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P-selectin Glycoprotein Ligand-1 Is the Major Counter-receptor for P-selectin on Stimulated T Cells and Is Widely Distributed in Non-functional Form on Many Lymphocytic Cells*

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Gloria Vachino‡§, Xiao-Jia Chang§, Geertruida M. Veldman‡, Ravindra Kumar‡, Dianne Sako‡, Lynette A. Fouser¶, Michael C. Berndt‖, and Dale A. Cumming‡

From the ‡Small Molecule Drug Discovery Group, and the ¶Department of Molecular Immunology, Genetics Institute, Cambridge, Massachusetts 02140 and the Department of Vascular Biology, Baker Medical Research Institute, Commercial Road, Prahran, Victoria, Australia

P-selectin glycoprotein ligand-1 (PSGL-1) is the high affinity counter-receptor for P-selectin on myeloid cells (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). Here we demonstrate that PSGL-1 is also widely distributed on T- and B-lymphocytic tumor cell lines, resting peripheral blood T and B cells, and on stimulated peripheral blood T cell and intestinal intraepithelial lymphocyte (IEL) lines. However, the majority of PSGL-1-positive resting peripheral blood lymphocytic cells and lymphoid tumor cell lines do not display significant P-selectin binding. In contrast, *in vitro* stimulated peripheral blood T cell and IEL lines avidly bind P-selectin, and PSGL-1 is the sole high affinity counter-receptor mediating this binding. During the course of *in vitro* stimulation, cell surface expression levels of PSGL-1 do not change as P-selectin binding increases. Rather, the activities of two glycosyltransferases reportedly involved in the production of functional PSGL-1 in myeloid cells are substantially higher in the stimulated T-lymphocytic lines than in resting T lymphocytes, consistent with the hypothesis that activation-dependent post-translational events contribute to the expression of functional PSGL-1 on lymphocytes.

The selectin family of adhesion molecules participates in the initial stages of leukocyte extravasation by tethering cells to the vascular endothelium (1). L-selectin, constitutively expressed on myeloid cells and the majority of lymphocytes, mediates cell binding to counter-receptors displayed on activated endothelia and high endothelial venules. Two other members of the selectin family, E- and P-selectin, are expressed on activated endothelia and are recognized by various leukocyte populations including neutrophils and monocytes (2) as well as by subpopulations of lymphocytes (3–8).

Selectin-mediated interactions have been studied frequently in the context of acute inflammation. However, the capacity of E- and P-selectin to bind primed and memory lymphocytes suggests that these adhesion molecules may also play a role in cell trafficking during chronic inflammation. E-selectin, for example, binds to memory T cells isolated from normal skin and activated T cells derived from dermatological lesions expressing the cutaneous lymphocyte antigen (4, 6, 9). P-selectin is detected on venules infiltrating chronically inflamed synovial membranes of rheumatoid arthritis (RA)[1] patients (10). Furthermore, T lymphocytes derived from the synovial fluid of RA patients (11) and atopic dermatitis lesions (9) display marked P-selectin binding activity. Damle *et al.* (11) and Rossiter *et al.* (9) have also demonstrated that in contrast to resting T cells, *in vitro* activated T cells avidly bind P-selectin. Therefore, primed T lymphocytes may access chronic inflammatory lesions *in vivo* through selectin-mediated interactions.

Although the counter-receptor for P-selectin on stimulated T lymphocytes has not yet been fully characterized, recent studies by Alon *et al.* (8) suggest that the T cell ligand is the same as or similar to the myeloid P-selectin ligand. This myeloid glycoprotein, cloned from an HL-60 cDNA library and designated P-selectin glycoprotein ligand -1 (PSGL-1), has undergone extensive characterization (12–14). PSGL-1, a member of the mucin-like selectin counter-receptors (15), is expressed as a homodimer with an approximate molecular mass of 220 kDa and displays multiple sialylated, fucosylated, *O*-linked poly-*N*-acetyllactosaminylated oligosaccharides (16). Both protein and carbohydrate components of myeloid PSGL-1 are necessary for P-selectin binding with the terminal tetrasaccharide sialyl-Lewis^x (SLe^x) comprising a critical epitope for binding (17, 18). Proper post-translational modification of PSGL-1 requires the combined activities of a number of glycosyltransferases which are all expressed in myeloid cells, including Core 2 GlcNAc-transferase, a 2,3-sialyltransferase, and a fucosyltransferase (12, 16).

In the present study we evaluate the expression and function of PSGL-1 in P-selectin binding to cells of lymphocytic lineage, including T- and B-lymphocytic tumor cell lines, resting peripheral blood T and B cells and stimulated peripheral blood T cell, and intestinal intraepithelial lymphocyte (IEL) lines. Our studies show that although the vast majority of lymphocytes express cell surface PSGL-1, they display considerable varia-

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§ To whom correspondence should be addressed. Tel.: 617-498-8919; Fax: 617-498-8993.

[1] The abbreviations used are: RA, rheumatoid arthritis; PSGL-1, P-selectin glycoprotein ligand-1; IEL, intestinal intraepithelial lymphocyte; sLe^x, sialyl Lewis x; Core 2, Core 2 β(1, 6)GlcNAc transferase; CHO, Chinese hamster ovary; CHO-PACE SOL, soluble PACE secreting CHO-cells; PACE, paired basic amino acid converting enzyme; EBV, Epstein-Barr virus; PBMC, peripheral blood mononuclear cells; IL, interleukin; mAb monoclonal antibody; FITC, fluorescein isothiocyanate; Lecγ1, P-selectin IgG1 Fc chimera; Ig, immunoglobulin; FACS, fluorescent-activated cell sorter; HBSS, Hanks' balanced salt solution; BCECF-am, 2′,7′,-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetomethyl ester; BSA, bovine serum albumin; PIPES, 1,4-piperazinediethenesulfonic acid; PA, protein A; PAGE, polyacrylamide gel electrophoresis; MOPS, 4-morpholinepropanesulfonic acid; α1,3-FT, α(1,3)-fucosyltransferase.

bility in P-selectin binding; only stimulated peripheral blood T cell and IEL lines show significant binding to P-selectin. The studies reported here further establish that PSGL-1 is the predominant counter-receptor for P-selectin on stimulated T-lymphocytic lines. We hypothesize that PSGL-1 undergoes cell activation-associated post-translational modifications which enable high affinity binding to P-selectin. Consistent with this hypothesis, we observe that the activities of certain glycosyltransferases are significantly higher in stimulated T cells than in resting T lymphocytes.

EXPERIMENTAL PROCEDURES

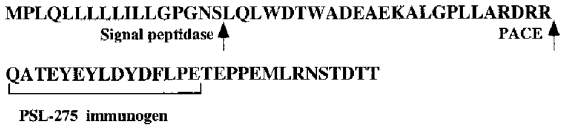
Cells and Cell Lines—The tumor cell lines MOLT-4 (CRL 1582), CEM (TIB 195), THP-1 (TIB 202), HUT 78 (TIB 161), U-937 (CRL 1593), WIL2-NS (CRL 8155), Ramos (CRL 1596), HL-60 (CCL 240), and CCRF-SB (CCL 120) were obtained from the ATCC (American Type Culture Collection, Bethesda, MD) and maintained in RPMI 1640, 10% fetal calf serum (Sigma).

