear that HECA-452 reactivity is indeed closely correlated with functional E selectin ligand activity. This was true for all lymphocyte clones thus far tested, a result consistent with data previously obtained on these T cells in static binding assays (Ros-

siter et al., 1994). The role of CLA in the binding of these cells has been difficult to confirm by antibody blocking experiments, since HECA-452 is an inefficient blocking antibody (L. Picker, personal communication; and Berg et al., 1992).

PSGL-1 or a Closely Related Molecule Is the Major P Selectin Ligand on T Cells

The P selectin ligand, PSGL-1, recently cloned from a cDNA library of HL-60, appears to be the sole P selectin ligand on myeloid cells and neutrophils (Sako et al., 1993). We asked whether the sialomucin ligand(s) for P selectin on T cells is homologous to PSGL-1, using a polyclonal anti-

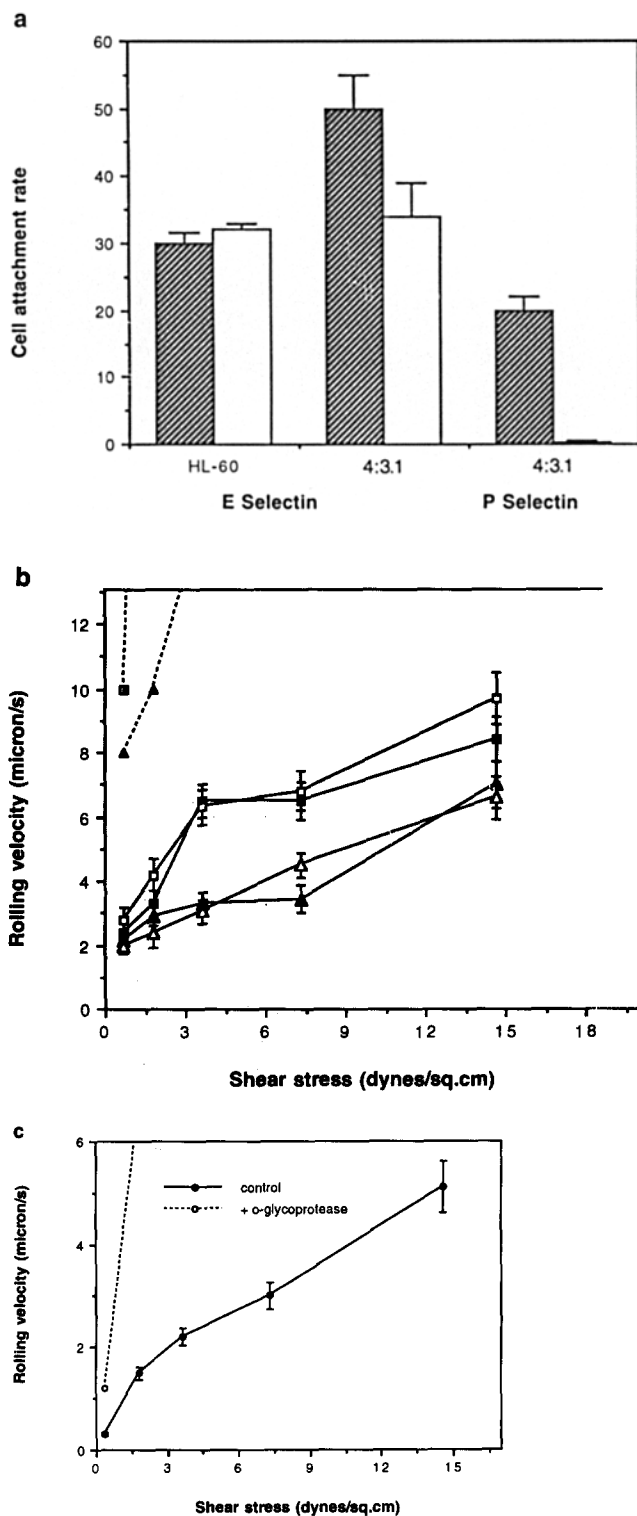


Figure 4. Attachment and rolling of CFTS 4:3.1 T clone on P but not on E selectin are mediated by sialomucins. (a) Effect of *O*-glycoprotease induced removal of surface mucin-like sialoglycoproteins on attachment of CFTS 4:3.1 under flow to E selectin and P selectin. HL-60 cell attachment to E selectin is shown for comparison. Cells ($1 \times 10^6/\text{ml}$) were infused at $0.73 \text{ dyne}/\text{cm}^2$ and attachment rates to P selectin at $650 \text{ sites}/\mu\text{m}^2$ or E selectin at $250 \text{ sites}/\mu\text{m}^2$ were determined as in Fig. 3. Bars show the standard error of the