Inflammatory Roles of P-Selectin

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Abstract

Polymorphonuclear leukocytes (PMNs) bind rapidly and reversibly to endothelial cells induced to express P-selectin, a glycoprotein that mediates adhesive intercellular interactions. In addition, PMNs adherent to endothelium expressing P-selectin demonstrate an intracellular Ca^{2+} transient, functionally up-regulate β_2-integrins (CD11/CD18 glycoproteins), become polarized in shape, and are primed for enhanced degranulation when subsequently stimulated with chemotactic factors. However, P-selectin induces none of these responses directly when used alone, when incorporated into model membranes, or when expressed by transfected cells. The absence of direct activation of the PMNs is not due to competing antiinflammatory effects of P-selectin; instead, purified P-selectin and P-selectin in membranes support agonist-stimulated PMN responses. Furthermore, tethering of PMNs to endothelial surfaces by P-selectin is required for priming to occur efficiently, as shown by experiments with blocking monoclonal antibodies. The priming event is directly mediated by the signaling molecule, platelet-activating factor (PAF), and is inhibited by blocking the PAF receptor on PMNs. Thus, P-selectin and PAF are components of an adhesion and activation cascade, but have distinct roles: P-selectin tethers and captures the PMN, whereas PAF mediates juxtacrine activation. In vivo, selectins may facilitate interaction of target cells with membrane-bound molecules that send intercellular signals, in addition to mediating rolling of leukocytes and other adhesive functions. (*J. Clin. Invest.* 1993. 92:559–570.) Key words: endothelium • inflammation • platelet-activating factor • polymorphonuclear leukocyte • selectins • signaling

Introduction

The selectins are novel molecules that mediate adhesive interactions among endothelium, leukocytes, and platelets (1–3). These interactions are required for physiologic inflammation and vascular homeostasis but, if dysregulated, contribute to inflammatory tissue injury and pathologic thrombosis (1). The three members of the selectin family are differentially expressed (1, 2). An integral membrane form of P-selectin is constitutively present in subcellular granules of platelets and endothelial cells and is rapidly translocated to the cell surface after agonist stimulation, where it mediates adhesive interactions with PMNs, monocytes, and some lymphocytes (1, 2). Molecular cloning of P-selectin predicted that there is also an alternatively spliced variant, lacking the transmembrane domain, that is a soluble, secreted form (4). E-selectin is restricted to endothelium, but is not constitutively present in resting endothelial cells; rather, it is synthesized and translocated to the plasma membrane after stimulation with certain cytokines. E-selectin, like P-selectin, mediates binding of PMNs, monocytes, and subsets of lymphocytes (1, 2). L-selectin is present on PMNs, monocytes, and most lymphocytes and is rapidly shed from the surface after cellular activation. It binds to constitutive and inducible ligands on endothelial cells (1–3).

Selectins recognize sialylated glycans that are attached to more complex structures on target cells (1–3). P-selectin and E-selectin bind to purified sugars, including sialyl Lewis^x (SLE^x) and sialyl Lewis^a, to glycolipids and to glycoproteins under various in vitro conditions (2, 3, 5). Characteristics of recognition sites for E- and P-selectin on myeloid cells have been described, and candidate structures for the ligands have been proposed (2, 3, 5–9). However, the "biologically relevant natural ligand(s)" (5) on PMNs and other leukocytes remain to be identified (2, 5).

In addition to tethering one cell to another in cell–cell adhesion (1), binding of selectins to their ligands may activate target cells or alter their functional states in other ways. E-selectin is reported to directly activate PMNs, indicated by functional up-regulation (1) of β_2-integrins (CD11/CD18 glycoproteins) (10, 11). A truncated, soluble, construct of E-selectin is a chemotaxin for PMNs (10). Presumably, these responses result from binding to the same unknown ligand that mediates intercellular adhesion, raising the possibility that it transduces intracellular signals.

It is unknown if P-selectin—which has a similar structure to E-selectin, and may recognize similar ligands (6, 9)—can activate PMN functional responses. This issue is important because PMN activation, including functional upregulation of CD11/CD18 integrins, occurs when they bind to endothelial cells that are stimulated by thrombin or histamine to transiently express P-selectin (12–14; and data presented below). However, in the latter situation P-selectin is coexpressed with platelet-activating factor (PAF),[1] a biologically active lipid that stimulates PMNs (1, 14, 15). This coordinate expression makes it difficult to determine if P-selectin directly induces

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Received for publication 22 December 1992 and in revised form 8 March 1993.

J. Clin. Invest.

0021-9738/93/08/0559/11 $2.00
Volume 92, August 1993, 559–570

1. *Abbreviations used in this paper:* CHO, Chinese hamster ovary; PAF, platelet-activating factor.

activation responses at the endothelial surface. An additional complicating feature is that the putative soluble form of P-selectin (3, 16) is proposed to inhibit CD11/CD18-dependent activation responses of PMNs, based on experiments in which the transmembrane form of the molecule was solubilized and presented in the fluid phase (17–19). Although a mechanism for this effect was not defined, these observations imply that P-selectin is recognized by a signal-transducing receptor and that it may have both proinflammatory and antiinflammatory roles, depending on its presentation. Yet both the soluble and transmembrane forms appear to bind to the same ligand on PMNs (16; Ushiyama et al., manuscript submitted for publication). Thus, identification of the functional alterations that result when P-selectin binds to PMNs is required to understand its roles in complex inflammatory events, and may be a key to defining the biologically relevant ligand. Here we examined responses of PMNs adherent to human endothelial cells that were stimulated to express P-selectin. We then determined its ability to induce these functional alterations using P-selectin presented in purified forms or incorporated into model membranes, and by cells transfected with a cDNA for P-selectin.

Methods

Cells

Primary, confluent, human umbilical vein endothelial cell monolayers and suspensions of purified human PMNs were prepared as described (12–15). Chinese hamster ovary (CHO) cells stably transfected with a cDNA for P-selectin were prepared and maintained using standard methods (Disdier et al., manuscript in preparation).

P-selectin

The transmembrane form of P-selectin was purified from human platelets (20, 21). To examine its effects when presented to PMNs under various conditions, experiments were done with (*a*) PMNs incubated with P-selectin in the fluid phase in concentrations at, or below, those that appear to saturate its high-affinity binding sites (1–5 μg/ml, 7–35 nM [21, 22]), using conditions described earlier (14), (*b*) purified P-selectin immobilized on plastic (20), (*c*) P-selectin incorporated into planar phospholipid membranes.

Planar membranes were prepared using minor modifications of the method of Mimms et al. (23). Egg phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) was diluted in chloroform/methanol (2:1 vol/vol), dried under nitrogen, and then placed under a vacuum (20 p.s.i.) for 2 h. The lipid film was redissolved in 25 mM Tris-HCl in 150 mM NaCl, pH 8.0 ("Tris-saline"), containing octyl-β-D-glucopyranoside (obtained from Calbiochem Corp., San Diego, CA) at a 15:1 molar ratio of detergent to lipid. In the standard preparations, the Tris-saline contained 10 μg/ml P-selectin, 40 μg/ml glycophorin (as a control transmembrane protein), or no added protein. In some experiments a mAb, W6/32, was substituted for glycophorin as a control protein because it binds to a determinant (HLA class I) on PMNs, whereas glycophorin was a nonbinding control. To do this, we acylated the W6/32 with palmitic acid using described methods (24), so that it would anchor in the membrane, before mixing with the phosphatidylcholine and detergent.

The detergent was removed by dialysis against Tris-saline for 36 h with three changes of dialysis fluid, leaving liposomes. Planar membranes were formed by fusion of liposomes on the surfaces of glass coverslips (25). Before coating with liposomes, each coverslip was boiled in detergent (Linbro 7× solution; Flow Laboratories, McLean, VA) for 15 min, rinsed extensively in deionized distilled water for 24 h, and stored at 4°C in ethanol. The liposomes were deposited on coverslips supported in the bottom of tissue culture wells, and allowed to adsorb and coalesce for 30 min at room temperature. The planar membranes that formed were then washed three times with buffer without being exposed to an air interface. These membranes were uniform and continuous, as shown by microscopic examination of membranes formed from fluorescently labeled phosphatidylcholine (obtained from Avanti). A second indication that the membranes were continuous was that there was no enhanced adhesion of PMNs stimulated with agonists (see below) to phospholipid membranes that did not contain protein. In contrast, stimulated PMNs adhere to glass in an enhanced fashion (this is mediated by CD11/CD18 integrins) and would be expected to adhere to exposed areas of glass not coated with lipid if the model membranes were not continuous.

Increasing the concentration of P-selectin from 2 to 10 μg/ml Tris-saline in the preparative mixture resulted in a concentration-dependent increase in binding of unstimulated PMNs to the planar membranes. This binding was specific for a P-selectin-mediated adhesive interaction (see Fig. 3).

Assays of PMN adhesion and functional alterations

ADHESION

The assays for adhesion of PMNs to endothelial monolayers stimulated with histamine, thrombin, or leukotriene C_4 (LTC_4), using PMNs radiolabeled with ^{111}In or using unlabeled PMNs and microscopy, have been described previously (12–15). We used minor modifications of the assay with radiolabeled PMNs to measure their binding to CHO cells transfected with P-selectin, to purified, immobilized P-selectin, and to P-selectin in model membranes.