Chinese hamster ovary cells deficient in dihydrofolate reductase (CHO-DUKX) and stable transfectants of CHO-P-selectin cells were maintained as described previously (17). Stable transfectants of CHO-PACE SOL cells expressing a soluble form of paired basic amino acid converting enzyme (PACE; 19) were maintained in modified 11-AAU media as described previously (20). PSGL-1 contains a cleavage site for PACE (12) evinced by the tetrapeptide consensus sequence RXRR (19). For co-culture experiments with lymphocytic cells, moderately confluent CHO-PACE SOL cells or CHO-DUKX cells were trypsinized, diluted 1:3, and plated in modified 11-AAU media. Shortly thereafter, 2×10^6 lymphocytic cells (*i.e.* B cell lines, CEM, or peripheral blood B cells) were added per 100-mm tissue culture plate, and these co-cultures were maintained overnight (except for peripheral blood B cells which were co-cultured for 4 h).

A peripheral blood T cell line was established by repeated allostimulation with an Epstein Barr virus-transformed cell line JY. A human CD8+, TCRα'62+ IEL line, IEL line A, was kindly provided by Richard S. Blumberg (Brigham and Women's Hospital, Boston, MA) and Steven P. Balk (Beth Israel Hospital, Boston, MA) and was established from normal jejunum (21). For the present study, both the T cell and IEL lines were stimulated bi-weekly with fresh irradiated human peripheral blood mononuclear cells (PBMC) from either whole blood or from a leukopak, 1 μg/ml purified phytohemagglutinin (Murex Diagnostics, Dartford, United Kingdom), 10 units/ml interleukin 2 (IL-2), and 10 units/ml interleukin 4 (IL-4) in 10% human AB+ serum (BioCell, Rancho Domingo, CA) in RPMI and subsequently fed with IL-2 and IL-4. Cells were analyzed for PSGL-1 expression and P-selectin binding near the end of the proliferative phase of the stimulation cycle. Cell viability was assessed prior to analyses by Trypan Blue (Sigma) exclusion and found to be routinely >95%.

PBMC were isolated from heparinized blood of normal donors by centrifugation over a Ficoll/Hypaque (Sigma) density gradient. B cells were enriched by adherence (22) to nylon wool (Wako BioProducts, Richmond, VA), and T cells were purified by adherence to anti-CD3 antibody immobilized on plastic (AIS MicroCELLector, Applied Immune Sciences, Inc, Santa Clara, CA). Short term (7 day) cultures of freshly isolated PBMC were maintained with 10 units/ml of IL-2 and with 0.1% phytohemagglutinin for the first 48 h.

Proteins—The anti-PSGL-1 polyclonal antiserum Rb3026 was generated by immunizing rabbits with a recombinant form of soluble PSGL-1 (sPSGL-1.T7) expressed in COS cells as described previously (12). Affinity purified Rb3026 antibody was prepared according to standard procedures (23) using sPSGL:Fc (a chimera of the extracellular portion of PSGL-1 and the Fc portion of human IgG_1) coupled to Affi-Gel (Bio-Rad). The anti-PSGL-1 murine monoclonal antibody (mAb) PSL-275 (IgG_1 isotype) was raised against a 15 amino acid peptide corresponding to the NH_2 terminus of mature (PACE-cleaved) PSGL-1 (Scheme I). Anti-sialyl Le^x mAb CSLEX-1 (mouse IgM) was obtained as ascites from the ATCC. FITC-labeled HECA-452 (rat IgM; 24) and a rat IgM control was generously provided by Dr. Louis J. Picker, University of Texas Southwestern Medical Center, Dallas, TX. Phycoerythrin-conjugated mAbs directed against CD3, CD14 and CD19 were purchased from Becton Dickinson. Isotype control mAbs were purchased from Fisher. The P-selectin-IgG chimera (Lecγ1) was produced by fusing the extracellular portion of P-selectin and the Fc portion of human IgG_1 as described previously (12). The protease mocarhagin was isolated from venom of the cobra, *Naja mocambique*



SCHEME I.

mocambique as described elsewhere.[2]

Flow Cytometry—Flow cytometric analyses were performed on a Becton Dickinson FACScan (Mountain View, CA). Cell surface antigens were detected as follows. Between $0.5–1.0 \times 10^6$ cells were washed in FACS buffer (RPMI, 2% fetal calf serum, 0.1% NaN_3), incubated with primary antibody for 30 min on ice, washed, and incubated with FITC-conjugated goat $F(ab')_2$ anti-mouse Ig antibody (Boehringer Mannheim) for an additional 30 min. For dual parameter flow cytometric analyses of PBMC, FITC-stained cells were subsequently incubated with phycoerythrin-conjugated mAbs.

P-selectin binding was detected by incubating cells for 30 min on ice with 4 μg Lecγ1 pre-complexed with protein A-FITC (Zymed, San Francisco, CA) in a 2:1 molar ratio. Parallel samples were incubated with protein A-FITC complexes of $huIgG_1$ at the same molar ratio. All stained cells were fixed in Hanks' balanced salts solution (HBSS) containing 1% formalin and analyzed by FACS. Prior to analyses, the viability of cell samples was assessed by Trypan Blue (Sigma) exclusion. During FACS analysis, dead cells and debris were excluded by appropriate scatter gates.

P-selectin Adhesion Assay—Adhesion assays were performed as described previously (17) with the following modifications. Stable CHO-P-selectin cells were plated in 48-well plates at 2×10^4 cells/well 24 h prior to assay. Tumor cells, peripheral blood, or intestinal lymphocytes, labeled with BCECF-am (2′,7′,-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; Molecular Probes, Eugene, OR) were added at a density of 2×10^5 cells/well and allowed to bind to CHO-P-selectin cells for 10 min in ice-cold HBSS on a rotating platform. Non-adherent cells were washed away, and adherent cells were quantitated using a microplate fluorometer (model 7620, Cambridge Technology Inc. Watertown, MA). In experiments testing the neutralizing activity of polyclonal antibody Rb3026, fluorescent-labeled cells were preincubated with Rb3026 serum or non-immune serum or with affinity purified Rb3026 on ice for 20–30 min, washed, and then added to adherent CHO-P-selectin cells. The effect of the mocarhagin protease was assessed by incubating fluorescent-labeled cells with 5 μg/ml mocarhagin in HBSS containing 2% fetal calf serum and 1 mg/ml bovine serum albumin (BSA) for 20 min at 37 °C. The cells were washed in HBSS and presented to adherent CHO-P-selectin cells as described above.

Northern Blot Analysis—RNA was extracted from tumor cell lines and analyzed on Northern blots as described previously (12, 25). Nitrocellulose filters were hybridized under stringent conditions with a ^{32}P-labeled cDNA probe for PSGL-1 comprising nucleotides 60–389 (12). Filters were subsequently stripped and rehybridized with a ^{32}P-labeled cDNA probe for actin.