mean. $\blacksquare$, Control; $\square$, *O*-glycoprotease. (b) Effect of *O*-glycoprotease treatments and neuraminidase on rolling velocities of 4:3.1 clone at different shear stresses on E selectin (coated at $250 \text{ sites}/\mu\text{m}^2$). Effect of enzyme treatments on HL-60 rolling on E

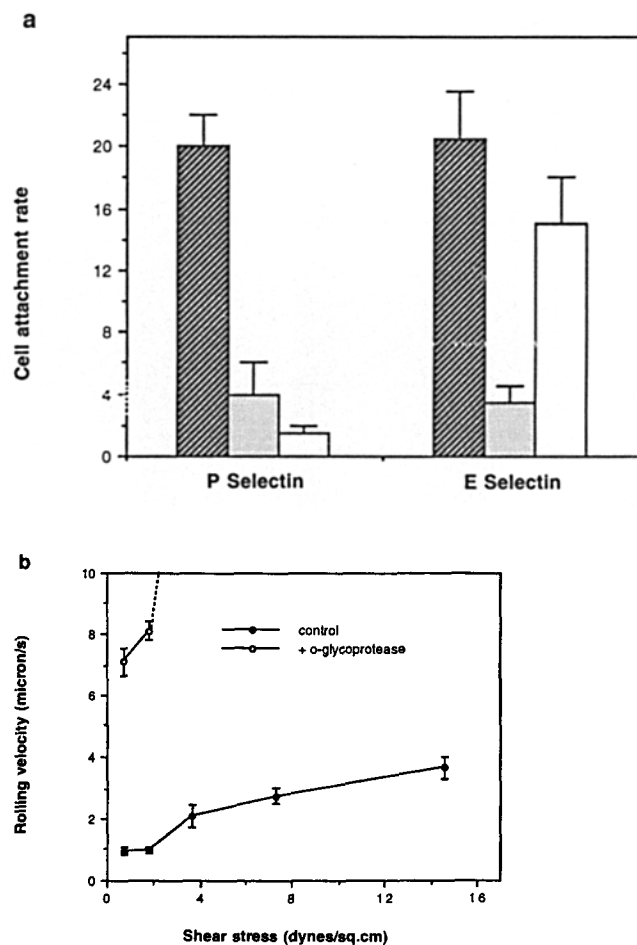


Figure 5. Different contribution of sialomucin and CLA ligands to CFT 4:1.7 T cell clone rolling adhesions on P and E selectins. (a) Attachment of 4:1.7 T clone to selectins under flow conditions following enzymatic treatments. Cells ($1 \times 10^6/\text{ml}$) were infused at $0.73 \text{ dyne}/\text{cm}^2$ and rates of attachment to P selectin (at $650 \text{ sites}/\mu\text{m}^2$) or to E selectin (at $500 \text{ sites}/\mu\text{m}^2$) were determined. The T clone attachment rate to the P selectin containing membrane was reduced two-fold at elevated shear ($1.8 \text{ dynes}/\text{cm}^2$). Enzymatic pretreatments were as described above. Error bars show the standard error of the mean. Results are representative of three experiments. $\blacksquare$, control; $\blacksquare$, + neuraminidase; $\square$, + *O*-glycoprotease. (b) Rolling of 4:1.7 T cells on P selectin at different wall shear stress. Effect of *O*-glycoprotease treatment on rolling velocity. Cells attached during flow were exposed to increasing shear stresses and their rolling velocities were calculated as described in previous figures. Each point represents the mean $\pm$ SEM of 20–30 cells.

