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P-SELECTIN (CD62) BINDS TO SUBPOPULATIONS OF HUMAN MEMORY T LYMPHOCYTES AND NATURAL KILLER CELLS

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ABSTRACT: P-selectin (CD62) is a Ca^{2+}-dependent lectin expressed on activated platelets and endothelium. Although P-selectin is known to function as a receptor for myeloid cells, previous studies indicated that P-selectin also bound to a subset of lymphocytes. Using a multi-color immunofluorescence assay we found that purified P-selectin bound to 12.2 $\pm$ 4.1% of peripheral blood lymphocytes and that P-selectin could mediate adhesion of activated platelets to lymphocytes. A subpopulation of $CD4^+$, $CD8^+$, and $CD16^+$ lymphocytes bound P-selectin. There was a marked preference for P-selectin binding to memory cells ($CD45RO^+$) in both the $CD4^+$ and $CD8^+$ populations. Binding to all cell types was Ca^{2+}-dependent and blocked by pretreatment of the cells with sialidase. These data suggest that P-selectin may play a role in the recruitment of specific lymphocyte populations to sites of inflammation.

P-selectin (CD62), formerly known as GMP-140 or PADGEM protein, is a receptor for myeloid leukocytes that is rapidly translocated from secretory granules to the surface of activated platelets (1,2) and endothelial cells (3). It is a member of the selectin family of adhesion receptors that mediate adhesion of leukocytes to the blood vessel wall (4). Although P-selectin was first characterized as a receptor for myeloid cells, a previous study showed that P-selectin bound specifically to highly purified, unfractionated peripheral blood lymphocytes, albeit at much lower levels than to myeloid cells (5). We therefore developed a multi-color immunofluorescence assay to examine which subpopulations of peripheral blood lymphocytes interact with P-selectin. We found that subpopulations of $CD4^+$ and $CD8^+$ T cells, and NK cells specifically bound P-selectin, whereas B cells did not. In addition, we have demonstrated that P-selectin interacted preferentially with memory T cells.

ABBREVIATIONS: HBSS, Hank's balanced salt solution; PHA, phytohemagglutinin; PE, phycoerythrin; MNC, mononuclear cells; mAb, monoclonal antibody; FCS, fetal calf serum.

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MATERIALS AND METHODS

Antibodies and proteins: Platelet P-selectin was purified as previously described (5). Anti-human P-selectin monoclonal antibodies S12, W40, and G1 (all IgG1) were prepared and characterized as previously described (3). G1 inhibits the interaction of P-selectin with myeloid cells, whereas S12 and W40 do not (2,3,5). Anti-human GPIIb/IIIa mAb, LJP4 (IgG1) (6) was a gift from Dr. Sam Burstein (University of Oklahoma). PE-streptavidin, FITC-Leu 2a (CD8), FITC-Leu 3a (CD4), FITC-Leu 11a (CD16), FITC-Leu M3 (CD14), FITC-Leu 18 (CD45RA), FITC-Leu 12 (CD19), Leu CD45RO, PerCP-Leu 2a (CD8), PerCP-Leu 3a (CD4), and PerCP-IgG1 were purchased from Becton-Dickinson (San Jose, CA). FITC-anti-murine IgG2a was obtained from Caltag (Oyster Point, CA). The control mAbs MOPC 21 (IgG1) and UPC 10 (IgG2a) were from Cappell-Organon Teknika (Malvern, PA). OKT3 (CD3) was from the American Type Culture Collection.

Cell isolation: Neutrophils and mononuclear cells (MNCs) were isolated from heparinized blood (10 U/ml) using MonoPoly Resolving Media (Flow Laboratories) as previously described (2). The cells were washed with HBSS (Gibco/BRL) and resuspended in HBSS/1%FCS/0.1% NaN_3. The neutrophils were >95% pure as assessed by Wright-Giemsa staining. No attempt was made to remove contaminating platelets from MNC suspensions. T cells were isolated from MNCs by rosetting with sheep erythrocytes at 29°C (7) and were >95% pure as judged by staining with OKT3. Under these conditions, most $CD2^+/CD3^-/CD16^+$ lymphocytes fail to form rosettes with sheep erythrocytes.

Activation of T cells: T cells were cultured in the presence of 10 μg/ml PHA (Sigma) in RPMI-1640/10% FCS for 7 days at 37°C in 5% CO_2. After 3 days the cultures were supplemented with 50 U/ml of recombinant interleukin-2 (Genzyme).