In experiments in which we inhibited PMN adhesion to surfaces bearing P-selectin or to activated endothelial cells, we used the blocking mAb against P-selectin, G1, as described (14, 20). S12, a mAb against P-selectin that does not block adhesion (14, 20), mAbs against irrelevant proteins, or nonimmune murine IgG were used as controls. None of the control immunoglobulins have inhibitory effects under the conditions of these experiments (14, 20; and unpublished observations). In experiments with endothelial cells, the monolayers were pretreated with the antibodies and the antibodies were also present in the PMN suspension during incubation of the PMNs on the monolayers (14). In experiments with P-selectin presented by transfected cells or with purified P-selectin (immobilized or in model membranes), pretreatment alone with G1 was sufficient to completely inhibit adhesion and, therefore, neither it nor the control antibodies were included in the PMN suspension.

FUNCTIONAL RESPONSES

To examine the effect of P-selectin on functional responses of PMNs, we incubated them on P-selectin–bearing surfaces (purified, or in planar membranes), with P-selectin in the fluid phase, or with P-selectin transfectants. A given activation response was evaluated with fluid phase P-selectin and with a minimum of one of the models for surface presentation. In key experiments, we evaluated the response with more than one of the surface presentations, since they are not equivalent (the density of P-selectin and the molecular makeup of the surrounding surface is different for P-selectin immobilized on plastic, incorporated into model membranes, and expressed on plasma membranes of cells). In each experiment control solutions that contained diluents and additives that were exactly the same as in the P-selectin preparation were included. In parallel incubations PMNs were stimulated with one or more agonists that induced the activation response in question, including PAF, FMLP, or PMA, as a positive control. In additional experiments PMNs were first incubated for various times with P-selectin or a control preparation and then stimulated with an agonist to determine if P-selectin modified the activation response under these conditions. The specific agonist(s), concentrations, and other conditions that we used in individual experiments were chosen to give a range in the magnitude of the activation response ("weak" and "strong" agonists) so that we would have an equal chance to detect inhibition or enhancement of the response by P-selectin (for example, see legend to Fig. 3).

Intracellular Ca^{2+} ($[Ca^{2+}]_i$). PMNs were loaded with the calcium-sensitive fluorescent dye, Indo-1, by incubation with the acetomethoxy

ester (Indo-1 AM) (10^{-5} M) for 1 h at room temperature in HBSS. They were then centrifuged (250 *g* for 5 min at 4°C) and resuspended in HBSS containing 5 mg/ml human serum albumin (HBSS/A). Alterations in $[Ca^{2+}]_i$ after treatment with saturating concentrations of fluid-phase P-selectin, or while adherent to P-selectin immobilized on support surfaces or in membranes, were measured as described (26).

Granular secretion. We developed "sandwich" ELISAs to monitor PMN secretion of primary (azurophilic) and secondary (specific) granular enzymes (Topham et al. manuscript submitted for publication). Lactoferrin was assayed as a marker for secretion from the secondary granules and elastase was assayed as an index of primary granular secretion.

Alterations in CD11/CD18 integrins. Functional up-regulation of CD11b/CD18 integrin was assayed by the binding of iC3b-coated erythrocytes to PMNs using minor modifications of a published method (10). Sheep erythrocytes that had been coated with iC3b (27) were layered over PMNs adherent to purified, immobilized P-selectin or control proteins; allowed to adhere for 30 min at room temperature; washed five times; and fixed for 15 min with 0.01 M $NaHPO_4$, 2% glutaraldehyde, and 1% sucrose (pH 7.4). In parallel incubations, some replicates of adherent PMNs were stimulated with PAF, in various concentrations, for 5 min before addition of the iC3b-coated erythrocytes. The cells were stained with Giemsa to facilitate counting. The attachment index, which is the number of erythrocytes bound per 100 PMNs, was determined microscopically at ×63 with oil immersion. The same assay was used when the response of PMNs adherent to P-selectin in planar membranes was examined.

In a second assay of CD11/CD18 integrin activation, we examined functional up-regulation of CD11a/CD18 with mAb NK1/L-16, which identifies an activation-dependent epitope (28). PMNs were incubated in control buffer, with fluid-phase P-selectin, or with an agonist known to activate PMNs for 30 min at 37°C and then put on ice. After being washed with cold HBSS/A containing 0.1% azide (HBSS/A/0.1% azide), the PMNs were resuspended in HBSS/A containing a 1:100 dilution of the anti-CD11a mAb, NKI L-16, or an equal volume of HBSS/A/0.1% azide, and kept on ice for 45 min. The PMNs were then washed with cold HBSS/A/0.1% azide, resuspended in a 1:100 dilution of FITC-conjugated goat anti-mouse IgG (Sigma Immuno Chemicals, St. Louis, MO), and kept on ice for 30 min. After washing again in cold HBSS/A/0.1% azide, the PMNs were resuspended in 1.0% paraformaldehyde and fluorescence was measured (Becton Dickinson & Co. FACScan) (29).

Quantitative up-regulation of CD11b/CD18 on PMN surfaces was analyzed by mAb binding and flow cytometry using the anti-CD11b mAb, 60.1 (0.01 mg/ml), or the anti-CD18 mAb, 60.3 (0.01 mg/ml), and conditions described above. Shedding of L-selectin was measured in parallel using binding of mAb Dreg 200.9 (0.01 mg/ml) (30).

Oxygen radical generation. Superoxide generation was measured as the fraction of cytochrome *c* reduction inhibited by superoxide dismutase (31). PMNs (1.0×10^7/ml) were incubated on immobilized P-selectin or control proteins, or were incubated with fluid phase P-selectin or controls. After a 20-min incubation at 37°C, cytochrome *c* was added (0.1 mM final concentration), followed by superoxide dismutase (200 μg/ml final concentration). In parallel, PMNs were treated with control buffer or an agonist (PAF, FMLP, or PMA). The PMNs were then centrifuged, and the reduced cytochrome *c* in the supernatant was measured by spectrophotometry (model DU-40; Beckman Instruments, Inc., Palo Alto, CA) at 550 nm. In some experiments PMNs were sequentially incubated with P-selectin or control proteins (20 min), followed by an agonist (30 min), and superoxide anion generation was measured.

Generation of biologically active lipids. LTB_4 and PAF synthesis were assayed as previously described (32).

Spreading and polarization. Electron microscopy (12, 13) was performed on PMNs adherent to planar membranes containing P-selectin or glycophorin, and on membranes without incorporated protein. After a 10-min incubation with the membranes, PMNs were fixed with Karnovsky's solution for 30 min, coated with atomic gold by vacuum evaporation, and examined with a model JSM35 scanning electron microscope (JEOL USA, Peabody, MA). In some experiments PAF was added to PMNs on membranes containing P-selectin or control membranes (10-min incubation), and they were fixed and their morphology was examined as above.

Results and Discussion

PMNs that adhere to endothelial cells stimulated to express P-selectin are activated and are primed for enhanced secretion. Endothelial cells stimulated with thrombin transiently express P-selectin and bind PMNs (14). We assessed the adherent PMNs for evidence of activation or functional alterations. We found that PMNs that adhered to thrombin-treated endothelial cells demonstrated a rise in the concentration of free cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) (Fig. 1). We also observed a CD11/CD18-dependent component of adhesion (14, 33; and unpublished experiments), which requires PMN activation (1, 34). Thus maximal adhesion of PMNs to endothelial cells stimulated with thrombin requires both P-selectin and CD11/CD18 integrins (14); involvement of CD11/CD18 proteins indicates that the PMNs had become activated. Adherent PMNs also developed polarized morphology, a third index of activation (12, 13; and below). This group of experiments showed that these responses, which are important in PMN migration, secretion, and other inflammatory events, can be induced by molecules expressed on the surface of stimulated endothelium.

We also found that PMNs adherent to endothelial cells stimulated with thrombin (2 U/ml for 5 or 10 min followed by washing) were "primed" for enhanced secretion of the primary granule enzyme, elastase, and the specific granule constituent, lactoferrin, when subsequently exposed to chemotactic factors. In five experiments PMNs first were incubated with buffer-

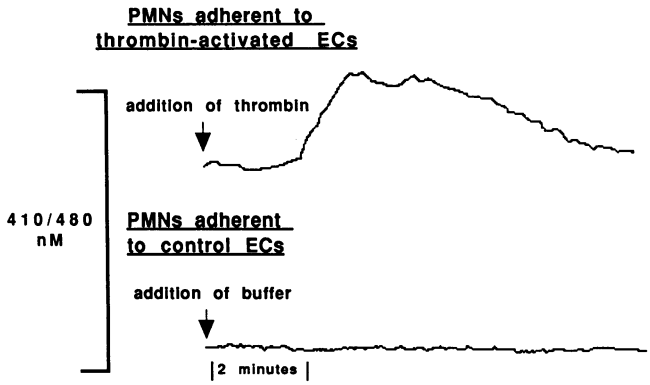

Figure 1. PMNs adherent to stimulated endothelial cells respond with an increase in $[Ca^{2+}]_i$. PMNs were loaded with the Ca^{2+}-sensitive dye Indo-1 and layered over confluent endothelial monolayers that had been grown on coverslips. The coverslips were mounted in wells of a custom spectrofluorimeter while the incubations with PMNs were done. Thrombin (2 U/ml; *upper trace*) or control buffer (*lower trace*) was added and the incubations continued for the period shown. Enhanced PMN adhesion to thrombin-stimulated endothelial cells occurs in this interval under these conditions (12). Fluorescence in the Indo-1–loaded PMNs was measured at 410 and 480 μM as described (26). The figure was traced from the original recording. In additional experiments thrombin added to Indo-1–loaded PMNs in the absence of endothelial cells did not induce a Ca^{2+} transient, indicating that the signal for an increase in intracellular Ca^{2+} came from the endothelial monolayers.