selectin (coated at $500 \text{ sites}/\mu\text{m}^2$) is shown for comparison. $\blacktriangle$, HL-60 control; $\triangle$, HL-60 + *O*-glycoprotease; $\triangle$, HL-60 + neuraminidase; $\blacksquare$, 4:3.1 control; $\square$, 4:3.1 + *O*-glycoprotease; $\square$, 4:3.1 + neuraminidase. (c) Effect of *O*-glycoprotease treatment on rolling of 4:3.1 clone on P selectin coated at $650 \text{ sites}/\mu\text{m}^2$. Velocities of cells which rolled transiently at the higher shear are indicated by dashed lines. *O*-glycoprotease treatments and rolling velocity analysis were performed as described in Fig. 2. Results are representative of two and four different sets of experiments, for 4:3.1 T clone and HL-60 cells, respectively.

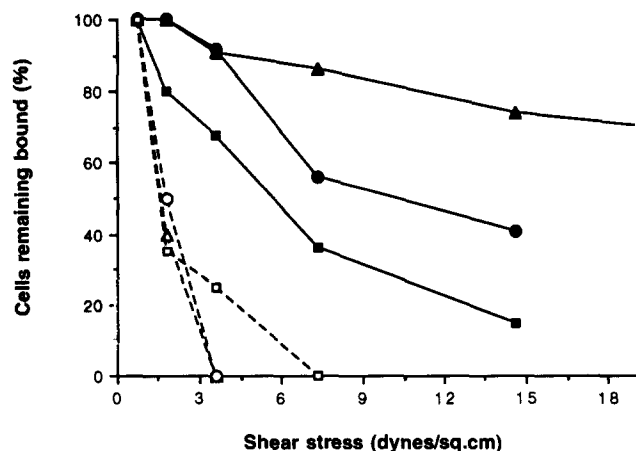


Figure 6. Relative resistance of T cells attached to P selectin to detaching shear stresses. Effect of *O*-glycoprotease pretreatment. Cells were infused through the chamber at 0.73 dynes/cm² for 2 min and then the flow rate was increased in stage increments every 20 s. The number of cells which attached to the P selectin-containing membrane after *O*-glycoprotease treatment was significantly lower than untreated cells. The percentage of cells that remained bound and rolling was determined after 10 s at each shear stress. Control (untreated) samples of each clone are shown in bold; *O*-glycoprotease treated cells are shown in open symbols. $\blacktriangle$, C4B5; --- $\triangle$ ---, + *O*-glycoprotease; $\bullet$, 4:1.7; -- $\square$ ---, + *O*-glycoprotease, $\blacksquare$, 4:3.1; -- $\square$ ---, + *O*-glycoprotease.

body (Rb 3026) specific for a functional selectin-binding glycoform of the recombinant PSGL-1 protein expressed in COS cells cotransfected with fucosyltransferase III (Sako et al., 1993). All T cell clones and lines thus far tested are positive by flow cytometry for PSGL-1 expression (not shown). Treatment of T cell clones and HL-60 cells with *O*-glycoprotease significantly reduced PSGL-1-specific staining (Fig. 7 a).

We next assessed the specific role of PSGL-1 in T cell binding to P selectin in a binding assay shown in Fig. 7 b. A significant, specific, and dose-dependent blocking of T cell binding to P selectin in the presence of Rb 3026 was reproducibly observed. The adhesion inhibition by this antiserum was not a result of cell agglutination by the polyclonal antibody, as judged by visual inspection. Rb 3026 did not influence T cell binding to E selectin (not shown). Collectively, these data demonstrate that T cell PSGL-1, or a closely related sialomucin, can function as a major P selectin ligand.

Finally, we turned our attention back to peripheral blood T cells. Two color flow cytometry analysis of peripheral blood T cells indicated that, surprisingly, the majority of T cells express immunoreactive PSGL-1 (judged by Rb 3026 binding), while only a small subset express CLA (Fig. 8). Treatment of T cells with *O*-glycoprotease completely abolishes Rb 3026 reactivity while not affecting HECA-452 reactivity. This indicates that all Rb 3026 immunoreactivity is associated with the *O*-glycoprotease sensitive sialomucin(s). The tethering of peripheral blood T cells to P selectin shown in Fig. 1 can be blocked by *O*-glycoprotease, while E selectin tethering cannot (not shown). These data indicate that peripheral blood T cells and the clones characterized in this study behave in a similar fashion with regards to selectin tethering.