Analysis of ^{35}S-labeled P-selectin Counter-receptor(s) on Stimulated Peripheral Blood T Cell and IEL Lines—The peripheral blood T cell and IEL lines were metabolically labeled with 1 mCi of [^{35}S]methionine (DuPont NEN) per 2×10^7 cells for 4 h in methionine-free MEM (ICN FLOW), washed with phosphate-buffered saline, resuspended in lysis buffer (10 mM PIPES, pH 7.5, 100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM ethylmaleimide, 2 mM benzamidine, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, and 1 μg/ml aprotinin) and lysed by sonication. Lysates were centrifuged at low speed (10 min at $500 \times g$) to remove the nuclear fraction and then at high speed (1 h at $100{,}000 \times g$) to isolate the membrane fraction. The high speed pellet was solubilized in 1% Thesit in lysis buffer supplemented with protease inhibitors for 30–45 min on ice and diluted 1:4 with IP buffer (Tris-buffered saline (TBS) pH 8.0, 5 mg/ml BSA, 2 mM $CaCl_2$). Nonspecific binding proteins and P-selectin binding proteins were precipitated from soluble membrane extracts by sequential incubation (24 h at 4 °C) with protein A (PA)-Sepharose beads precomplexed with $HuIgG_1$ and Lecγ1, respectively. The Sepharose beads were removed by centrifugation, washed twice with IP buffer, twice with IP buffer containing 0.5% Triton X-100, and twice with TBS. Membrane proteins affinity captured by $HuIgG_1$ and Lecγ1 were eluted from the beads with 5 mM EDTA. These eluates were

[2] C. M. Ward, R. K. Andrews, D. V. Vinogradov, and M. C. Berndt, manuscript in preparation.

either precipitated with trichloroacetic acid and analyzed directly by SDS-PAGE on 8% gels, or reprecipitated with Rb3026 or preimmune IgG precomplexed with PA-Sepharose, or recalcified and reprecipitated with Lecγ1 precomplexed with PA-Sepharose.

Construction of cDNAs for Soluble PSGL-1 Mutants—Two mutants of PSGL-1 with alterations in the PACE consensus sequence were generated from a previously described construct of soluble PSGL-1(pED.sPSGL-1.T7) (12) by site directed mutagenesis. In one construct, a point mutation was introduced at codon 61, substituting an alanine for an arginine and in the other, the recognition site for enterokinase cleavage (DDDDK) was substituted for the PACE cleavage site RDRR. The constructs were validated by DNA sequencing.

Glycosyltransferase Assays—Cell lysates were prepared from resting peripheral blood T cells and from the stimulated peripheral blood T cell and IEL lines as follows. Cells were washed with phosphate-buffered saline and lysed in 100 mM sodium cacodylate, pH 6.0, 150 mM NaCl, 25% glycerol, and 1% Nonidet P-40 (150 μl/10^7 cells) on ice for 15 min. Cell debris was removed by centrifugation, and the supernatants were stored at −80 °C. Protein concentrations were determined using the BCA Protein assay (Pierce).

The α(1,3)-fucosyltransferase assay was performed essentially as described previously (26). Briefly, in a 50-μl reaction volume 30–100 μg of cell lysate protein was incubated with 50 mM MOPS buffer, pH 7.0, 5 mM $MnCl_2$, 100 mM NaCl, 2 nmol of GDP-[^{14}C]fucose (20,000 counts/min/nmol; Sigma) and 1 μmol of the acceptor substrate LacNAc (Sigma) for 1 h at 37 °C. The reaction was stopped by the addition of 1 ml of ice-cold water, applied to a 1-ml column of Dowex 1X4 (Cl-form, Bio-Rad). The radiolabeled product was eluted with 3 ml of water and counted in a scintillation counter.

The Core 2 transferase assay was performed as described by Higgins *et al.* (27). Briefly, in a 50-μl reaction volume 30–100 μg of cell lysate protein was incubated in 50 mM sodium cacodylate, pH 6.7, 0.1% Triton X-100, 0.1% BSA, 0.1 M GlcNAc, 10 nmol UDP-[^{3}H]GlcNAc (20,000 counts/min/nmol) and 2 mM βD Gal(1–3) αD-GalNAc-P-nitrophenol (Sigma) as a substrate for 1 h at 37 °C. The reaction was terminated by the addition of 1 ml of ice-cold water and applied to a C18 Sep-pak column (Water-Millipore). The column was washed with 5% acetonitrile and the product eluted with 20% acetonitrile and counted in a scintillation counter. All assays were carried out in duplicate. Control reactions carried no substrate, and specific activity was determined as pmol/min/mg.

RESULTS

PSGL-1 Is Expressed by T- and B-lymphocytic Tumor Cell Lines—The expression of PSGL-1 transcripts by lymphocytic cell lines was examined by Northern blot analysis. The 2.5-kilobase PSGL-1 transcript previously detected in myeloid cell lines (HL-60, U-937, and THP-1) (12) is expressed in cells of T-lymphocytic (CEM, MOLT-4, and HUT 78) and B-lymphocytic (Ramos, SB) lineage. A third B cell line WIL2-NS expressed detectable but much lower levels of this 2.5-kilobase transcript, while a human hepatoma line HepG2 did not express detectable PSGL-1 mRNA (Fig. 1).

Cell surface expression of PSGL-1 in lymphoid cell lines was assessed using PSL-275, a monoclonal anti-PSGL-1 antibody that was raised against a 15 amino acid peptide of PSGL-1 (Scheme I). This peptide juxtaposes a PACE cleavage site, defined by the consensus sequence RXRR (19). Fig. 2*A* shows that soluble PSGL-1 (WT) expressed in COS cells could be immunoprecipitated by PSL-275. However, when PACE processing of recombinant PSGL-1 was prevented by either disrupting the consensus sequence with a point mutation (R61) or substituting RDRR with the consensus sequence for enterokinase cleavage (EK), PSL-275 was unable to bind PSGL-1. In contrast, a polyclonal anti-PSGL-1 antibody Rb3026 was able to immunoprecipitate both the wild type and mutant forms of PSGL-1. These data thus show the specificity of PSL-275 for PACE processed PSGL-1.

Flow cytometric analysis of PSL-275 binding by lymphocytic cell lines revealed that greater than 90% of T-lymphocytic cells bound PSL-275 although the mean fluorescence intensities varied among the different T cell lines; CEM and MOLT-4 cells were comparable to myeloid cells while HUT 78 was approximately half a log-fold brighter (Fig. 2*B*). In contrast, the B-lymphocytic cell lines did not appreciably bind PSL-275. Given the specificity of this monoclonal antibody for PACE-cleaved PSGL-1, we speculated that the inability of the B cell lines to bind PSL-275 was due to expression of unprocessed PSGL-1. Indeed, following overnight co-culture of B cell lines with PACE-secreting CHO cells (CHO-PACE SOL; 20), greater than 95% of SB and Ramos bound PSL-275 (Fig. 2*C*) in amounts comparable to myeloid cells (Fig. 2*B*), while simultaneous co-culture with the parental cell line CHO-DUKX had no effect on PSL-275 binding. In contrast to Ramos and SB, co-culture of WIL2-NS with CHO-PACE SOL cells only minimally affected PSL-275 binding (Fig. 2*C*). However, low cell surface expression of PSGL-1 on WIL2-NS is consistent with the low level of PSGL-1 mRNA expressed in this cell line (Fig. 1). The T cell line CEM, which constitutively expresses PSL-275-reactive PSGL-1, was not affected by co-culture with CHO-PACE SOL cells (data not shown).