treated or thrombin-treated endothelial monolayers. Then we added the chemotactic peptide FMLP (0.3 μM final concentration) and measured secretion of lactoferrin after a further 10-min incubation. We found that the release of lactoferrin was significantly higher ($P < 0.05$) from PMNs incubated with thrombin-activated endothelial cells than from those incubated with control monolayers. This effect was even more apparent when we examined the concentration–response relationship for FMLP-stimulated lactoferrin secretion. PMNs that were stimulated while adherent to thrombin-activated endothelium were more responsive: concentrations of FMLP that were below threshold for PMNs on control endothelial cells were effective in inducing secretion from PMNs incubated on monolayers stimulated with thrombin (Fig. 2, *top*). The release of elastase was also enhanced (see below). There was no enhancement of granular secretion from PMNs adherent to thrombin-treated endothelial cells in the absence of FMLP (Fig. 2, *top*). These experiments demonstrated that thrombin-activated endothelial cells prime PMNs for enhanced secretion. Priming may augment secretory responses of PMNs after they migrate to extravascular sites (35) or, alternatively, can potentiate endothelial injury by PMNs if they are stimulated at the luminal surface (36).

The priming of PMNs was not restricted to endothelium activated by thrombin: in two experiments PMNs adherent to endothelial cells treated with LTC_4 secreted increased concentrations of lactoferrin upon subsequent stimulation with FMLP. Adhesion of PMNs to endothelial cells activated with LTC_4 (13), like adhesion to endothelium activated by thrombin (14), is inhibited by antibodies to P-selectin (our unpublished observations). Both LTC_4- and thrombin-activated endothelial cells primed PMNs for enhanced secretion in response to LTB_4 in addition to FMLP. Therefore PMNs adherent to endothelium expressing P-selectin are primed for enhanced granular secretion, and stimulation of this enhanced secretion can be induced by inflammatory agonists of different classes.

P-selectin is required, but not sufficient, for the priming response. We next explored the role of P-selectin in the priming response. mAb G1, which identifies an epitope in the ligand recognition domain of P-selectin (21), inhibited priming of the adherent PMNs (73% inhibition; Fig. 2, *bottom*). In parallel, G1 inhibited PMN adhesion to thrombin-activated endothelial cells by 75%. This degree of inhibition of adhesion is similar to that in earlier experiments (14). A control immunoglobulin did not inhibit adhesion or priming. This result indicated that P-selectin is required for efficient priming of PMNs at the surfaces of endothelial cells stimulated with thrombin. It is consistent with our earlier experiments demonstrating that P-selectin is required for maximal adhesion (14).

We then asked if P-selectin induces an increase in $[Ca^{2+}]_i$ in PMNs, because this appears to be a key component of the priming mechanism (35), because there is an increase in $[Ca^{2+}]_i$ when PMNs adhere to thrombin-stimulated endothelial monolayers (Fig. 1), and because $[Ca^{2+}]_i$ is influenced by adhesive interactions (34–38). Therefore, we incorporated P-selectin into model membranes and evaluated the binding of PMNs to these surfaces and the levels of $[Ca^{2+}]_i$ in adherent PMNs that were preloaded with Indo-1. There was enhanced binding of PMNs to planar phospholipid membranes containing P-selectin compared with that to membranes containing glycophorin or phospholipids alone (five- to eightfold increase

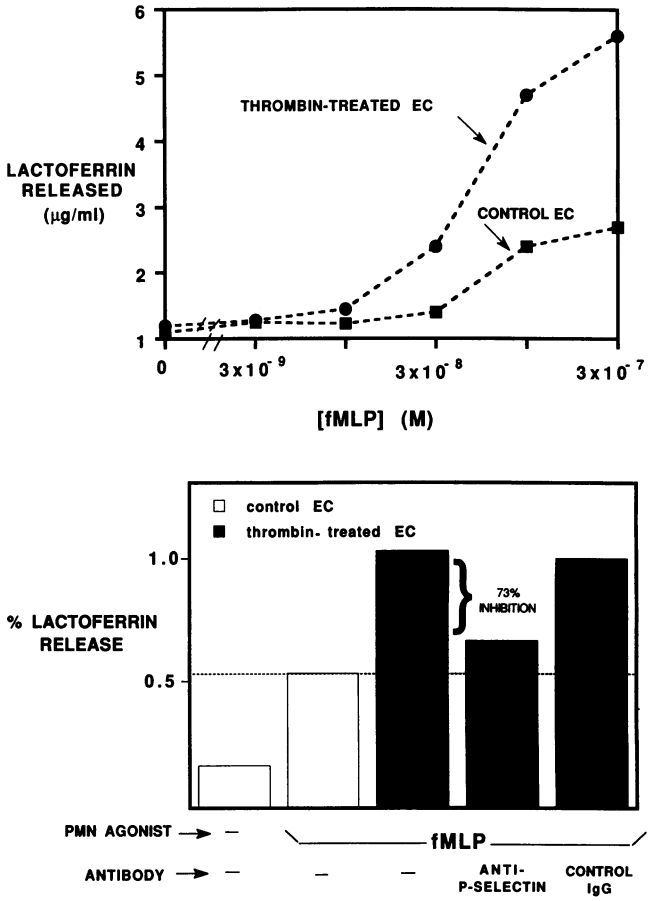

Figure 2. PMNs are activated and primed at the endothelial surface; P-selectin is required for efficient priming. *Upper panel:* Adhesion of PMNs to endothelium stimulated by thrombin primes them for enhanced secretion of lactoferrin when they are subsequently stimulated with a chemotactic factor. Confluent endothelial monolayers in 16-mm wells were incubated with thrombin (2 U/ml for 10 min at 37°C in 5% CO_2, 95% air) to induce P-selectin expression, or with control buffer, and then washed. 0.5 ml of PMNs (5.5×10^6 cells/ml in HBSS/A) was added to each monolayer. The PMNs were incubated with the endothelial cells (*EC*) for 5 min at 37°C in 5% CO_2, 95% air, a period that allows maximal adhesion. FMLP was added in the concentrations shown and the PMNs and endothelial monolayers were incubated together for another 10 min under the same conditions. The solutions were aspirated from each monolayer and assayed for lactoferrin content by ELISA. *Lower panel:* An anti-P-selectin mAb, G1, inhibits priming of PMNs by endothelium stimulated with thrombin. Endothelial cell monolayers were incubated with buffer alone or buffer containing thrombin (2U/ml) as described. Control buffer, an anti-P-selectin mAb (G1; 10 μg/ml), or a control murine IgG (10 μg/ml), was added before addition of thrombin or control solution in parallel incubations. After 10 min PMNs containing the same concentrations of the indicated IgG, or control buffer, were added, and incubated for 5 min, FMLP (100 nM) was then added, the incubations were continued another 10 min, and the concentration of lactoferrin secreted by the PMNs was measured by ELISA. The bracket indicates the percent inhibition of the priming response. A second experiment yielded a similar result.

in multiple experiments), indicating that it can tether PMNs to a membrane in the absence of other accessory molecules. This enhanced binding was inhibited by the anti-P-selectin mAb, G1 (Fig. 3, *left*). However, while there was avid adhesion of PMNs to model membranes containing P-selectin, there was