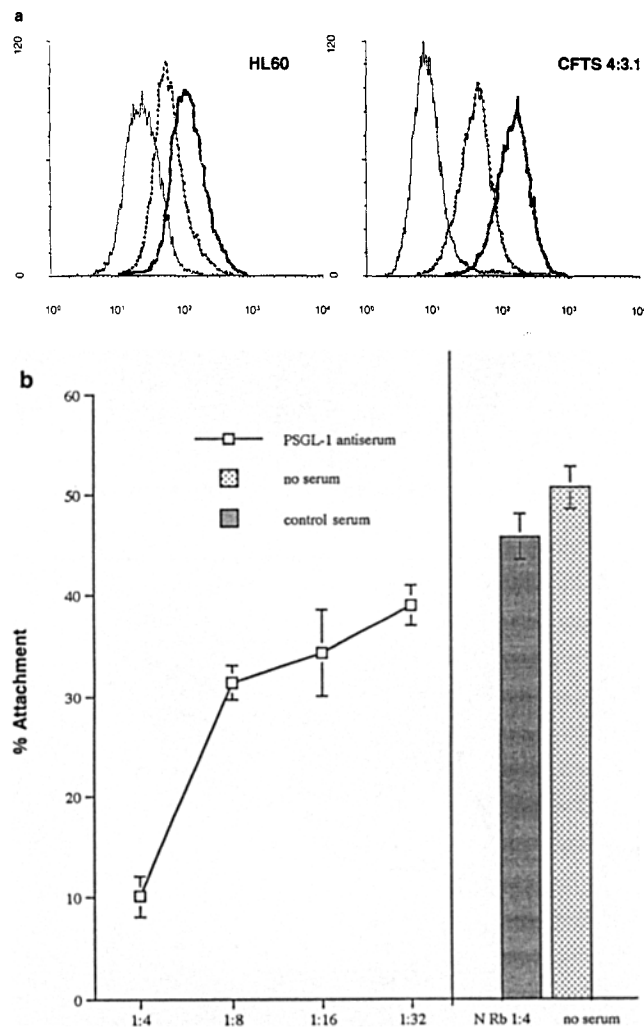


Figure 7. PSGL-1 is a major P selectin ligand in T cells. (a) PSGL-1 staining of CFTS 4:3.1 clone and HL-60 cells before and after treatment with *O*-glycoprotease. Cells were stained with anti-PSGL-1 rabbit antiserum (Sako et al., 1993) or control rabbit serum (1:100 dilution) washed and labeled with a secondary FITC-goat anti-rabbit antibody. Heavy line, before treatment; dotted line, after treatment; pole line, control Ab. (b) A dose-response blocking of CFTS 4:3.1 T cell adhesion to P selectin substrate in the presence of anti-PSGL-1 antiserum. ⁵¹Cr-labeled lymphocytes were preincubated at 4°C with various dilutions of PSGL-1-specific antiserum (Rb 3026) or NRb in binding medium. The cell suspension was added to multiwell plates precoated with P selectin or control BSA and the plates were gently rotated at room temperature for 30 min. Unbound cells were separated from bound cells by inverting the plates, as described previously (Rossiter et al., 1994). Adhesion is expressed as percentage of radioactivity bound. Background adhesion to human IgG was 4%.