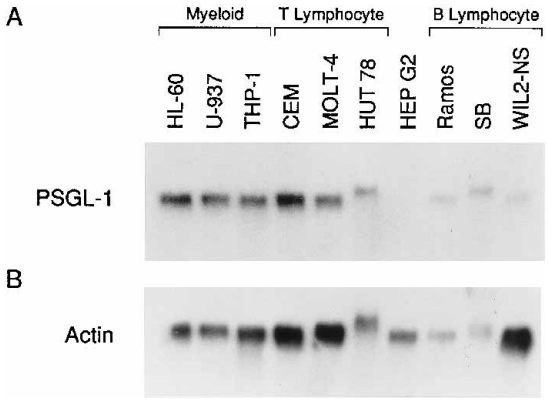


FIG. 1. **Northern blot analysis of PSGL-1 mRNA**. Blots containing RNA prepared from HL-60, U-937, THP-1, CEM, MOLT4, HepG2, Ramos, and WIL2-NS (3 μg of poly(A)$^+$ RNA/lane) and from HUT-78 and SB (10 μg of total RNA/lane) were hybridized with a ^{32}P-labeled probe comprising nucleotides 60–389 of PSGL-1 (*A*) or with a probe for actin (*B*).

Surface Expression of PSGL-1 on Lymphocytic Tumor Cell Lines Does Not Correlate with P-selectin Binding—The observation that the majority of lymphoid cell lines constitutively express cell surface PSGL-1 led us to assess the P-selectin binding capacity of these cells. Binding activity of each cell line was measured using both flow cytometry and a cell adhesion assay as described under "Experimental Procedures." FACS analysis revealed that the T cell lines CEM and MOLT-4 bound very low levels of Lecγ1 (soluble P-selectin-IgG chimera), whereas Lecγ1 binding by HUT 78 cells was heterogeneous (Fig. 3*A*). Lecγ1 was not bound appreciably by B-lymphocytic cell lines (Fig. 3*A*) even after co-culture with CHO-PACE SOL cells (data not shown).

The adherence of lymphocytic tumor cell lines to CHO cells expressing P-selectin corroborated the flow cytometry data (Fig. 3*B*). Among the lymphocytic cell lines, HUT 78 displayed the most adherence to CHO-P-selectin cells reflecting perhaps the minor population of HUT 78 cells, identified by flow cytometric analysis, that bind higher levels of Lecγ1 (Fig. 3*A*). Nevertheless, all interactions between HUT 78 and P-selectin were much less pronounced than those between myeloid cells and P-selectin even though HUT 78 cells express more PSGL-1 protein (Fig. 2*B*). Therefore, despite the apparently adequate expression of cell surface PSGL-1, the majority of lymphocytic tumor cell lines display insignificant P-selectin binding activity relative to myeloid cells.

Peripheral Blood Lymphocytes Express Cell Surface PSGL-1 but the Majority Do Not Bind P-selectin—Cell surface expres-

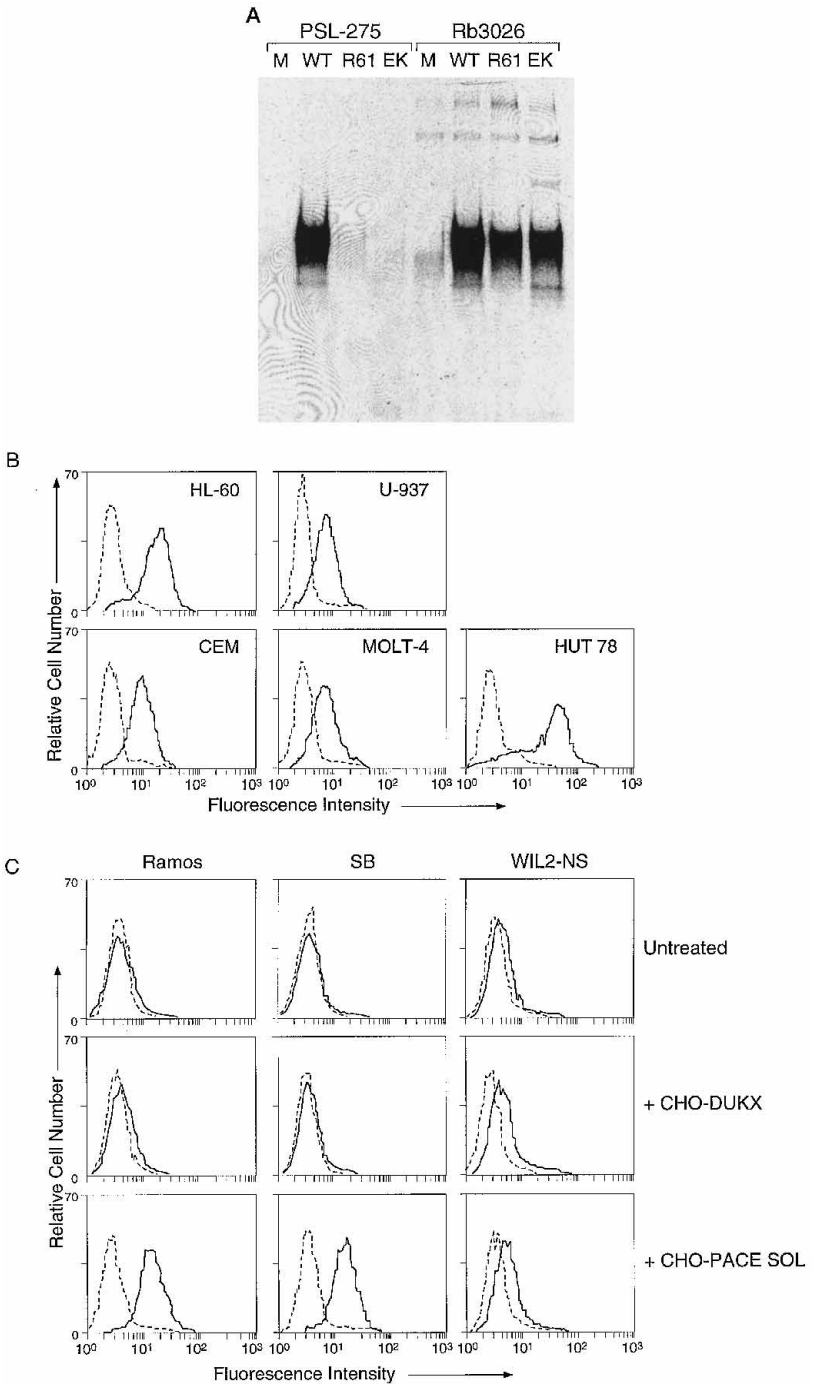


FIG. 2. **PSL-275 recognition of recombinant, myeloid, and lymphocytic PSGL-1.** *A*, [^{35}S]methionine-labeled conditioned media from "mock" transfected COS cells (*M*) or COS cells expressing recombinant soluble PSGL-1 (*WT*) or PACE mutants of soluble PSGL-1 (*R61* and *EK*) were immunoprecipitated with PSL-275 and Rb3026. Immunoprecipitates were separated on SDS-PAGE under reducing conditions and analyzed by autoradiography. *B* and *C*, cell surface PSGL-1 expression was determined by flow cytometric analyses, measuring the binding of PSL-275 (—) or control murine IgG_1 (- - -) followed by FITC-conjugated anti-murine Ig antibody myeloid cell lines HL-60 and U-937 and T-lymphocytic cell lines CEM, MOLT-4 and HUT 78 (*B*), and B-lymphocytic cell lines Ramos, SB, and WIL2-NS cultured for 18 h with media alone (untreated) (*C*), or co-cultured with CHO-DUKX or CHO-PACE SOL cells.

sion of PSGL-1 protein and P-selectin binding by resting peripheral blood T and B lymphocytes were subsequently examined. Dual parameter flow cytometric analysis of PBMC revealed that PSGL-1 expression on resting peripheral blood B lymphocytes differs from the B-lymphocytic cell lines SB and Ramos. Between 30–50% of CD19+ peripheral blood B cells bound PSL-275 (Fig. 4*A*). A 4 h co-culture period of resting B cells with CHO-PACE SOL cells had no effect on either the percentage or fluorescence intensity of PSL-275-reactive B cells (data not shown). SB cells similarly co-cultured for 4 h showed a marked increase in PSL-275 binding. Attempts to measure P-selectin binding by B cells using flow cytometry were hampered by high nonspecific binding of protein A-FITC. Therefore, the cell-based adhesion assay was used exclusively to assess B cell/P-selectin interactions. As observed with the B cell tumor lines, the binding of resting B cells to adherent CHO-P-selectin cells was insignificant (data not shown).