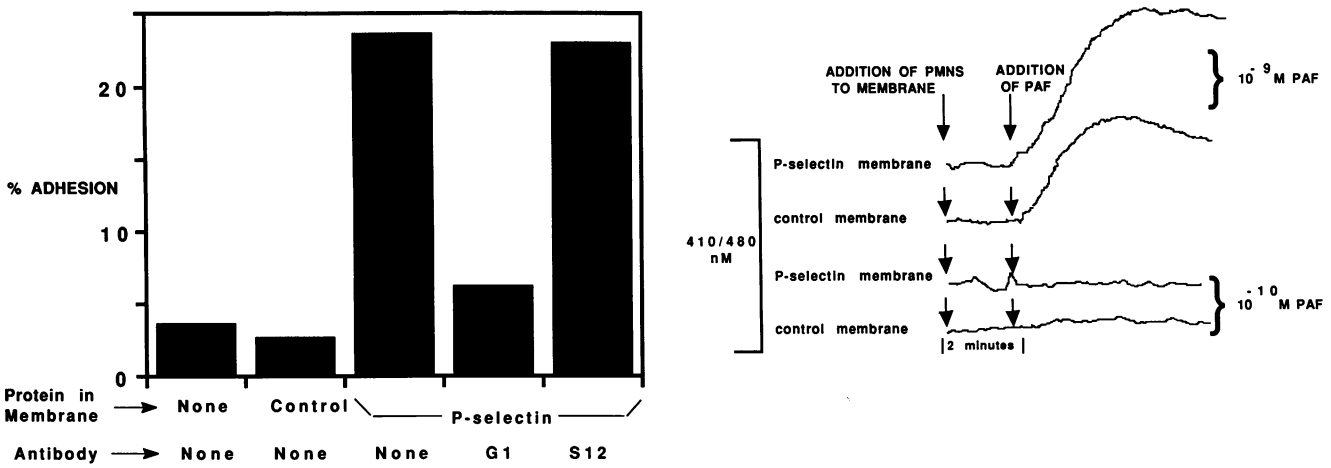

Figure 3. P-selectin incorporated into planar membranes tethers PMNs and supports, but does not induce, increased $[Ca^{2+}]_i$. *Left panel:* P-selectin was incorporated into planar phospholipid membranes mounted on glass coverslips as described in Methods. Additional membranes were made that contained no protein, or that contained the control protein, glycophorin. ^{111}In-labeled PMNs (5.5×10^6 cells/ml in 0.5-ml volumes) were layered over the membranes and incubated for 10 min at 37°C in 5% CO_2, 95% air. In some replicates the membranes were pretreated (10 min) with the blocking anti-P-selectin mAb, G1 (10 μg/ml), or with mAb S12 (10 μg/ml), an anti-P-selectin that does not block adhesion. After incubation, nonadherent PMNs were removed by aspiration, loosely adherent PMNs were removed with a wash of one incubation volume of buffer, the membranes and bound PMNs were solubilized, and the fraction of adherent PMNs was determined (12). *Right panel:* Control membranes, or membranes containing incorporated P-selectin, were placed in the chamber of a spectrofluorimeter, PMNs loaded with Indo-1 were added, and fluorescence was recorded for 2 min. At the end of that period PAF was added to the bath at final concentrations at (10^{-9} M; *upper two traces*), or below (10^{-10} M; *lower two traces*), the K_D for the PMN receptor for PAF ($\sim 10^{-9}$ M; see reference 47). These conditions were chosen to determine if P-selectin enhances submaximal stimulation by PAF at the lower concentration or, conversely, inhibits the rise in $[Ca^{2+}]_i$ induced by concentrations of PAF that approach those that saturate the receptor. The threshold concentration of PAF that induced an increase in $[Ca^{2+}]_i$ varied from preparation to preparation of PMNs, but was not consistently altered by adhesion to P-selectin. A second experiment gave the same result, as did an experiment in which PMNs were treated in suspension with liposomes containing P-selectin.

no increase in $[Ca^{2+}]_i$ in adherent PMNs preloaded with Indo-1 (Fig. 3, *right*). When subsequently stimulated with PAF, the increase in $[Ca^{2+}]_i$ in PMNs incubated on membranes containing P-selectin was equivalent to that in control membranes or, in some experiments, was slightly greater (Fig. 3, *right*). Thus P-selectin incorporated into a membrane did not directly stimulate a $[Ca^{2+}]_i$ transient. This finding contrasts with the observation that adhesion of PMNs to uncoated glass (39), or to surfaces coated with albumin (35), is sufficient to initiate an increase in $[Ca^{2+}]_i$. The absence of a direct stimulation of $[Ca^{2+}]_i$ was not, however, due to an inhibiting effect of P-selectin, because adhesion of PMNs to P-selectin in model membranes did not inhibit increases in $[Ca^{2+}]_i$ that were then stimulated by PAF or FMLP (Fig. 3, *right;* and data not shown).

The experiments in Fig. 3 argued that P-selectin on the surface of an activated endothelial cell does not itself stimulate an increase in $[Ca^{2+}]_i$ (Fig. 1) or prime PMNs for enhanced degranulation (Fig. 2). To more directly ask this question, we examined CHO cells stably transfected with P-selectin for their ability to prime PMNs. We first characterized the adhesive interaction between the two cell types to verify its specificity. PMNs adhered avidly to P-selectin-transfected cells compared to wild-type CHO cells (Fig. 4). The mean adhesion was fivefold higher to the transfected cells than to wild-type cells in multiple experiments. This adhesion required Ca^{2+} in the buffer, was completely inhibited by treatment of PMNs with saturating concentrations of fluid phase P-selectin, and was inhibited by the blocking mAb G1; it was not inhibited by mAb S12 (20), a nonblocking mAb against P-selectin, by the anti-CD18 mAb, 60.3 (33), or by the anti-L-selectin mAb, LAM 1.1 (40) (Fig. 4). Each of these results is characteristic of binding of radiolabeled P-selectin to its high affinity ligand on PMNs, and of PMN adhesion to purified, immobilized P-selectin (7, 14, 20, 21). Thus there was a specific, P-selectin-dependent adhesive interaction between the transfected cells and PMNs. The adhesion was also inhibited by treatment of the PMNs with chymotrypsin (threshold inhibition < 0.01 U/10^6 PMNs, complete inhibition at 0.1 U/10^6 PMNs; not shown). This result is consistent with other evidence that proteases cleave the ligand for P-selectin on myeloid cells (2, 9, 21, 41).

These experiments documented a specific, P-selectin–dependent adhesive interaction between PMNs and the transfected CHO cells. We then examined the ability of P-selectin expressed by the transfected cells to influence PMN secretory responses. There was no direct secretion of lactoferrin by PMNs incubated on the P-selectin transfectants, and no priming for enhanced lactoferrin secretion, when the adherent PMNs were stimulated with FMLP (not shown, $P = 0.8$). This was consistent with the data in Fig. 3 but, to explore this issue further, we also examined superoxide anion generation, because it was previously reported that endothelial cells activated by thrombin prime PMNs for enhanced O_2 radical generation (42) and because priming for granular secretion and for superoxide production are tightly linked. Similar to our results with granular secretion, we found that adhesion of PMNs to P-selectin–transfected CHO cells neither directly induced superoxide generation nor primed them for enhanced superoxide anion generation ($P = 0.48$ and 0.24, respectively) when they were stimulated with FMLP or PMA (Fig. 4, *right*). These results again suggested that P-selectin does not act alone to prime

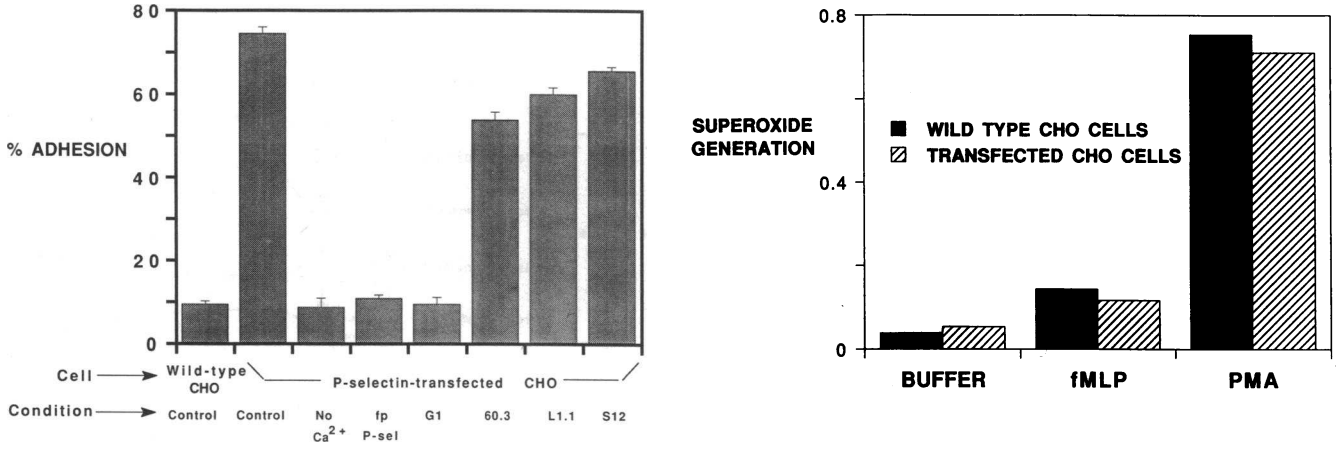

Figure 4. CHO cells transfected with P-selectin cDNA mediate specific adhesion of PMNs and support secretory responses. *Left panel:* Transfected cells support specific adhesion. ^{111}In-labeled PMNs were incubated with wild type CHO cells, or CHO cells stably transfected with P-selectin cDNA, for 10 min at 37°C in 5% CO_2, 95% air and adhesion was determined. In parallel incubations the adhesion of PMNs in Ca^{2+}-free buffer, or adhesion of PMNs that had been pretreated with 5μg/ml solubilized P-selectin in the fluid phase (*fp P-sel*) for 10 min at 37°C, to P-selectin–transfected CHO cells was measured. In additional parallel incubations CHO transfectants were pretreated with mAb G1 (blocking anti–P-selectin) or S12 (nonblocking anti–P-selectin) for 10 min, before addition of PMNs, or PMNs were pretreated with mAb 60.3 (anti-CD18), or with mAb LAM 1.1 (anti–L-selectin as the $F(ab')_2$ fragment), for 10 min before addition to P-selectin–transfected CHO cells. *Right panel:* Transfected cells support, but do not induce, superoxide anion secretion. PMNs (1.0×10^7 cells/ml) were layered over CHO cells and incubated at 37°C. After 20 min of buffer alone, a weak agonist for superoxide generation (10^{-7} M fMLP), or a strong agonist (10^{-7} M PMA), was added and the cells were incubated at 37°C for an additional 30 min. Superoxide generation was determined by the reduction of cytochrome *c* measured by absorbance at 550 mM.

