P-Selectin and Platelet-activating Factor Mediate Initial Endotoxin-induced Neutropenia

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Summary
Polymorphonuclear neutrophil (PMN) accumulation within damaged tissues, a hallmark of acute inflammation, is dependent upon initial adhesion to endothelial cells. In vitro studies suggest that P-selectin and platelet activating factor (PAF) are key molecules in this process by promoting the initial adhesion of PMN to endothelial cells. We report in vivo studies in which intravenous administration of lipopolysaccharide (LPS) to anesthetized rats caused a very rapid onset (<5 min) of neutropenia, in association with induction of surface expression of P-selectin on microvascular endothelial cells in kidney, liver and lung; analogous induction of P-selectin expression by cultured endothelial cells was observed in response to LPS stimulation in vitro. In addition, treatment with an antibody (Ab) to P-selectin (or use of a PAF antagonist) blocked development of neutropenia in vivo for at least 15 min post-LPS injection, and Ab treatment was shown to block PMN accumulation in tissues. These studies document roles for P-selectin and PAF in the early adhesion of PMN to endothelial cells in vivo.

Inflammation is usually a beneficial process leading to destruction or isolation of injurious agents and tissue repair. However, in situations such as gram-negative sepsis, this response may be excessive, and cellular infiltration, endothelial damage, and vascular leakage can lead to extensive tissue injury. Accumulation of leukocytes, particularly PMN, is an important and early component of this inflammatory response (1). A three-step mechanism for the binding of PMN to endothelial cells is proposed (2), whereby reversible adhesion through binding to selectin molecules is followed by PMN activation and activation-dependent binding. The nature of the molecules involved in these interactions appears to depend upon the type and length of exposure of the inflammatory stimulus. P-selectin (also known as CD-62, GMP-140, or PADGEM) is normally located in α granules of platelets and the Weibel-Palade bodies of endothelial cells, but is rapidly expressed on the surface of stimulated endothelial cells (3, 4). Surface-expressed P-selectin mediates the “rolling” of PMN, whereby the cells are slowed and brought into close vicinity of the endothelium, allowing further adhesive interactions to occur (5). In vitro, P-selectin expression is rapidly induced by thrombin or histamine, peaks at 5–10 min after stimulation and, because of reinternalization, is largely absent by 20 min (3, 6), suggesting that a transient appearance in membrane P-selectin may be of significance in some of the earliest events of inflammation.

Platelet activating factor (PAF) is a potent, biologically-active phospholipid implicated in many inflammatory reactions, including endotoxic shock (1) and endotoxin-induced lung injury (7). Like P-selectin, PAF expression by endothelial cells is maximal within 5–10 min of stimulation and returns to background levels shortly thereafter (6, 8). PAF contributes to PMN adhesion and activation, and PAF-primed PMNs damage endothelial cells (1, 9). Moreover, treatment of endothelial cells with the combination of an anti-P-selectin Ab and PAF-antagonist completely blocks PMN adhesion in vitro (6). This observation, plus the rapid and transient nature of their surface-bound expression on endothelial cells, have led to the concept that P-selectin and PAF act synergistically in promoting the early adhesion of PMN to endothelium. Specifically, rapid endothelial expression of P-selectin could mediate the initial tethering of PMN to endothelial cells, independently of CD11/CD18 integrins, whereas associated endothelial PAF expression induces juxtacrine activation of surface CD11/18 molecules on PMN and their high affinity binding to endothelial-expressed ICAM molecules (2, 6, 9). Targeting of P-selectin and PAF may thereby prove therapeutic efficacy in blocking initial PMN adhesion to endothelium in vivo.

To this end, since LPS is present within the cell wall of gram-negative bacteria, is implicated in the pathogenesis of septic shock (10), and is known to induce a marked neutropenia...
upon intravenous administration (11), we investigated the role of P-selectin and PAF in early PMN adhesion to endothelium in vivo, using a rat model of systemic endotoxemia.