The expression of PSGL-1 on resting peripheral blood CD3+ T cells was similar to CD14+ monocytes. The majority of both these cell populations bound PSL-275 (Fig. 4*A*), and expression levels of PSGL-1, as measured by the mean fluorescence intensities of PSL-275-reactive CD3+ and CD14+ cells, were comparable (data not shown). However, in contrast to monocytes, only a small percentage (less than 20%) of CD3+ T cells bound Lecγ1 (Fig. 4*B*). Thus, analogous to the T cell lines CEM and MOLT-4, the majority of resting CD3+ T cells do not display marked P-selectin binding despite adequate expression of PSGL-1 at the cell surface.

Short term propagation of peripheral blood T cells *in vitro* led to augmented P-selectin binding activity. Between Day 0 and Day 7 of *in vitro* culture, the percentage of T cells binding Lecγ1 increased from less than 20% to approximately 50% (data not

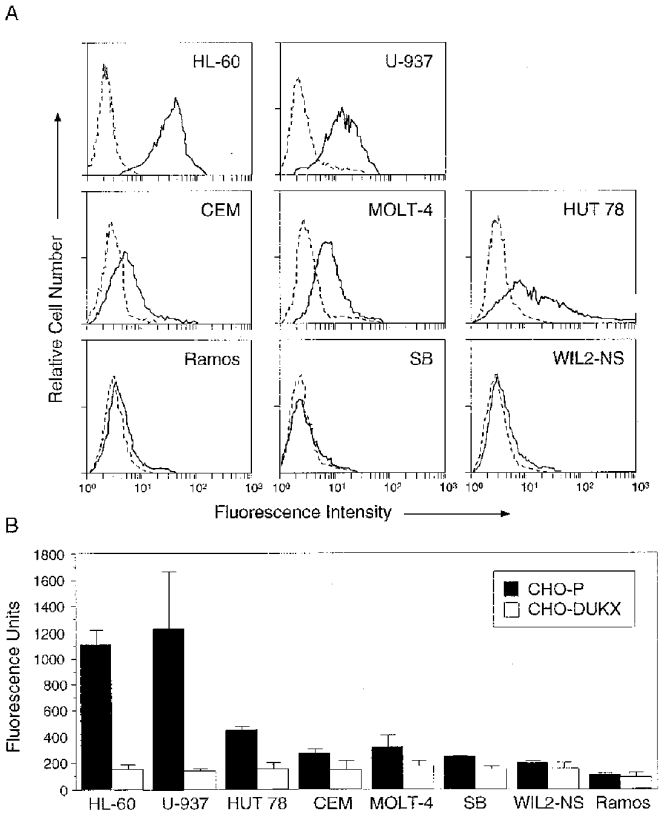


FIG. 3. **P-selectin binding by myeloid and lymphocytic cell lines.** *A*, flow cytometric analysis of myeloid cell lines HL-60 and U-937, T cell lines CEM, MOLT-4, and HUT 78 and B cell lines, Ramos, SB and WIL2-NS reacted with the P-selectin-IgG chimera Lecγ1 (—) or control human IgG_1 (- - -) precomplexed with Protein A-FITC. *B*, cell adhesion of fluorescent-labeled tumor cells to adherent CHO-DUKX or CHO-P-selectin cells. Bound cells were quantitated using a microplate fluorometer. The level of fluorescence intensity per cell was similar for all tumor cell lines tested.

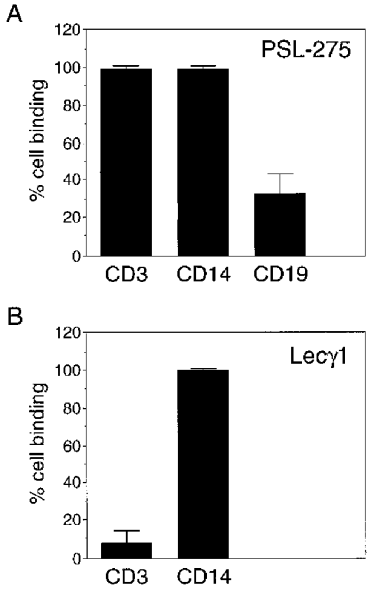


FIG. 4. **Flow cytometric analysis of PSGL-1 expression and P-selectin binding by subpopulations of resting peripheral blood mononuclear cells.** *A*, PBMC were incubated with mAb PSL-275, followed by FITC-conjugated anti-murine Ig antibody. The samples were subsequently reacted with phycoerythrin-conjugated antibodies against the cell-surface markers CD3 (T cells), CD14 (monocytes) or CD19 (B cells). *B*, PBMC were incubated with Lecγ1 precomplexed with Protein A-FITC, followed by incubation with phycoerythrin-conjugated anti-CD3 or anti-CD14 antibodies. Samples were analyzed by dual parameter flow cytometry. The data represent the mean of five samples from different donors.

shown). Surprisingly, however, this increment in binding activity was not accompanied by a change in the mean fluorescence intensity of PSL-275-reactive T cells (data not shown) indicating that PSGL-1 expression levels remain constant during stimulation.

In Vitro Stimulation of Peripheral Blood T Lymphocyte and IEL Lines Increases P-selectin Binding While Cell Surface Expression of PSGL-1 Remains Constant—To further evaluate the lack of correlation between P-selectin binding and PSGL-1 expression during T cell stimulation, T cells of two diverse ontogenies, peripheral blood T cells and IELs, were propagated *in vitro* for several weeks. Binding of Lecγ1 and PSL-275 was assessed on days 10, 24, and 38. Fig. 5*A* shows that the percentage of peripheral blood T lymphocytes binding Lecγ1 increased from 50% on day 10 to greater than 97% by day 38 while the majority of IELs consistently displayed Lecγ1 binding throughout this period. In both T cell populations, the mean fluorescence intensity of Lecγ1-binding cells increased by at least one order of magnitude. Yet, despite this marked increment in P-selectin binding by both IELs and peripheral blood T cells, expression levels of cell surface PSGL-1 did not increase during the course of stimulation. Fig. 5*B* shows that the mean fluorescence intensity of PSL-275-reactive cells did not vary appreciably over time in culture.