PMNs for enhanced secretion. However, we considered alternative explanations including insufficiant site density, expression of an inhibitor of priming or secretion by the transfected cells, or an aberrant glycosylation or folding pattern of P-selectin by the CHO cells that allowed it to tether the PMNs but not mediate signaling functions. To resolve this question, we exposed neutrophils for various times (2, 5, or 15 min) to purified immobilized P-selectin, or to immobilized fibrinogen as a control, added FMLP (0.1 or 1.0 μM), PAF (1 μM), or PMA (10 ng/ml), and measured degranulation after a 10-min incubation. There was no direct release of lactoferrin from PMNs, and no priming for enhanced lactoferrin secretion in response to any of the agonists, on the P-selectin–coated surfaces compared to the control surfaces; similarily, there was no enhanced secretion of superoxide from PMNs adherent to immobilized P-selectin. We found the same result with PMNs adherent to P-selectin incorporated into model membranes (Fig. 5). These experiments supported our conclusion that P-selectin does not directly prime PMNs for enhanced secretion. In additional experiments, immobilized P-selectin neither primed nor stimulated synthesis of PAF ($n = 5$).

The experiments described above indicated that P-selectin does not directly induce an increase in intracellular Ca^{2+}, granular secretion, generation of 0_2 radicals, or synthesis of biologically active lipids by PMNs, and does not directly mediate priming of the PMNs for enhanced secretory responses. An alternative possibility is that P-selectin indirectly enhances these responses by altering another critical molecular component. The CD11/CD18 integrins of PMNs influence $[Ca^{2+}]_i$ in adherent PMNs (see above); this facilitates secretion of oxygen radicals and granule contents (34, 35, 37, 38, 43). Thus, if P-selectin caused a quantitative increase in CD11/CD18 integrins on the PMN surface it could indirectly facilitate priming and other activation responses. To examine this issue, we incubated PMNs on immobilized P-selectin or an immobilized control protein, eluted them with EDTA-containing buffer, and measured surface binding of the anti-CD18 mAb, 60.3, by flow cytometry. In parallel, we measured binding of an anti–L-selectin antibody, because L-selectin is shed from activated PMNs as CD11b/CD18 is quantitatively upregulated (1, 30, 40). Adhesion of PMNs to immobilized P-selectin did not increase surface expression of CD11/CD18 integrins, nor did it decrease binding of the anti–L-selectin mAb. In a second ex-

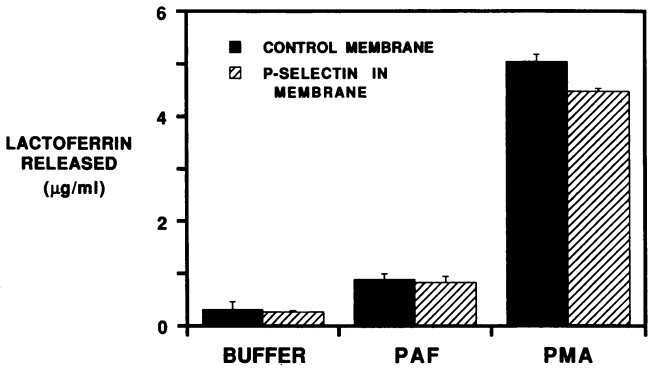

Figure 5. P-selectin in planar membranes supports granular secretion from PMNs but does not prime them. Planar membranes containing P-selectin and control membranes without P-selectin were mounted in wells, PMN suspensions (5.5×10^6/ml in HBSS/A) were layered over them, and control buffer, a weak degranulating agonist (PAF 10^{-7} M), or a strong degranulating agonist (PMA 10^{-7} M), was added. After a 1-h incubation at 37°C in 5% CO_2, 95% air, the suspensions were aspirated, remaining PMNs were removed by centrifugation, and the concentration of lactoferrin was measured by ELISA. The bars indicate the means of two determinations and the error bars indicate the ranges.

periment, we pretreated PMNs for 30 min at 37°C with fluid-phase P-selectin in a concentration (5 μg/ml) that saturates the high affinity binding sites on the leukocytes (21) (also see Fig. 4, *left*, which documents that this concentration is saturating), and determined its effect on the surface adhesion molecules. There was no increase in CD11b/CD18 (Fig. 6, *top*) or shedding of L-selectin (not shown). A similar result, under different experimental conditions, was reported by Moore et al. (21).

We then asked if P-selectin alters CD11/CD18 integrin function independent of quantitative upregulation (1, 34). To do this, we examined the ability of PMNs adherent to P-selectin to bind erythrocytes coated with iC3b, which is recognized by the CD11b/CD18-integrin; this assay can be used even when high level adhesion to immobilized P-selectin precludes other assays of CD11/CD18-integrin-mediated adhesiveness (10). Adhesion of PMNs to purified, immobilized P-selectin did not enhance their binding of iC3b-coated erythrocytes (Fig. 7), indicating that it does not directly activate CD11b/CD18. We found a similar result when the same experiment was done

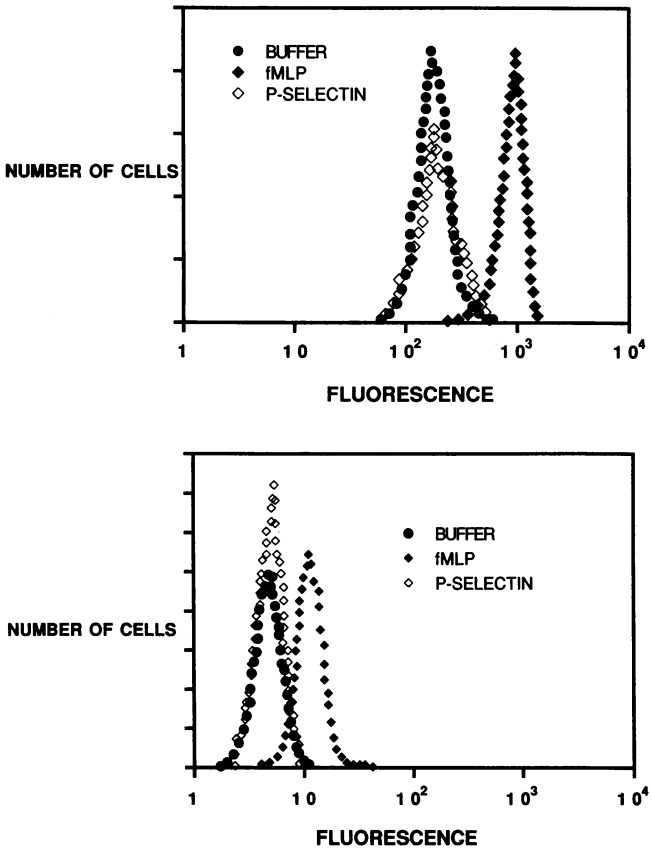

Figure 6. Surface phenotype of PMNs incubated with saturating concentrations of purified, fluid-phase P-selectin. PMNs were incubated with buffer alone, buffer containing P-selectin (5 μg/ml), or with FMLP (10^{-6} M) for 30 min at 37°C. They were then incubated with an antibody to CD11b/CD18 (mAb 60.1, *top panel*) or an antibody that identifies an activation-dependent epitope on CD11a/CD18 (NKI-L16, *bottom panel*) and analyzed by flow cytometry (Methods). In parallel incubations, binding of an antibody to L-selectin (Dreg 200.9) to PMNs pretreated with P-selectin was not reduced; in contrast, binding to FMLP-stimulated PMNs was decreased, indicating shedding of L-selectin (not shown).

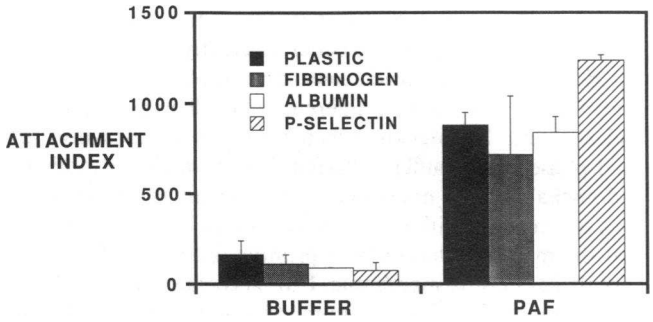

Figure 7. Purified, immobilized P-selectin supports functional up-regulation of CD11b/CD18 integrin, but does not directly stimulate this activation response. Plastic wells were washed and coated with P-selectin (2.5 μg/ml), with control proteins (fibrinogen and albumin) in the same concentrations, or were left uncoated. PMNs were layered onto the surfaces, incubated for 10 min, and then were treated with buffer or PAF for an additional 5 min. Sheep RBCs that had been coated with iC3b were placed over PMNs and allowed to adhere for 30 min. Nonadherent RBCs were washed away and the attachment index (the number of RBCs bound per 100 PMNs) was determined by microscopy. The figure represents the mean of two experiments and the error bars represent the range.

with P-selectin in model membranes. Thus, these experiments indicate that P-selectin does not positively regulate CD11b/CD18, and therefore differs from E-selectin (9). This result is consistent with recent observations that the two selectins recognize different, albeit similar, ligands on myeloid cells (2, 9, 41, 44). In additional experiments we found that purified P-selectin does not cause functional up-regulation of CD11a/CD18 on PMNs using NKI-L16, an antibody that defines an activation-dependent epitope on this integrin (28). PMNs were treated with a saturating concentration of fluid-phase P-selectin (see above), or with FMLP as a positive control. There was no increase in binding of NKI-L16 to PMNs incubated with P-selectin, whereas there was enhanced binding of the antibody to PMNs treated with FMLP in parallel (Fig. 6, *bottom*). Thus, using independent assays of integrin function we found that P-selectin does not directly activate CD11a/CD18 or CD11b/CD18. These results are similar to the finding that P-selectin in model membranes mediates rolling of PMNs in a flowing system but does not alone induce adhesion strengthening that is dependent on activation of CD11/CD18 integrins (45), and with our earlier findings using PMN adhesion to endothelial cells as the assay of CD11/CD18 activation (14).