It is clear that only a subset of PSGL-1 positive T cells bind to P selectin. It is likely that only specific glycoforms of PSGL-1 mediate binding to P selectin, and that glycosylation patterns, rather than surface-expression of PSGL-1 per se correlate with ligand binding activity. These results are consistent with the observation that transfection of COS cells with PSGL-1 cDNA alone did not result in P selectin binding. Cotransfection with fucosyltransferase was required for the acquisition of binding activity. The PSGL-1 sialomucin appears to be restricted to leukocytes, however, since fibro-

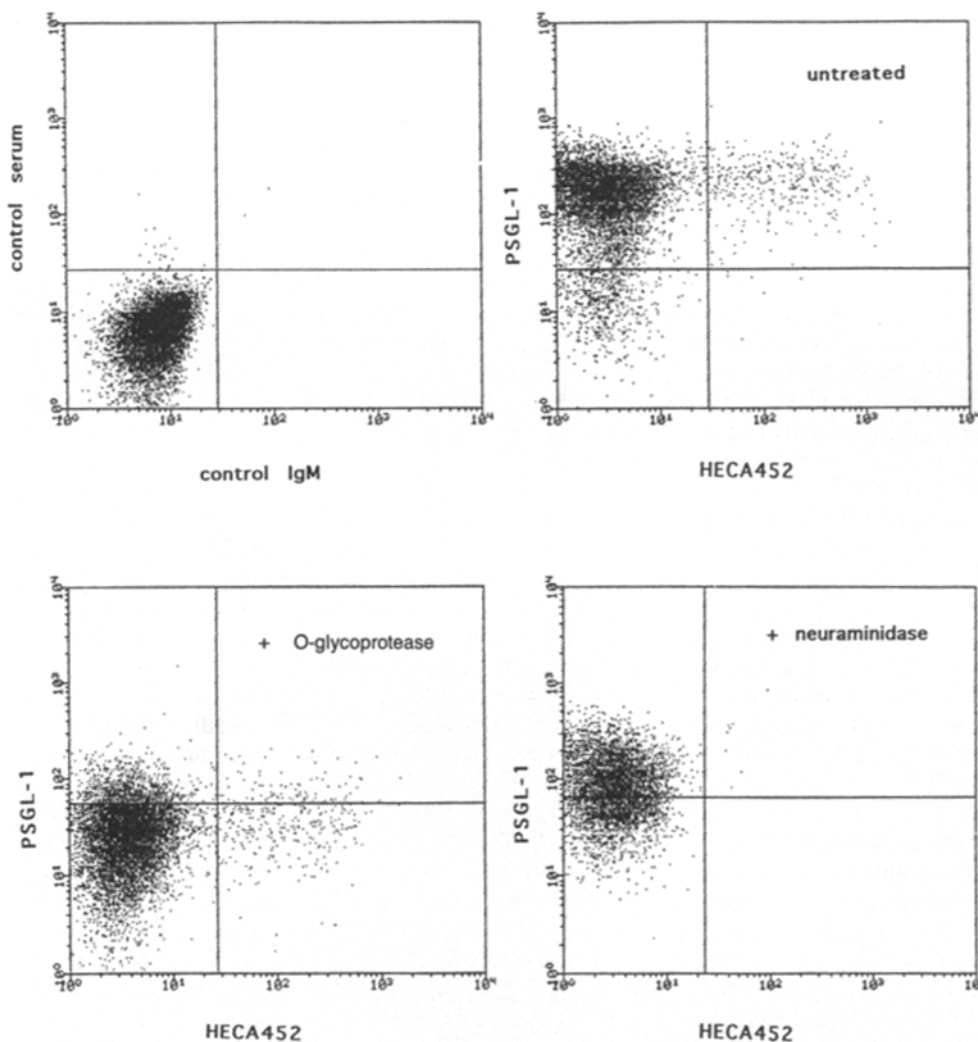


Figure 8. Distinct subsets of T lymphocytes express CLA and PSGL-1. Two color FACS analysis of cells stained with FITC-labeled HECA 452 and anti-PSGL-1 antiserum followed by PE-conjugated secondary antibody was performed before and after enzyme treatments with either *O*-glycoprotease or neuraminidase as described in Materials and Methods.

blasts and keratinocytes are not Rb 3026 positive (not shown). It should be noted that these latter cells do express multiple sialomucins that do not react with Rb 3026.