The Major High Affinity Counter-receptor for P-selectin on Stimulated Peripheral Blood T Cell and IEL Lines Is Immunocross-reactive with PSGL-1—The increase in P-selectin binding activity in the absence of increased PSGL-1 expression led us to hypothesize that during the process of lymphocyte activation, either PSGL-1 is functionally up-regulated or another ligand for P-selectin is induced. To address these issues, counter-receptors for P-selectin from stimulated T cell membranes were characterized by their electrophoretic mobility on SDS-PAGE, reactivity with anti-PSGL-1 antibodies, and sensitivity to a protease which cleaves PSGL-1 and abrogates binding to P-selectin.

SDS-PAGE analysis of the membrane counter-receptor for P-selectin affinity captured by immobilized Lecγ1 from [^{35}S]methionine-labeled IELs and T cells (on day 38 of *in vitro* culture), identified a single major component with the electrophoretic characteristics previously observed for myeloid PSGL-1 (12). That is, the major counter-receptor for P-selectin on both stimulated peripheral blood T cells and IELs is a homodimeric protein of ~220 kDa under non-reducing (Fig. 6*A*) and ~110 kDa under reducing conditions (Fig. 6*B*). The molecular species precipitated by Lecγ1 from IELs does, however, have a slightly retarded electrophoretic mobility relative to peripheral blood T cells. Examination of these two cell populations at earlier points during stimulation revealed the same distinction in electrophoretic mobility of the single predominant molecular species captured by Lecγ1 (data not shown). Nevertheless, a polyclonal anti-PSGL-1 antibody Rb3026, (but not preimmune antibody), was able to reprecipitate the Lecγ1 affinity captured material from both stimulated IELs (Fig. 6*C*) and peripheral blood T cells (data not shown) indicating that the P-selectin counter-receptor of both T cell populations is immunocross-reactive with PSGL-1.

The significance of differences in the electrophoretic mobilities of IEL and peripheral blood T cell PSGL-1 is not clear. Such differences are also evident among other cell lines. For example, the PSGL-1 of the T cell line CEM co-migrates with that of U-937, while the PSGL-1 of the B cell line SB has a notably higher molecular weight (data not shown). Yet, the data in Fig. 3 indicate that neither CEM nor SB markedly bind P-selectin

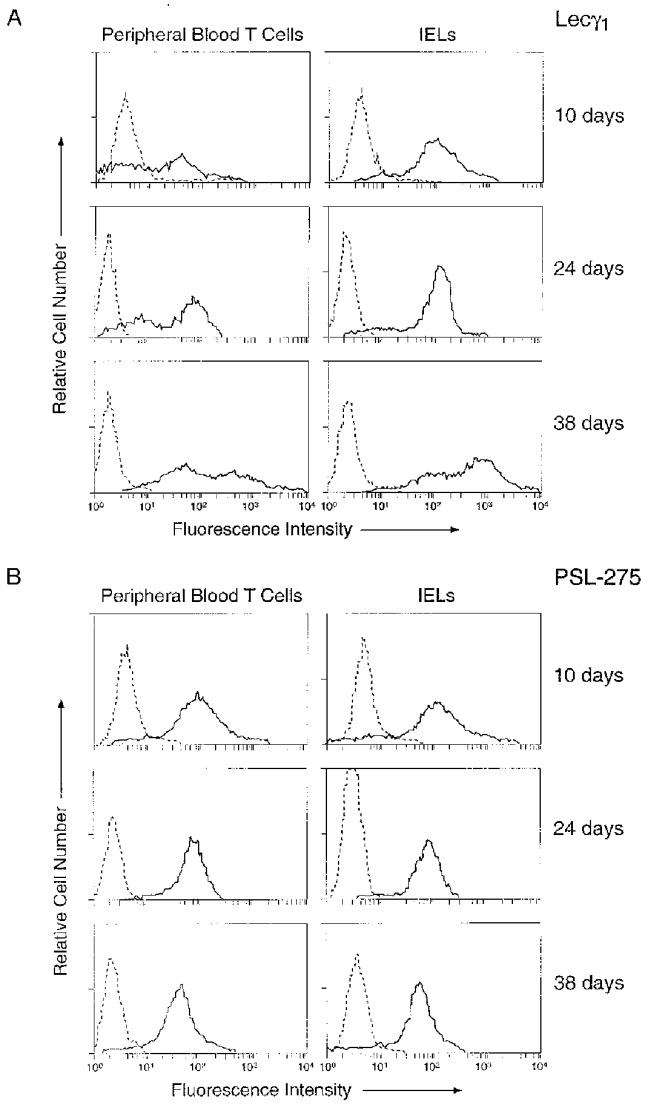


FIG. 5. **Flow cytometric analysis of P-selectin binding and PSGL-1 expression of *in vitro* stimulated peripheral blood T cell and IEL lines.** Peripheral blood T lymphocyte and IEL lines were propagated and stimulated *in vitro* as described under "Experimental Procedures." At 10, 24, and 38 days, cells were withdrawn from culture and incubated with Lecγ1 (—) or control human IgG_1 (- - -) precomplexed with Protein A-FITC (*A*) or mAb PSL-275 (—) or control murine IgG_1 (- - -) (*B*).

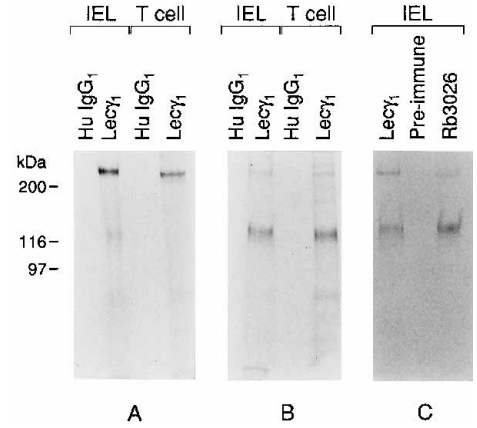


FIG. 6. **Analysis of the counter-receptor for P-selectin on *in vitro* stimulated T lymphocytes and IELs.** IELs and T cells were stimulated in culture for 38 days as described under "Experimental Procedures." [^{35}S]Methionine-labeled IEL and T cell membrane proteins affinity precipitated with Lecγ1 (or control human IgG_1) were eluted with EDTA and analyzed by SDS-PAGE under non-reducing (*A*) or reducing conditions (*B*). *C*, Lecγ1 affinity precipitated proteins from ^{35}S-labeled IEL membranes were eluted with EDTA, recaptured with Lecγ1, preimmune rabbit IgG, or the anti-PSGL-1 polyclonal antibody Rb3026 and analyzed by SDS-PAGE under reducing conditions.

suggesting, therefore, that there is no direct correlation between the electrophoretic mobility of PSGL-1 and its ability to mediate P-selectin binding.

To demonstrate that the P-selectin binding of IELs and T cells is mediated by PSGL-1 expressed at the cell surface, intact lymphocytes were treated with a metalloprotease displaying narrow substrate specificity. Mocarhagin, a protease derived from the cobra *N. mocambique mocambique* cleaves specifically near the amino terminus of mature PSGL-1 as the sole apparent proteolytic event on neutrophils and HL-60 cells.[3] This cleavage results in the loss of both P-selectin binding and the PSL-275-reactive epitope. Thus, binding to CHO-P-selectin cells by mocarahgin-treated HL-60 cells, stimulated peripheral blood T cells, and IELs was reduced to 4, 9, and 6%, respectively, that of untreated cells. Similarly, binding of PSL-275 by these cells, as measured by flow cytometry, was reduced to that of the isotype control (3–5%) following proteolysis by mocarhagin. The comparable behavior displayed by myeloid and lymphocytic cells in response to mocarhagin treatment further supports the finding that binding to P-selectin is mediated by the same cell surface ligand in both cell types. Thus, the increased P-selectin binding activity that accompanies lymphocyte activation is associated with an increase in PSGL-1 functionality rather than with the expression of another novel P-selectin ligand.