Although P-selectin did not directly induce functional up-regulation of CD11/CD18 integrins, it supported and moderately enhanced CD11b/CD18 integrin function when adherent neutrophils were stimulated with PAF (Fig. 7). Since P-selectin does not quantitatively increase CD11b/CD18 on the PMN plasma membrane (Fig. 6, *top*), this implies that P-selectin may enhance functional up-regulation of the integrin if the PMN is stimulated by an agonist. This result is consistent with our earlier observation of enhancement of agonist-stimulated adhesion of PMNs to unactivated endothelial cells, which is mediated by CD11/CD18 integrins, by fluid phase P-selectin (14).

Priming of PMNs adherent to endothelial cells stimulated by thrombin is due to juxtacrine activation by PAF. The results with purified P-selectin and with transfected cells demonstrated that it does not prime PMNs for enhanced granular

secretion. However, the results in Fig. 2 indicated that it is required for this response to occur efficiently in PMNs adherent to activated endothelial cells. This implies that the signal for priming comes from a different molecule expressed by the endothelium. The signaling factor was not released into the fluid phase, since buffer collected from thrombin-stimulated endothelial cells did not contain priming activity (not shown). This is consistent with earlier experiments (12), and indicated that the molecule that induces priming is associated with the endothelial plasma membrane. PAF is coexpressed with P-selectin on endothelial cells stimulated with thrombin (14) and can bind to its receptor on PMNs and activate them when associated with the endothelial surface (15). Therefore, we asked if blocking the PAF receptor on PMNs inhibited their priming for enhanced granular secretion when they were incubated with thrombin-stimulated endothelial monolayers, as in Fig. 2. WEB 2086, a specific competitive antagonist for the PAF receptor on PMNs (46), blocked enhanced elastase release (Fig. 8), demonstrating that EC-associated PAF primes the leukocytes for enhanced secretion of primary as well as specific (Fig. 2) granule contents. In four of five experiments pretreatment of PMNs with the receptor antagonist also completely blocked priming for enhanced secretion of lactoferrin from PMNs adherent to thrombin-stimulated endothelial cells (see Fig. 2), and in a fifth experiment there was partial inhibition ($P < 0.05$ when the means were compared). In an additional experiment, blockade of the neutrophil PAF receptor completely inhibited the primed secretion of lactoferrin from PMNs adherent to endothelial monolayers activated with LTC_4.

The experiments described above, and others (14), indicated that PAF is the signaling molecule expressed by thrombin-stimulated endothelial cells that induces functional alterations in PMNs. PAF ligates a receptor that is linked via G-proteins to $[Ca^{2+}]_i$ regulatory mechanisms and activation of protein kinases (reviewed in reference 47). PAF in purified form or incorporated into model membranes can induce $[Ca^{2+}]_i$ transients, activation of CD11/CD18 integrins, and is a potent priming agent (12, 14, 15, 33, 47). Experiments indicating that PAF expressed by stimulated endothelial cells can mediate priming and induce $[Ca^{2+}]$ transients were also reported by others (11, 42). However, the role played by P-selectin was previously unrecognized. The ability of mAb G1 to inhibit the priming effect (Fig. 2), even though P-selectin does not directly mediate priming (Figs. 4 and 5), indicates that tethering of PMNs to the endothelial surface by P-selectin is necessary for an optimal priming response and that it facilitates their interaction with membrane-bound PAF (14). These experiments also show that inhibition of functional responses of target cells by antibodies that block tethering molecules, such as selectins, may indicate that adhesion mediated by them is required for the target cell to effectively interact with a second molecule that signals the functional event, rather than that the tethering molecule itself directly induces the response. The ability of anti-E-selectin antibodies to inhibit CD11b/CD18 activation and migration by PMNs adherent to cytokine-activated endothelium (10, 11, 48) may in part reflect such a mechanism, since endothelial cells stimulated with these cytokines express several signaling molecules, in addition to E-selectin (1, 49).

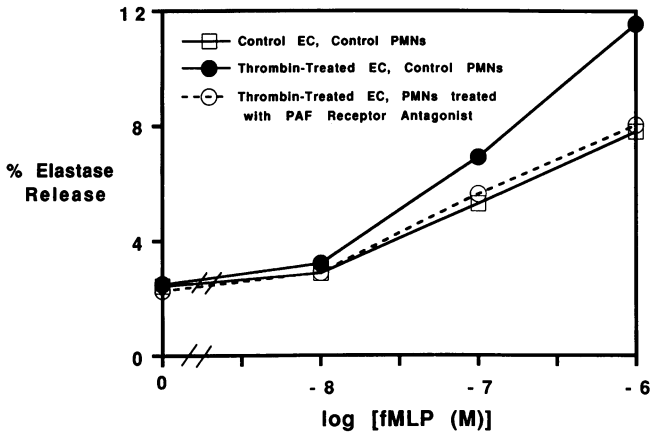

Figure 8. Blocking the PAF receptor on PMNs inhibits priming of granular secretion induced by thrombin-stimulated endothelium. The conditions for stimulation of the endothelial monolayers with thrombin, incubation with PMNs, and stimulation of the PMNs with FMLP were the same as in Fig. 2 except that the incubation period after addition of FMLP was 15 min, and PMNs were preincubated with control buffer or the competitive PAF receptor antagonist, WEB 2086 (4 μM), for 10 min before addition to the monolayers. In additional incubations (not shown) WEB 2086 blocked degranulation directly stimulated by PAF but not by other agonists (FMLP, PMA), documenting its specificity for the PAF receptor on PMNs under these conditions.

To further examine the roles of P-selectin and PAF, we determined the effect of P-selectin on polarization and spreading of PMNs. PMNs allowed to settle onto planar phospholipid membranes that contained no incorporated protein remained rounded, as predicted from an earlier study showing that surface receptors are not redistributed (50). PMNs on membranes containing glycophorin or W6/32, the control proteins, also were round. Similarly, PMNs adherent to P-selectin incorporated into membranes remained rounded and did not polarize or spread (Fig. 9 *A*). This indicates that P-selectin alone does not provide a signal for shape change. We found the same result in multiple experiments in which PMNs adhered to purified, immobilized, P-selectin. In contrast, when PMNs adherent to P-selectin in model membranes were stimulated with PAF, there was dramatic polarization and spreading (Fig. 9 *B*). Thus, this confirmed that surface-associated P-selectin can both tether PMNs and support their activation by PAF. Furthermore, while it is known that PAF can induce polarization of neutrophils in suspension or when they settle onto protein-coated surfaces (46), Fig. 9 demonstrates that polarization in response to PAF can occur when they are first captured by P-selectin. This result contrasts with an earlier report indicating that purified, immobilized P-selectin inhibits spreading of PMNs stimulated with a chemotactic factor (18; also see below), but it is consistent with our observation that PMNs polarize on the surfaces of endothelial cells that are induced to coexpress P-selectin and PAF (12, 13). PMNs adherent to P-selectin in model membranes also undergo shape change when stimulated with PMA (45).