Materials and Methods

Reagents and Cells. LPS and lactoperoxidase were purchased from Sigma Chemical Co. (St. Louis, MO). RPMI 1640 and FCS were obtained from Whittaker Bioproducts (Walkersville, MD), and 125I was supplied by Amersham Radiochemicals (Amersham, UK). Thrombin was purified as described (12). Details of a blocking, affinity-purified rabbit anti-human P-selectin Ab and control, nonimmune rabbit IgG (13), plus a method for their pepsin digestion and purification as F(ab')2 fragments, were reported previously (14). Anti-rat P-selectin mAb (RP-3) (15) was a gift of Dr. F. Sendo (Yamagata University School of Medicine, Yamagata, Japan). The synthetic PAF analogue, CV-3988 (16), was obtained from Takeda Chemical Industries (Osaka, Japan) and dissolved in 0.9% saline just before injection. HL-60 cells, obtained from American Type Culture Collection (Rockville, MD), were cultured under standard conditions, and platelets were isolated from citrated plasma (13).

Effect of Anti-P-selectin Ab on Rat P-selectin In Vitro. The ability of anti-P-selectin Ab to block rat P-selectin was tested by study of neutralization of rat platelet rosette formation with HL-60 cells (13). Briefly, thrombin-activated human or rat platelets (2 x 10^9/ml) were preincubated with specific Ab or control Ig for 20 min before addition to HL-60 cells (2 x 10^5/ml); platelet rosetting was evaluated on a cell suspension by scoring a minimum of 100 cells/assay.

Induction of P-selectin on Endothelial Cells In Vitro. Human umbilical endothelial cells were cultured in 25-cm^2 flasks (Costar Corp., Cambridge, MA) according to Jaffe et al. (17). Actively growing, subconfluent cultures (second passage, ~10,000 cells/well), or in two experiments, confluent endothelial cell cultures, were washed six times in buffer (PBS/0.5% human serum albumin), stimulated with thrombin (1 U/ml) or LPS (1 mg/ml) for 10 min at room temperature, and washed six times in buffer. Surface-radiolabeling using 125I-lactoperoxidase, immunoprecipitation with anti-P-selectin or control Ab, and analysis by gel electrophoresis and autoradiography were performed as described by Goding (18). Briefly, endothelial cells were iodinated, scraped, and solubilized using 0.1 M-Tris/0.1% EDTA/0.1% NP-40 (1 h, 4°C), nuclei pelleted by centrifugation, and the endothelial cells lysate precleared by two incubations with nonimmune serum, followed by specific Ab, six washes, SDS-PAGE (10% gels run under reducing conditions), and autoradiography overnight at -70°C.

In Vivo Model of Endotoxemia. Specific-pathogen-free male Brown Norway rats (100–200 g) were obtained from the Animal Resource Center (Willetton, WA). To assess the effects of endotoxin administration on circulating PMN, rats were anesthetized with ether and injected with LPS (5 mg/kg i.p.). The effects of P-selectin Ab were determined by intravenous injection of whole or F(ab')2-fragment of anti-P-selectin Ab or control rabbit Ig before LPS administration. An Ab concentration of 2.5 mg/kg was used, based upon preliminary dose/response data. The PAF antagonist, tested at doses from 5–40 mg/kg, was injected pre-LPS, either alone or in combination with anti-P-selectin Ab. Blood samples (0.5 ml) were collected in sterile glass tubes containing 10 μM EDTA just before LPS injection, and thereafter at 5-min intervals for 20 min, to allow serial analysis of PMN levels and, as appropriate, measurement of the concentration of rabbit Ig. Data were collected from five rats/treatment group per time-point.

Quantitation of Circulating PMN. Acridine orange/ethidium bromide (0.1%) was added to aliquots of blood, to assist in recognition of leukocytes vs. RBC, and total leukocyte counts were performed using a Neubauer hemocytometer under a phase microscope equipped for fluorescent microscopy. Differential cell counts were performed on hematoxylin and eosin-stained, methanol-fixed blood smears; 500 leukocytes/smear were counted, and total PMN numbers were determined by reference to total leukocyte counts. Given variable PMN counts, results were expressed as a percentage of the PMN count for that animal pre-LPS injection. The statistical significance of data was assessed using the Instat program (GraphPad Software, San Francisco, CA); p <0.05 in the nonparametric Mann-Whitney U-test was considered significant.