Enzyme digestions: Cells (2 x 10^7/ml) were treated with 1 U/ml *Arthrobacter ureafaciens* sialidase (Calbiochem) for 1 h at 37°C in 0.15 M NaCl, 50 mM acetate, pH 6.0, 1% HSA, 0.02% NaN_3, 20 μM leupeptin, 30 μM antipain, and 0.64 mM benzamidine. Cells (2 x 10^7/ml) were treated with 41 U/ml TPCK-trypsin (Worthington) for 10 min at 37°C in HBSS/10 mM MOPS, pH 7.5, after which DFP was added to 2 mM to inactivate the trypsin. Following enzyme digestion the cells were washed twice with HBSS/1% FCS/0.1% NaN_3.

Immunofluorescence: Leukocytes (10^6) were incubated in 50 μl of purified P-selectin (10 μg/ml) for 30 min. Bound P-selectin was detected by sequential incubation of cells with 50 μl of biotin-S12 (10 μg/ml) followed by 20 μl of PE-streptavidin (neat). Each incubation was at 4°C for 30 min in HBSS/1% FCS/0.1% NaN_3, between which the cells were washed with HBSS/1% FCS/0.1% NaN_3. In some experiments, fluorescent-labeled mAbs to lymphocyte subsets were included with PE-streptavidin. CD45RO was detected by an additional incubation

with FITC-anti-IgG2a. After the last wash, the cells were fixed with 1% paraformaldehyde. MAbs of the appropriate subclasses were used as negative controls. In certain experiments the cells were incubated with P-selectin in the presence of EDTA or anti-P-selectin mAbs. Twenty thousand cells were analyzed with a Becton Dickinson FACScan flow cytometer formatted for three color analysis using electronic compensation. Forward and orthogonal light scatter were used to set lymphocyte analysis gates. The FL2 threshold defining P-selectin binding cells was set so that 99% of the cells analyzed were below this threshold when P-selectin binding was assessed in the presence of 20 μg/ml of the inhibitory mAb G1.

RESULTS

Binding of P-selectin to neutrophils: Neutrophils have been shown to bind to P-selectin in a Ca^{2+}-dependent manner using a variety of assays (1,3,5). Therefore, we first examined P-selectin binding to neutrophils to optimize and validate the immunofluorescence assay. Neutrophils were incubated with saturating concentrations of P-selectin and bound P-selectin was detected with biotin-S12, an anti-P-selectin mAb that does not block P-selectin binding to target cells. Figure 1 shows that virtually all neutrophils bound P-selectin and that binding was

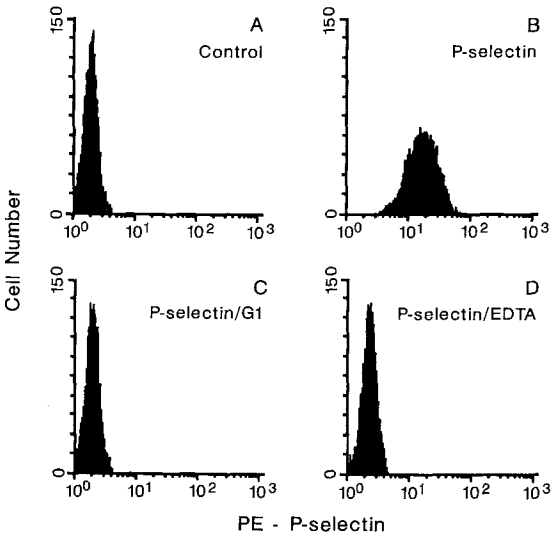

FIGURE 1. P-selectin binding to neutrophils. Neutrophils were assayed for P-selectin binding by incubating cells (10^6) with either diluent alone (A), P-selectin alone (B), or P-selectin in the presence of 20 μg/ml of G1 (C) or 5 mM EDTA (D). Bound P-selectin was detected as described in Methods. A set of histograms representative of more than fifty independent experiments is shown.