Inflammatory roles of P-selectin. There are several important conclusions from these experiments. They show that coexpression of P-selectin and PAF by endothelial cells induces functional responses of PMNs (Figs. 2 and 8), in addition to mediating adhesion (14). A fluid-phase chemotactic factor is not required, demonstrating that responses such as priming can be initiated in a spatially specific manner at the endothelial

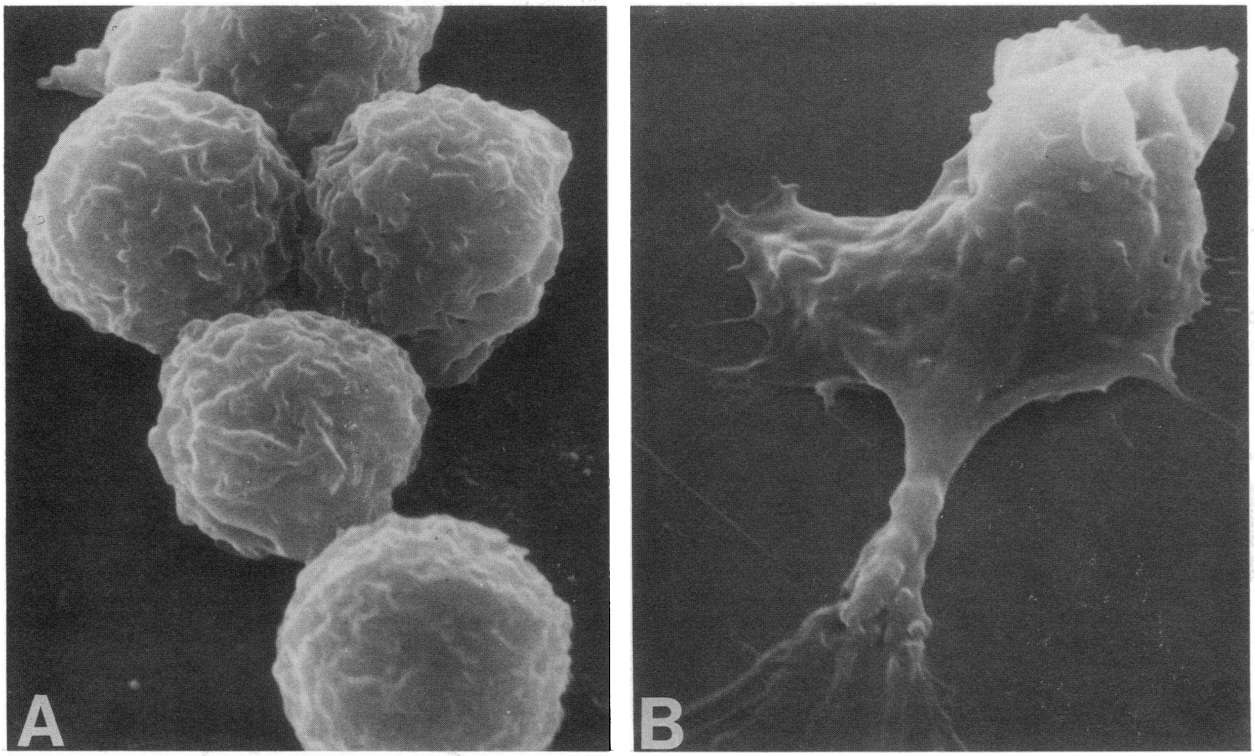

Figure 9. Stimulated PMNs polarize and spread while tethered by P-selectin to membrane surfaces. Planar membranes containing P-selectin and control membranes were prepared as described in Methods. PMNs were layered onto the planar membranes and, in some wells, were then stimulated with (PAF 10^{-6} M) for 10 min. The membranes and PMNs were fixed and viewed by scanning electron microscopy. (*A*) PMNs adherent to planar membranes with incorporated P-selectin remained round with minimal or no spreading. (*B*) When PMNs adherent to P-selectin in membranes were examined after stimulation with PAF, 95% of cells had undergone partial or complete shape change including elongation, spreading, or, as shown, development of well-defined lamellopodia and uropods.

surface. Antibody inhibition experiments indicated that P-selectin is involved in the priming process (Fig. 2), but experiments with purified P-selectin and transfected cells showed that its role is to facilitate interaction with PAF (Fig. 8), rather than to directly mediate priming (Figs. 4 and 5). We conclude that P-selectin is essential, but not sufficient, for optimal priming under these conditions. Similarly, purified P-selectin and transfected cells gave a uniform result in assays of other functional responses and indicate that P-selectin tethers PMNs but does not directly activate them; however, it supports $[Ca^{2+}]_i$ fluxes, CD11b/CD18 activation, and shape change induced by the signaling molecule, PAF (Figs. 3, 7, and 9, and text), or by other agonists, such as FMLP. Using different presentations of P-selectin and a variety of different assays of function, we found that the responses of PMNs exposed to P-selectin were equivalent to those of control PMNs or, in some cases, somewhat enhanced (Figs. 3 and 7, and data not shown). We saw no consistent evidence for inhibition of any PMN response, whether the P-selectin was presented in saturating concentrations in the fluid phase, immobilized in high density on plastic, or was incorporated into model membranes or expressed in the plasma membranes of transfected cells. This issue is important because of the earlier reports that P-selectin has inhibitory effects (16–19; and below). The latter observations raised the possibility that a role of P-selectin is to blunt, or modulate, responses of PMNs to PAF or other juxtacrine signals at the endothelial surface, akin to its putative role as a soluble inhibitor acting in the fluid milieu (16–19). Concomitant, or sequential, positive and negative signals are important regulatory mechanisms in a variety of intercellular interactions (51), lending credence to this idea. However, our experiments indicate that, instead, P-selectin in artificial surfaces and at the surfaces of endothelial cells facilitates PMN responses rather than blunting them. Thus, the coexpression of P-selectin and PAF is similar to the "adhesion and activation cascade" of costimulatory molecules that regulates degranulation of cytotoxic T lymphocytes and other responses of T cell subsets (52, 53), but the role of P-selectin appears to be distinct: to capture and tether the PMN, allowing stimulation by the juxtacrine signaling molecule, PAF, to occur (Fig. 10). Therefore, P-selectin has an additional function—to facilitate interaction of PMNs with other surface molecules—besides mediating rolling (45) and static (14) adhesive interactions of PMNs and endothelium.

While our experiments were aimed at defining roles of membrane-associated P-selectin, we also examined the effect of P-selectin presented to the PMN in the fluid phase to directly address the question of whether it inhibits PMN responses under this condition (16–19). In the first series of experiments, PMNs were loaded with Indo-1 and then treated with saturating concentrations (see above) of P-selectin, or with the same concentration of a control protein, fibrinogen. Neither P-selectin nor fibrinogen induced a $[Ca^{2+}]_i$ transient when presented to PMNs under these conditions. However, when PMNs were subsequently stimulated with PAF or FMLP, a $[Ca^{2+}]_i$ transient was induced; the magnitude of the increase in $[Ca^{2+}]_i$ in

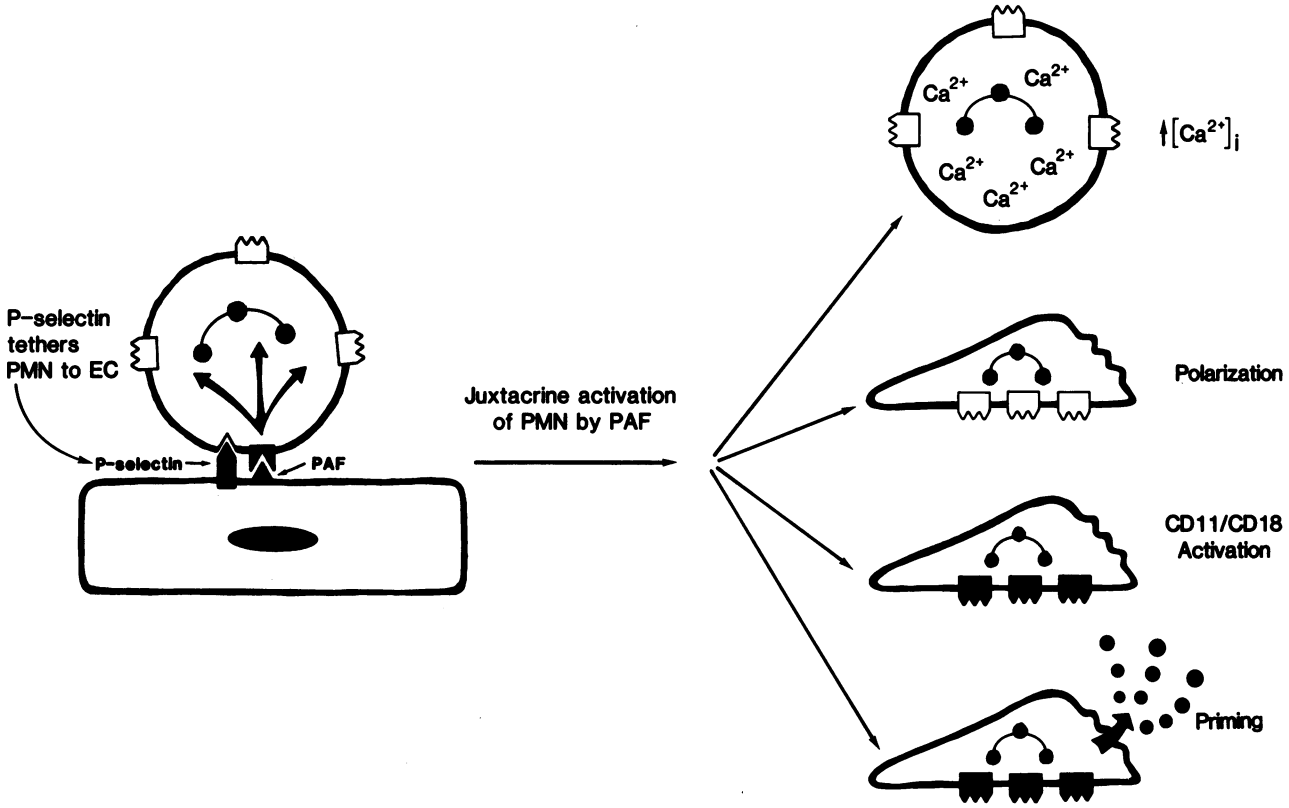

Figure 10. P-selectin and PAF coexpressed by activated endothelial cells mediate an adhesion and juxtacrine activation cascade. P-selectin tethers the PMN to the plasma membrane of the endothelial cell without activating it. This facilitates interaction with the signaling molecule, PAF. Juxtacrine stimulation of the PMN by PAF induces transient CD11/CD18 activation, which strengthens adhesion, and also induces $[Ca^{2+}]_i$ elevation, polarization, and priming for enhanced secretory responses.