Detection of Rabbit Ig by ELISA. Serial circulating levels of rabbit Ig in Ab-injected rats were determined by ELISA, as described (14), using appropriate peroxidase-conjugated goat antibodies (The Jackson Laboratories, Bar Harbor, ME) to detect either whole or F(ab')2-Ab fragments.

Immunohistologic Studies. After rats were killed under anesthesia, lung, liver, and kidney tissues were removed from normal or LPS-injected rats (5, 10, 15, or 20 min post-injection), plus rats given rabbit Ab (anti-P-selectin or control Ig) or PAF antagonist before LPS injection. Tissues were snap-frozen in liquid nitrogen and stored at -70°C. P-selectin expression and PMN infiltration were assessed in cryostat sections stained using a peroxidase-antiperoxidase method (19). Since preliminary studies showed a close correlation between morphologic detection and RP3 mAb labeling, PMN infiltration was determined by counting RP3+ cells (20 high power fields/tissue section, 3 levels within each tissue) and comparing data between groups (n = 3/group), as described for circulating PMN.

Results and Discussion

Anti-P-selectin Ab Blocks Rat P-selectin Function In Vitro. Anti-P-selectin Ab blocked the binding of thrombin-activated human rat platelets to HL-60 cells in vitro (Fig. 1), whereas control rabbit Ig had no effect, indicating the ability of an anti-human P-selectin Ab to block rat P-selectin, as was also reported by Mulligan et al. (20).

LPS Stimulates Surface Expression of P-selectin by Cultured Endothelial Cells. Treatment of endothelial cells in vitro (whether subconfluent or confluent) with thrombin or LPS resulted in the surface expression of a 140-kD protein as shown by SDS-PAGE analysis (Fig. 2). Analogous results were seen by immunofluorescent staining of cultured endothelial cells

Figure 1. Neutralization of rat P-selectin in vitro by anti-P-selectin Ab. Rosetting of HL-60 cells by thrombin-activated human (shaded bars) or rat platelets (open bars) in the presence of buffer alone (A), Fab fragments of anti-P-selectin at 5 μg/ml (B), or 100 μg/ml (C), or Fab fragments of rabbit Ig at 100 μg/ml (D). Results are representative of three experiments.
Poolings in various organs.

but not after its surface expression by stimulated endothelial
cells. Endothelial cells were treated with tissue culture me-
dium alone (lane A), or medium plus thrombin (lane B), or LPS
(lane C), followed by surface radiolabeling, lysis, immuno precipita-
tion with affinity-purified rabbit anti-P-selectin Ab, separation by
10% SDS-PAGE under reducing conditions, and autoradiography.
Molecular weight markers were myosin (200,000), β-galactosi-
dase (116,000), phosphorylase B (97,000), BSA (66,000), OVA
(45,000).

findings suggest that the PMN were removed from the cir-
culation by adherence to rapidly induced endothelial adhesion
molecules, or simply by pooling within highly vascular-
ized tissues in the absence of increased endothelial adhesivity,
or a combination of the two.

Adherence of PMN to P-selectin in vivo was shown by
the effect of injection of anti-P-selectin Ab immediately be-
fore LPS administration, which resulted in the mainte-
ance of PMN levels at ≥90% of baseline for the first 15 min (Fig.
4). Results were significantly greater than in rats receiving
LPS plus control Ig (Mann-Whitney U-test, p <0.01 at 5
and 10 min), and comparable effects were seen using an
F(ab')2 fragment of the anti-P-selectin Ab (not shown).
Treatment with control rabbit Ab (Fig. 4) did not significantly
differ from treatment with LPS alone (Mann-Whitney U-test; p >0.2 at all time points). Regardless of anti-P-selectin Ab
administration, PMN counts in LPS-injected animals pro-
gressively declined after 15–20 min. This effect was not due
to clearance of whole or F(ab')2 Ab from the circulation
since ELISA measurements showed persistence of rabbit Ig
levels at ≥5 µg/ml at all times (not shown). These data sug-
gest that the rapid onset of neutropenia induced by LPS ad-
ministration is due to adhesion of PMN to P-selectin expressed
on the surface of endothelial cells, but that P-selectin expres-
sion is not the mechanism for persistence of neutropenia be-
yond 15–20 min post-LPS injection in this model. Anti-P-
selectin Ab treatment had no effect on LPS-induced TNF
production in vivo as assessed by assay of serum samples (not
shown).