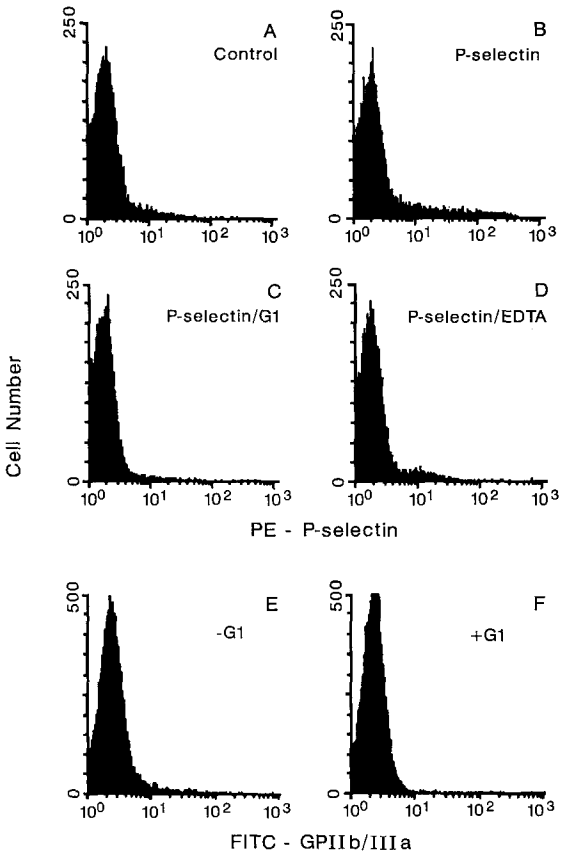

FIGURE 2. P-selectin binding to lymphocytes. Mononuclear cells were assayed for P-selectin binding by incubating cells (10^6) with either diluent alone (A), P-selectin alone (B), or P-selectin in the presence of 20 μg/ml of G1 (C) or 5 mM EDTA (D). Bound P-selectin was detected as described in Methods. A set of histograms representative of 10 independent experiments is shown. Alternately, mononuclear cells (10^6) were stained with a FITC-conjugated mAb directed against platelet GPIIb/IIIa (LJP4) alone (E) or in the presence of 20 μg/ml of G1 (F). Only cells falling within the lymphocyte gate were analyzed.

blocked by EDTA and by G1, an anti-P-selectin mAb which blocks the interaction of P-selectin with myeloid cells. In addition, P-selectin binding to neutrophils was abolished by pretreatment of neutrophils with either trypsin or sialidase (data not shown). These results are consistent with a previous study using a steady-state radioligand binding assay (5).

Binding of P-selectin to lymphocytes: MNCs were assayed for P-selectin binding as described above. We observed that 12.2 $\pm$ 4.1% (Mean $\pm$ SD, n=10) of lymphocytes bound P-selectin. Figure 2 shows a representative experiment. Binding was abolished by the anti-P-selectin mAb G1 (Fig. 2C), but not by W40, an isotype-matched anti-P-selectin mAb that does not inhibit binding of P-selectin to myeloid cells (data not shown). P-selectin binding was also blocked by EDTA (Fig. 2D). Lymphocytes stained with biotin-S12 and PE-streptavidin alone exhibited

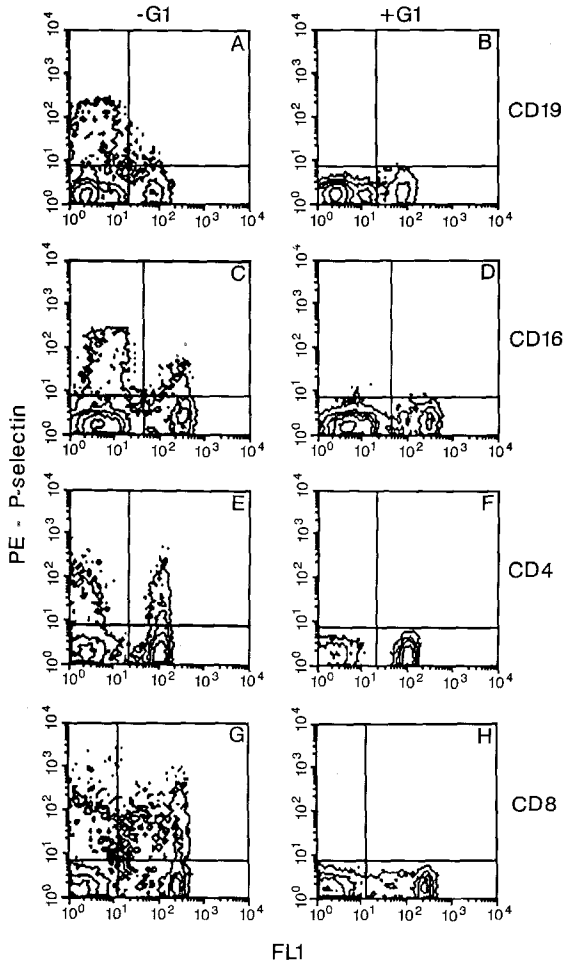

FIGURE 3. P-selectin binding to lymphocyte subsets. Mononuclear cells were assayed for P-selectin binding by incubating cells (10^6) with P-selectin in the presence (B, D, F, H) or absence of 20 μg/ml of G1 (A, C, E, G). Bound P-selectin was detected as described in Methods. FITC-conjugated mAbs against CD19 (A, B), CD16 (C, D), CD4 (E, F), or CD8 (G, H) were included with PE-SA. A set of contour plots representative of 8-18 independent experiments is shown. Only cells falling within the lymphocyte gate were analyzed.