Discussion

The present study provides the first evidence that subsets of T cells tether and roll on P and E selectins. Our use of cloned T cells in addition to peripheral blood T cells in this study enabled us to analyze interactions of homogeneous populations with purified selectins in well defined flow conditions, using a parallel plate flow chamber system (Lawrence and Springer, 1991, 1993). We show that, under continuous flow conditions using P or E selectin reconstituted at physiological site densities in artificial membranes, all T cells that tethered to these selectins remained attached and rolled on the selectins at high shear stresses.

Three main observations made in this study indicate that CLA, previously implicated in E selectin binding (Picker et al., 1991; Shimizu et al., 1992), does not serve as a P selectin ligand. First, high levels of CLA as measured by FACS analysis on a subset of peripheral blood T cells, the 4:3.1 T clone, and HL-60 remained intact after *O*-glycoprotease treatments which remove surface sialomucins, but were not

sufficient to support rolling adhesions on P selectin. Second, attachment and rolling on E selectin was not significantly influenced on any cell treated with the *O*-glycoprotease, suggesting (at best) only a minor contribution of mucin-like ligands to E selectin binding. Finally, HECA-452 reactivity, the working definition of CLA expression on different T clones, did not correlate with the level of P selectin binding activity; in fact, T cells that lacked any CLA reactivity or E selectin binding expressed the highest levels of P selectin ligand as assessed by function (C4B5).

We did not find any CLA-related carbohydrates to be associated with the *O*-glycoprotease-sensitive sialomucins on T cells or HL-60, because even the most exhaustive *O*-glycoprotease treatments did not alter their level by FACS analysis. The insensitivity of CLA expression to *O*-glycoprotease digestion was confirmed both with the 4:1.7 clone (not shown), which expresses very low levels of CLA, and with peripheral blood T cells (Fig. 8). Although a previous report indicated that HL-60-derived PSGL-1 decorated with SLe^x can support cell adhesion of cells transfected with either P or E selectin (Sako et al., 1993), we could not demonstrate any contribution of cell surface PSGL-1 or any other sialomucin to leukocyte rolling adhesions of E selectin.

All T cells tested were positive for PSGL-1 immunoreac-

tivity, as judged by a polyclonal antibody Rb 3026 specific for the recombinantly expressed sialoglycoprotein. PSGL-1 immunoreactivity was abrogated by *O*-glycoprotease treatment, both on peripheral blood T cells, T cell clones, and HL-60 cells, indicating that the RB 3026 immunoreactivity is sialomucin associated. Furthermore, the anti-PSGL-1 antibody blocked binding of T cells to P selectin in a dose dependent fashion. This blocking was highly reproducible, though it required relatively high concentrations of antibody. While it is likely that T cells express authentic PSGL-1, it should be pointed out that this molecule was defined in HL-60 cells and neutrophils, and we cannot rule out the possibility that a closely related but distinct molecule on T cells is the authentic P selectin ligand. If so, this T cell P selectin ligand must be a sialomucin (based on the sensitivity of immunoreactivity to *O*-glycoprotease) and must share P selectin binding epitopes with HL-60-derived PSGL-1, based on the blocking ability of Rb 3026. The future cDNA cloning of the P selectin ligand from one of our T cell clones should resolve the question of structural differences between myeloid and lymphoid PSGL-1 glycoforms.

We were initially surprised that virtually all peripheral blood T cells reacted with Rb 3062 by FACS analysis. It now appears that the protein backbone of PSGL-1 is nearly ubiquitous on all leukocytes, although it is not found on fibroblasts and keratinocytes. It appears that PSGL-1 immunoreactivity is necessary but not sufficient for P selectin binding function. Precedence exists for this dichotomy, in that immunoreactivity of CD34, the sialomucin endothelial ligand of L selectin is also widespread, while only a small subset of cells that express CD34 also have the potential to glycosylate this molecule appropriately to express L selectin carbohydrate ligand (Baumhueter et al., 1993). There is also good evidence that fucosylation of PSGL-1 is necessary for function, based on the requirement that PSGL-1 cDNA must be co-transfected with α 1-3/4 fucosyltransferase cDNA into COS cells to confer a P selectin binding phenotype (Sako et al., 1993). Fucosyltransferases are also required for the biosynthesis of E selectin carbohydrate ligands (Lowe, 1990), but the finding that T cells lacking E selectin ligands express functional ligands for P selectin suggests the possibility that different types of glycosyltransferases are required for the generation of E and P selectin ligands on T lymphocytes.