Glycosyltransferase Activity in in Vitro Stimulated T-lymphocytic Cell Lines Differs from Resting T Cells—While several mechanisms might account for the up-regulation of PSGL-1 binding to P-selectin, the time course for acquiring P-selectin reactivity in T cells suggests altered post-translational modifications of PSGL-1 during lymphocyte activation. Appropriate glycosylation of PSGL-1 is known to be essential for binding to P-selectin (12, 14), and two critical glycosyltransferases have been implicated: a fucosyltransferase capable of generating Lewis x-type structures (12) and Core 2 transferase[4] (16). Thus, the activities of these two transferases were measured in resting T cells and in stimulated peripheral blood T cell and IEL lines over time in culture. Both transferases were elevated above those of resting T cells throughout the period of stimulation. Table I compares the activities of resting T cells with those of stimulated T-lymphocytic cell lines and shows that in contrast to stimulated T lymphocytes, resting T cells express no detectable Core 2 transferase activity. Also, fucosyltransferase activity, which is low in resting T cells, is increased 5-fold in stimulated T lymphocytes. Thus, increased P-selectin binding activity by T lymphocytes coincides with increased activities of fucosyltransferase and Core 2 transferase. However, although these observations are intriguing, a causal relationship between PSGL-1 function and transferase activity cannot be established from these data alone.

Even if these transferases are involved in lymphocyte PSGL-1 modification, the precise nature of the carbohydrate structures that confer P-selectin binding activity upon stimulated T cell PSGL-1 remains unclear. Monoclonal antibodies such as CSLEX-1 and HECA-452, which recognize the SLe^x

[3] M. C. Berndt, L. C. Dunlop, M. De Luca, J. Flannery, R. Ettling, D. A. Cumming, and G. M. Veldman, manuscript in preparation.

[4] R. Kumar, R. Camphausen, and D. A. Cumming, manuscript in preparation.

TABLE I
α(1,3)-Fucosyltransferase and Core 2 β(1,6)GlcNAc transferase activities in resting and stimulated T cells and IELs

The activity of each transferase was measured as described under "Experimental Procedures." The specific activities represent the mean and standard deviations of duplicate readings from freshly isolated T cells of three donors and from stimulated T lymphocytic cell lines on day 38 of *in vitro* culture.

Cells	α1,3 FT activity	Core 2 transferase activity
	pmol/min/mg	
Resting peripheral blood T cells	8 ± 5	Not detectable
Stimulated peripheral blood T cell line	45 ± 11	10 ± 2
Stimulated IEL line	41 ± 4	15 ± 3

TABLE II
Effect of polyclonal anti-PSGL-1 antibody Rb3026 on stimulated T cells, IELs, and HL-60 cells binding to CHO-P-selectin cells

Fluorescent-labeled lymphocytes or HL-60 cells were preincubated with Rb3026 and added to monolayers of CHO-P-selectin cells as described under "Experimental Procedures." Adherent cells were quantitated using a microplate fluorometer.

Antibody	% cells binding to CHO-P-selectin cells		
	Stimulated IEL line	Stimulated peripheral blood T cell line	HL-60
Non-immune rabbit serum	100 ± 15	100 ± 17	100 ± 12
Rb3026 serum 1:15 dilution	79 ± 21	72 ± 21	20 ± 3
Rb3026 affinity purified			
14 μg/ml	82 ± 8	ND[a]	32 ± 3
28 μg/ml	85 ± 9	ND	ND
56 μg/ml	87 ± 15	ND	ND
104 μg/ml	62 ± 9	ND	ND

[a] Not determined.

epitope on myeloid cells, do not bind appreciably to stimulated T lymphocytes (data not shown). Furthermore, the anti-PSGL-1 polyclonal antibody Rb3026 which neutralizes myeloid PSGL-1 binding activity, recognizes lymphocyte PSGL-1 but does not block the binding of T cell lines to P-selectin. Table II shows that preincubation of HL-60 cells with Rb3026 markedly reduces their binding to CHO-P-selectin cells while similar treatment of stimulated T- lymphocytic cells has minimal effects even at high concentrations of affinity purified antibody. Since PSGL-1 is the sole high affinity counter-receptor for P-selectin on both myeloid cells (12, 13) and lymphocytes (Fig. 6*C*), the differential ability of Rb3026 to block PSGL-1-mediated binding of P-selectin likely reflects cell-lineage differences in glycosylation of PSGL-1.

DISCUSSION

The present study assessed the expression and function of PSGL-1 on cells of T- and B-lymphocytic lineage and found no direct correlation between PSGL-1 expression and binding to P-selectin. On the contrary, PSGL-1 is expressed on the vast majority of lymphocytic cells while P-selectin binding is evident in only some lymphocytic populations. In the present study only *in vitro* stimulated peripheral blood T cell and IEL lines displayed significant P-selectin binding. PSGL-1 is the major high affinity counter-receptor for P-selectin on these chronically stimulated T lymphocytic cells, and, thus, the functionality of lymphocyte PSGL-1 appears to be activation-dependent.

P-selectin binding by stimulated and memory T lymphocytes has previously been reported by others. For example, Moore and Thompson (3) demonstrated that among the subpopulation of freshly isolated peripheral blood T lymphocytes that bind P-selectin, a significant percentage is of the CD45RO+ memory phenotype. In addition, our data show modest P-selectin binding by the T lymphoma cell line HUT 78. This cell line produces IL-2 constitutively (28), a behavior characteristic of stimulated T cells. The most striking display of stimulation-associated P-selectin binding, however, is seen in T lymphocytes propagated *in vitro,* where the degree of P-selectin binding reflects the extent of stimulation. This binding may be a general consequence of chronic T-cell stimulation as it occurs during both antigen-independent (this report) and alloantigenic (11) stimulation.

The correlation between P-selectin binding and T lymphocyte stimulation supports the notion that PSGL-1 is functionally up-regulated during activation. Such up-regulation also provides an explanation for the lack of correlation between PSGL-1 expression and P-selectin binding among lymphocytic cells in general. Several alternative explanations for this lack of correlation can be excluded based on our data. For example, differences in P-selectin binding do not reflect differences in the density of PSGL-1 at the cell surface, as the expression levels of PSGL-1 on lymphocytes that bind poorly to P-selectin do not differ appreciably from those on myeloid cells. In fact, HUT 78 cells binding PSL-275 stain brighter than PSL-275-reactive HL-60 cells, yet most HUT 78 cells bind less well to P-selectin. Furthermore, the density of PSGL-1 on the surface of peripheral blood T cell and IEL lines undergoing *in vitro* stimulation does not change as P-selectin binding increases.