some orange fluorescence (Fig. 2A) that was inhibited by G1 (Fig. 2C). To determine whether this represented binding of contaminating activated platelets, cells were stained with a FITC-conjugated mAb directed against platelet GPIIb/IIIa (LJP4). Binding of this mAb to the cells (Fig. 2E) confirmed that lymphocytes interacted with platelets and/or platelet microparticles. The ability of the anti-P-selectin mAb G1 to block this interaction (Fig. 2F) demonstrated that adhesion of activated platelets to lymphocytes was mediated by P-selectin.

To assess which lymphocyte subsets bound P-selectin, MNCs were analyzed using two-color immunofluorescence. Less than 2% of $CD19^+$ B cells bound P-selectin (n=8). However, P-selectin bound to 16.4 $\pm$ 10.4% of $CD16^+$ NK cells (n=14), 6.0 $\pm$ 5.0% of $CD4^+$ T cells

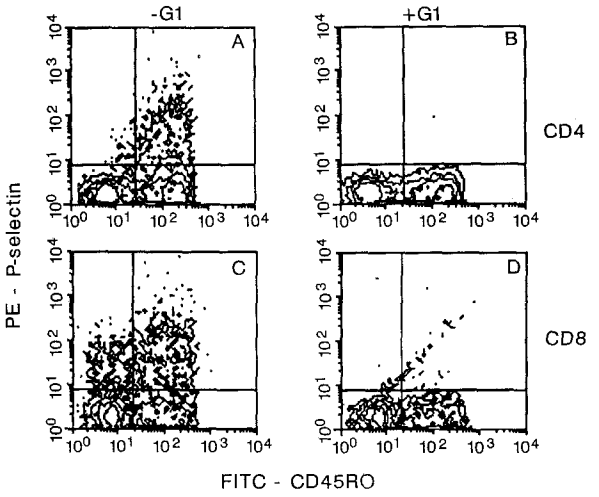

FIGURE 4. P-selectin binding to naive and memory T cells. Purified T cells were assayed for P-selectin binding by incubating cells (10^6) with P-selectin in the presence (B, D) or absence of 20 μg/ml of G1 (A, C). Bound P-selectin was detected as described in Methods. PerCP-conjugated mAbs against CD4 (A, B) or CD8 (C, D) and a mAb against CD45RO were included with PE-SA. Bound CD45RO was detected with FITC-anti-mouse IgG2a. In panels A and B, only $CD4^+$ cells were analyzed, whereas in panels C and D, only $CD8^+$ cells were analyzed. A set of contour plots representative of 6 independent experiments is shown.

(n=18), and 16.6 $\pm$ 8.2 % of $CD8^+$ T cells (n=18)(Mean $\pm$ SD). Figure 3 shows a representative two color analysis of P-selectin binding to lymphocyte subsets. P-selectin binding to all lymphocyte subsets was abolished by G1, an anti-P-selectin mAb that inhibits the interaction of P-selectin with myeloid cells (Fig. 3, panels B, D, F, and H).

We next examined P-selectin-binding to purified T cells. The CD4/CD8 ratio of purified T cells was 1.77 $\pm$ 0.30, whereas the CD4/CD8 ratio of P-selectin binding cells was 0.58 $\pm$ 0.23 (Mean $\pm$ SD, n = 6). Thus, both the percentage and absolute number of $CD8^+$ cells which bound P-selectin are higher than that of $CD4^+$ cells, by factors of 2.8 and 1.5, respectively.

We next examined P-selectin-binding T cells with respect to their expression of markers identifying naive (CD45RA) and memory (CD45RO) subsets (8). Three-color immunofluorescence analysis of T cells demonstrated that there was a strong preference for P-selectin binding to memory cells in both the $CD4^+$ and $CD8^+$ populations (Fig. 4). Among $CD4^+$ cells, 9.3 $\pm$ 4.5% (Mean $\pm$ SD; n = 6) of memory cells but only 1.7 $\pm$ 1.3% of naive cells bound P-selectin. Among $CD8^+$ cells, 26.7 $\pm$ 7.5% of memory cells but only 9.8 $\pm$ 4.5% of naive cells bound P-selectin. The CD45RO/CD45RA ratio in total $CD4^+$ cells was 1.2 $\pm$ 0.7, while that in the P-selectin binding subset was 11.6 $\pm$ 7.1. The CD45RO/CD45RA ratio in total $CD8^+$ cells was 0.6 $\pm$ 0.3, while that in the P-selectin-binding subset was 1.7 $\pm$