Taken together, these results indicate that a subset of human T cells express a functional P-selectin sialomucin ligand which mediate their rolling adhesions on this selectin under conditions of physiologic flow, and that the major T cell P selectin ligand is similar or identical to PSGL-1. Cell associated PSGL-1 does not appear to be either necessary or sufficient for T cell or HL-60 cell binding to E selectin (Figs. 3 a and 4 a), which strictly correlates with the expression of the SLe^x-containing molecule, CLA. Conversely, the presence of this putative E selectin ligand on a subset of T cells is not sufficient for their rolling adhesions on P selectin. The presence of two structurally distinct ligands, specific for different endothelial selectins and subject to differential regulation on different subsets of T cells, that support rolling adhesions of T cells under physiological flow conditions is novel and has not been reported previously. This observation suggests that there is heterogeneity among T cells with regards to their preferential interaction with one endothelial

selectin over another. The analysis of peripheral blood T cells, as well as the clones, indicates that T cells can express either one or both of these selectin ligands.

It would appear that recruitment of circulating lymphocytes to endothelial sites of inflammation (as opposed to secondary lymphoid tissues) is primarily regulated by endothelial selectins recognizing counter-receptors on memory T cells, rather than by L selectin, since at least some of the memory T cells which recirculate through these sites lack L selectin (Picker et al., 1994). L selectin on C4B5 and on peripheral blood T cells was resistant to *O*-glycoprotease, under conditions which removed all P selectin ligands on these cells (data not shown), suggesting it has no direct contribution to P selectin binding. To the extent that some memory T cells bearing L selectin could interact with putative L selectin ligands on non-lymphoid endothelial sites of inflammation (von Andrian et al., 1991), this may represent yet a third potential adhesion pathway that can be used by T cells to initiate tethering to these peripheral endothelial sites. Coexpression of L selectin and endothelial selectin ligands (e.g., CLA, active PSGL-1) may permit memory T cells to recirculate between peripheral tissue, blood, and lymph nodes (Picker et al., 1994).

The potential of circulating peripheral blood T cells to interact with activated endothelial cells by adhering and rolling on E and P selectins, both of which in turn are subject to specific regulation in many pathophysiologic states of inflammation (Weller et al., 1992; Hahne, et al., 1993; Mulligan et al., 1993), points out a novel mechanism by which T cells may recirculate through peripheral tissues. It suggests that like monocytes and granulocytes (Lawrence and Springer, 1993), T lymphocytes may also use rolling adhesions on endothelial selectins at peripheral sites of inflammation as the antigen independent primary event that is prerequisite for subsequent multi-step activation, stable adhesion, and *trans*endothelial migration (Shimizu et al., 1992; Bevilacqua and Nelson, 1993; Springer, 1994).

While P selectin expression has been identified with immediate inflammatory events (e.g., platelet degranulation, endothelial cell Weibel-Palade body membrane fusion), evidence is accumulating that it can be expressed on endothelial cells at sites where inflammation is prolonged or chronic (Weller et al., 1992; Grober et al., 1993). It is therefore possible that different subsets of T cells home to different anatomical sites based in part upon their selectin ligand surface profile. Just as CLA positive T cells may preferentially home to skin or to chronic sites where E selectin expression is up-regulated, P selectin ligand-expressing T cells may home more efficiently (for example) to inflamed synovium (where P selectin expression is chronically elevated) or preferentially recirculate through any anatomical sites where endothelial P selectin expression is transiently induced. It is furthermore likely that differential expression of specific P and E selectin ligands would add to the diversity of potential adhesive interactions that regulate memory T cell recirculation through peripheral tissues. It is these memory T cells that have been activated by environmental antigens in the immediate or remote past, and are most likely to find their antigen in the context of tissue specific inflammation induced by injury or microbial infection, rather than in peripheral lymph nodes. Tethering of memory T cells mediated by en-

endothelial selectins, followed by integrin-mediated firm adhesions and cytoskeletal mobilization, culminates in successful extravasation.

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