Another possible explanation for the lack of correlation between PSGL-1 expression and P-selectin binding is that another counter-receptor for P-selectin exists on lymphocytes and is induced during activation. However, analysis of stimulated T cell lines, employing the methodology previously utilized to identify the P-selectin counter-receptor on myeloid cells (*i.e.* affinity capture with immobilized P-selectin from detergent-solubilized cell membranes; 12, 13) revealed that the major high affinity ligand on stimulated peripheral blood T cell and IEL lines displays similar electrophoretic mobility to myeloid PSGL-1 and is recognized by polyclonal anti-PSGL-1 antibodies. It is important to note that this method of affinity capture precludes the detection of low affinity interactions. However, while other proteins may participate in P-selectin binding, PSGL-1 appears to be the sole high affinity counter-receptor for P-selectin on *in vitro* stimulated T lymphocytes.

Given these observations, it is reasonable to postulate that altered post-translational processing of PSGL-1 is responsible for the increase in P-selectin binding during T cell stimulation. Changes in glycosylation seem especially likely since previous studies have demonstrated the importance of appropriate *O*-linked glycosylation of PSGL-1 for P-selectin binding (12, 13). While many glycosyltransferases are involved in the biosynthesis of *O*-linked oligosaccharides, these studies suggested that at least two enzymes are critical: a fucosyltransferase capable of forming sialylated Lewis × (SLe^x) or related carbohydrate epitopes and Core 2 transferase which is required for the addition of blood group antigens such as SLe^x to *O*-linked oligosaccharides (29). Our evaluation of fucosyltransferase and Core 2 transferase activities revealed substantially elevated specific activities of both enzymes in stimulated T-lymphocytic cells. This is in agreement with findings by Piller *et al.* (30), who reported an activation-associated increase of Core 2 transferase activity in lymphocytes. While these observations are consistent with the notion that altered glycosylation regulates the ability of PSGL-1 to bind P-selectin, additional experimentation is clearly required both to further elucidate the nature of activation-associated glycosylation and to explore whether other post-translational modifications play an important role

in modulating the ability of PSGL-1 to bind to P-selectin.

The display of carbohydrates on lymphocyte glycoproteins differs from those on myeloid cells (31, 32). For example, most stimulated T cells do not appreciably bind CSLEX-1 or HECA 452, two monoclonal antibodies which recognize the SLe^x epitope on myeloid glycoproteins. Yet Ohmori *et al.* (31) have shown that these stimulated T cells do express a form of SLe^x readily detectable by another anti-SLe^x mAb designated 2F3. These authors have hypothesized that differences in SLe^x antigenicity between cells of diverse ontogeny may reflect the linkage, length, and modifications of core structures which in turn are determined by the precise repertoire of cellular glycosyltransferases. Thus, while stimulated T lymphocytes and myeloid cells may both express glycosyltransferases involved in SLe^x biosynthesis, the antigenicity of carbohydrate moieties could differ. Additional data consistent with this suggestion come from the differential neutralizing capacity of the polyclonal anti-PSGL-1 antibody (Rb3026). This antibody, which was generated against recombinant soluble PSGL-1 produced by COS cells co-expressing an α(1,3/1,4)FT, effectively blocks myeloid cell binding to CHO-P-selectin cells but had only minimal effects on stimulated peripheral blood T cell or IEL binding. These results differ from those reported by Alon *et al.* (8) who found that Rb3026 significantly blocked P-selectin binding by a chronically stimulated T cell line. While differences in assay conditions may be responsible for these contrasting findings, the most notable distinction is the nature of the T cell lines studied. Alon *et al.* (8) investigated the effects of Rb3026 on a T cell line established from skin lesions of a patient with atopic dermatitis. These T cells bind HECA 452, similar to other skin-homing T-lymphocytes and myeloid cells. Thus, the presentation of carbohydrates expressed on skin T cells appears to differ from the display of oligosaccharides on the peripheral blood T cell and IEL lines examined in the present study. If at least part of the neutralization epitope for Rb3026 is carbohydrate in nature, then the differential effects of this anti-PSGL-1 antibody may reflect differences in oligosaccharide presentation among cells.

Consistent with the contention that T cells from different tissues express diverse carbohydrates, we have noted a distinction in the electrophoretic mobility of PSGL-1 between IELs and peripheral blood T cells. Fig. 6 shows that IEL PSGL-1 migrates at a slightly higher apparent molecular weight than peripheral blood T cell PSGL-1. Both cell lines had been propagated *in vitro* for 38 days at the time of analysis; however, the disparate electrophoretic mobilities of IEL/T cell PSGL-1 are evident irrespective of time in culture (data not shown). Based on these data and observations that the PSGL-1 expressed by the B cell line SB has a higher molecular weight than the PSGL-1 of U937 or the T cell line CEM (data not shown), we postulate that cell lineage-specific post-translational modifications influence the electrophoretic mobility of PSGL-1. However, there is no correlation between PSGL-1's electrophoretic characteristics and its ability to mediate P-selectin binding.

A possible role for PSGL-1 on stimulated and/or memory peripheral blood T cells is suggested by studies that have assessed the distribution of P-selectin in T cell-associated pathologies. P-selectin has been detected on chronically inflamed rheumatoid arthritic synovial endothelium (10) and can be induced on mesenteric lymph node high endothelial venules (33, 34). Potentially then, primed T cells may access synovial tissue or the cortex of lymph nodes through PSGL-1/P-selectin interactions.

It is more difficult to postulate a role for PSGL-1 on IELs. These cells are located at the basolateral surface of the epithelial layer throughout intestinal tissue. Although still controversial, recent studies in mice suggest that IELs do not originate in the thymus. Rather, IEL progenitors are derived from fetal liver and adult bone marrow and are educated within intestinal tissue (35). In support of this, studies of parabiotic mice suggest that IELs do not generally traffic but are retained within the epithelial layer, perhaps by the novel IEL-specific integrin $\alpha_E\beta_7$ (35, 36). Whether P-selectin is expressed on gut epithelia or associated cells and whether PSGL-1 is present on freshly isolated IELs has yet to be determined. It is possible, however, that cell trafficking during intestinal inflammation might be initiated via selectin-mediated interactions.

The studies reported here also provide an assessment of B cell PSGL-1 cell surface expression and P-selectin binding. The B cell tumors SB and Ramos express PSGL-1 that has not been processed by PACE. In the peripheral blood only 30–50% of CD19+ B lymphocytes express cell surface PSGL-1, all of which appears to be PACE cleaved. None of the B cell tumors or resting peripheral blood B cells displayed P-selectin binding activity. However, Postigo *et al.* (37) recently demonstrated that P-selectin binding by B lymphocytes is also activation-dependent and that PMA-stimulated but not resting tonsillar B cells bind P-selectin. It remains to be determined whether P-selectin binding by stimulated B cells is mediated through PSGL-1.

In recent years, a number of cell adhesion molecules have been identified whose adhesive capabilities are determined by the tissue in which they are expressed. For example, GlyCAM-1, CD34, and MAdCAM-1 function as L-selectin ligands when expressed in the high endothelial venules of peripheral and/or mesenteric lymph nodes but not in other tissues; *e.g.* CD34 in extralymphoid endothelia (38–40). As an explanation for the differential functionality of these molecules, it has been postulated that post-translational modifications critical for L-selectin binding (*i.e.* sulfation) occur preferentially in lymph node high endothelial venules (41–43). Thus, in addition to PSGL-1, the function of other cell adhesion molecules appear to be regulated by post-translational events which in turn are determined by cell lineage and/or activation state. Such events could provide an additional mechanism for the regulation of lymphocyte trafficking.

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