Table 1. Effect of EDTA or Sialidase on P-selectin Binding to Lymphocytes

	Percent of Cells Binding P-selectin					
	- EDTA	+ EDTA *	n	- Sialidase	+ Sialidase ¶	n
CD16	14.7 ± 9.9	1.8 ± 1.4	4	14.7 ± 7.3	4.1 ± 2.6	5
CD4	4.6 ± 2.6	0.6 ± 0.4	4	5.7 ± 2.7	1.5 ± 0.4	3
CD8	11.9 ± 2.6	1.2 ± 0.9	4	18.0 ± 3.3	4.6 ± 2.2	3

*Mononuclear cells were assayed for P-selectin binding by incubating cells with P-selectin in the presence or absence of 5 mM EDTA.

¶Mononuclear cells were either sham-treated or sialidase-treated as described in Methods and then incubated with P-selectin.
Bound P-selectin was detected as described in Methods. FITC-conjugated mAbs against CD4, CD8, and CD16 were included with PE-SA. The data represent the Mean ± SD of the indicated number of experiments.

0.7. This demonstrates that for both $CD4^+$ and $CD8^+$ subsets, P-selectin bound preferentially to memory ($CD45RO^+$) cells.

Effect of EDTA or sialidase on P-selectin binding to lymphocytes: P-selectin binding to myeloid cells is Ca^{2+}-dependent and blocked by sialidase treatment of the cells (5). We found that P-selectin binding to all lymphocyte subsets was also Ca^{2+}-dependent and blocked by treatment of the lymphocytes with sialidase (Table 1).

Effect of T cell activation on P-selectin binding: T cells were assayed for P-selectin binding before and after seven days of culture in the presence of PHA. Under these conditions virtually all $CD45RA^+$ cells lost expression of CD45RA and became $CD45RO^+$. However, no significant effect on the percentage of $CD4^+$ or $CD8^+$ cells which bound P-selectin was observed (data not shown).

DISCUSSION

We have demonstrated that subpopulations of helper and suppressor/cytotoxic T cells, and NK cells specifically bound P-selectin. In contrast, very few B cells bound P-selectin. In addition, we have shown that a subset of lymphocytes bound activated platelets in a P-selectin-dependent fashion. Like P-selectin binding to myeloid cells (5), binding of P-selectin to $CD4^+$, $CD8^+$, and $CD16^+$ lymphocytes was Ca^{2+}-dependent and markedly diminished by sialidase treatment of the cells. Although lymphoid and myeloid cells share these basic requirements for interaction with P-selectin, further studies are required to determine if the ligand(s) on these cells are the same.

We also examined P-selectin-binding T cells with respect to their expression of markers identifying naive and memory subsets. We found that P-selectin showed a strong preference for binding to memory ($CD45RO^+$) cells in both the $CD4^+$ and $CD8^+$ populations. However, the percentage of CD4 or CD8 cells which bound P-selectin was unaffected when naive T cells were induced to express CD45RO by PHA *in vitro*. This indicates that *in vitro* T cell stimulation does not induce the expression of P-selectin ligand(s) and that CD45RO and P-selectin ligand expression are not coordinantly upregulated.

Lobb et al. reported that subpopulations of $CD4^+$ and $CD8^+$ T cells and NK cells bound to immobilized E-selectin and that $CD4^+$ memory cells bound preferentially to E-selectin (9). By panning on E-selectin-transfected COS cells Picker et al. observed a marked enrichment of T cells which express the cutaneous lymphocyte antigen (CLA) (10). CLA is present on $\approx$ 15% of both $CD4^+$ and $CD8^+$ cells and is exclusively expressed on memory cells (11). Using highly purified $CD4^+$ T cells, Shimizu et al. observed that memory, but not naive, $CD4^+$ T cells bound to E-selectin (12). Taken together, these studies indicate that E-selectin interacts preferentially with $CD4^+$ memory cells. In contrast, we observed that both the percentage and the absolute number of $CD8^+$ cells which bound P-selectin was substantially higher than for $CD4^+$ cells. Further studies will be required to determine if P-selectin and E-selectin bind to overlapping populations within these subsets.

In conclusion, P-selectin binds to a sialylated ligand(s) on subpopulations of NK cells and memory T cells. At present, the physiologic significance of P-selectin binding to lymphocytes is unclear. Nevertheless, the data demonstrate that P-selectin mediates adhesion of lymphocytes to activated platelets and suggests that P-selectin may be involved in the preferential migration of memory T cells into inflammatory sites.

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