neutrophils treated with P-selectin was the same as, or greater than, the increase in $[Ca^{2+}]_i$ in PMNs preincubated with fibrinogen (Fig. 11). This result, consistent in seven experiments, was similar to our experiments with P-selectin in model membranes (Fig. 3) and demonstrated that fluid-phase P-selectin does not globally inhibit signal transduction mechanisms. We also examined the effect of fluid phase presentation of P-selectin on CD11/CD18 expression and activity. Saturating concentrations of P-selectin did not inhibit quantitative upregulation of CD11b/CD18 when the PMNs were stimulated by FMLP (Fig. 11) and, in parallel incubations in the same experiment, did not inhibit expression of the NKI-L16 epitope on CD11a/CD18 (not shown). We previously showed that fluid-phase P-selectin does not inhibit agonist-stimulated PMN adhesion to resting EC (14), which is mediated by CD11a/CD18 and CD11b/CD18. Therefore, P-selectin in the fluid phase inhibited neither quantitative nor functional upregulation of PMN integrins. We also found that fluid-phase P-selectin supported, rather than inhibited, superoxide generation by agonist-stimulated PMNs ($n = 4$; $P = 0.77$ when PMNs pretreated with P-selectin were compared to fibrinogen-pretreated PMNs) (Fig. 11), granular secretion ($n = 5$; $P = 0.25$ for PMNs pretreated with P-selectin vs. fibrinogen), synthesis of PAF ($n = 2$), and synthesis and secretion of LTB_4 ($n = 1$) (not shown). Thus, solubilized P-selectin presented in the fluid phase behaved like the membrane-anchored or purified, immobilized molecule (Figs. 3–5 and 7). It is not clear why Gamble, Wong, and co-workers found inhibitory effects of solubilized P-selectin (17, 18) and we did not. A possible explanation is that maximal inhibition reported in some of their studies was seen at concentrations of fluid-phase P-selectin severalfold higher (10–20 μg/ml; 17, 18) than those calculated to saturate the specific binding sites on PMNs (1–5 μg/ml; 21, 22). In contrast, our experiments were done at, or in some experiments below, saturating concentrations. Experimental concentrations of 1–20 μg/ml considerably exceed the range that has been reported in normal human plasma (15; Ushiyama et al., manuscript submitted for publication) 36–250 ng/ml; however, it is currently unknown if higher concentrations are found in plasma or extravascular fluids in humans with inflammatory diseases. It is possible that at higher concentrations P-selectin binds to multiple low-affinity structures bearing sialyl LE^X or other sialylated glycans, in addition to the high-affinity ligand (2, 3, 5, 7), resulting in a different pattern of PMN behavior. One such structure may be the CD11b/CD18 integrin itself, which bears sialyl Le^X (54) and mediates or influences the responses that were reported to be inhibited by fluid phase P-selectin (17, 18).

P-selectin likely has complex roles in inflammatory syndromes. Genetically altered animals deficient in P-selectin have impaired leukocyte responses, indicating that it is important in physiologic inflammation (T. Mayadas, R. Hynes, and D. Wagner, personal communication of unpublished observations). It was recently shown to be expressed in vivo in models of inflammatory tissue injury (55–57) and to influence leukocyte-dependent thrombosis (58). In these conditions P-selectin will act, in sequence or in parallel, with juxtacrine signals such as PAF, or with other mediators. The experiments reported

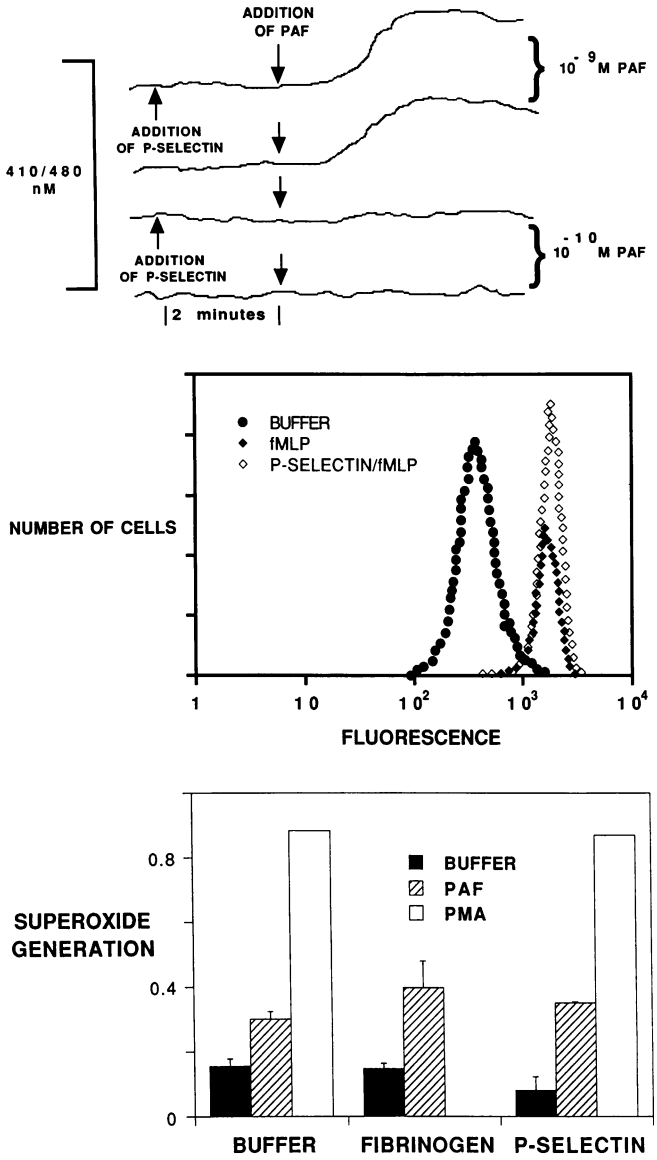

Figure 11. Solubilized, purified, P-selectin in the fluid phase supports PMN activation responses. *Upper panel:* $[Ca^{2+}]_i$. PMNs preloaded with INDO-1 were added to the wells of a spectrofluorimeter. P-selectin (2 μg/ml) was added to some wells, and the suspensions were incubated at 37°C for 2 min. PAF (at a subthreshold concentration, 10^{-10} M, or above the threshold, 10^{-9} M) was then added and $[Ca^{2+}]_i$ was recorded (Methods). The transients were traced from the recordings in one of seven experiments (see text). In some experiments PMNs were treated with fibrinogen, as an additional control, in parallel with the P-selectin incubations (not shown). There was no consistent difference in the $[Ca^{2+}]_i$ transients in PMNs pretreated with fibrinogen, P-selectin, or buffer alone. *Middle panel:* Quantitative upregulation of CD11b/CD18. PMNs were incubated with: buffer alone; buffer for 5 min at room temperature, followed by FMLP for 30 min; or with P-selectin (2 μg/ml) for 5 min followed by FMLP (10^{-6} M) for 30 min at 37°C. They were then incubated with mAB 60.1 and analyzed by flow cytometry as described in Methods. *Lower panel:* Superoxide generation. PMNs (10^6/ml) were incubated with buffer alone, P-selectin (4 μg/ml), or fibrinogen (4 μg/ml) for 20 min at room temperature. PAF (10^{-8} M in the first experiment, 10^{-7} M in the second), which is a weak agonist for superoxide generation, or PMA (10^{-7} M), a strong agonist, was added and the cells were incubated for an additional 30 min at 37°C. Superoxide anion was measured as described in Methods. The bars indicate the means of determinations in three experiments with PAF and one with PMA.

here provide a basis for evaluating roles of P-selectin that involve the PMN, a vanguard cell in the acute inflammatory response.

Acknowledgments

The authors thank Donelle Benson, Susan Cowley, Holly Nichols, Margaret Vogel, and Linda Wilcox for excellent technical assistance; Linda Jara, Michelle Bills, and Mary Cushing for preparation of the figures and manuscript; and the staff of the LDS Hospital Labor and Delivery Service for invaluable help in collecting umbilical samples. We also thank Magali Disdier for the contribution of transfected cells; Carl Figdor, T. K. Kishimoto, and Tom Tedder for the gifts of antibodies; John Fenton for the gift of purified thrombin; and John Bohnsack, Mark Elstad, and Kevin Moore for helpful discussions.

This work was supported by the Nora Eccles Treadwell Foundation, the Department of Veterans Affairs Medical Research Program, Established Investigator Awards (Drs. Prescott and Zimmerman) from the American Heart Association (85-204 and 87-22), grants from the National Institutes of Health (HL-44525, HL-43689, HL-07636, HL-34363, and HL-45510), a Research Career Development Award (01733) from the National Institutes of Health (Dr. McEver), and a Physician Scientist Award (HL-02726) from the National Institute of Health (Dr. Lorant). Diane Lorant was supported by a training grant (HL-07636) and Matthew Topham by an institutional Basic Sciences Research Support Grant from the National Institutes of Health while some of this work was being done.

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