Table 1. Anti-P-selectin Ab Blocks PMN Accumulation in
Kidney and Liver

| Treatment group   | Liver     | Kidney   |
|-------------------|-----------|----------|
| Nil               | 0.3 ± 0.5*| 0.1 ± 0.3*|
| LPS               | 8.7 ± 5.6 | 14.6 ± 7.7 |
| LPS/control Ig    | 9.5 ± 7.1 | 12.9 ± 6.2 |
| LPS/anti-P-selectin Ab | 0.5 ± 0.4* | 0.6 ± 0.6*|

All values indicate mean ± SD PMN/high power field in normal rats or 15 min post-injection with LPS ± Ab, as indicated, using 20 fields/rat and 3 rats/group.

*p <0.0001 compared with LPS or LPS/control Ig-treated groups.
Figure 3. Immunoperoxidase localization of P-selectin and PMN in cryostat sections of rat liver (a–d) and kidney (e–h). (a) No labeling for P-selectin was seen in rat liver before injection of LPS, whereas (b) at 5 min post-LPS injection, P-selectin was demonstrated on sinusoidal capillaries as well as arterioles (granular labeling, lower right) and venules. (c) Sections of livers from rats injected with LPS and anti-P-selectin Ab lacked significant PMN infiltration (one RP-3+ PMN is present, lower center) at 15 min post-LPS injection; but (d) corresponding sections from rats receiving LPS plus control Ig showed widespread PMN infiltration. Similarly to liver, (e) sections of kidneys from normal rats lacked P-selectin expression, including glomeruli (G), whereas (f) within 5 min of LPS injection, widespread microvascular labeling for P-selectin was detected, including G capillaries, intertubular capillaries, and arterioles; the contracted arteriole at center top of the panel shows dense surface labeling in association with multiple adherent leukocytes. (g) Treatment with an anti-P-selectin Ab blocked PMN infiltration, as assessed by labeling with RP3 mAb at 15 min post-LPS injection, whereas (h) many PMN were detected within renal interstitial areas and glomeruli of corresponding rats injected with LPS plus control Ig. (Hematoxylin counterstain; all panels ×400 original magnification, except panel f, ×630.)
Figure 4. Effect of pretreatment with anti-P-selectin Ab or control Ig on LPS-induced neutropenia. Circulating PMN counts (mean ± SEM) are shown (n = 5/time-point), expressed as a percentage of each animal's pretreatment PMN count. Development of LPS-induced neutropenia (□—□) was blocked by pretreatment with anti-P-selectin Ab (○—○) but not control Ig (■—■). Results at 5 and 10 min post-LPS injection were highly significant (* p < 0.01, Mann-Whitney U-test) in anti-P-selectin Ab-treated animals compared with the control Ab-treated group, whose PMN counts fell in close similarity to rats receiving LPS alone. Results at 15 and 20 min post-LPS showed only marginally significant (p = 0.056) effects.

combinations was precluded by the limited availability of the polyclonal Ab. In addition, these studies do not preclude a role for the indirect action of endotoxin via effects on complement, platelets, histamine release, PMN deformability, or other factors (23, 24).

These studies show that LPS causes a rapid and profound degree of neutropenia in vivo, in association with stimulation of endothelial cell surface P-selectin expression. Targeting of either P-selectin or PAF maintains circulating neutrophils levels and provides initial protection of the host against the potent effect of LPS. However, consistent with the transient expression of these molecules shown by previous in vitro studies, targeting of P-selectin or PAF does not prevent progressive development of neutropenia after 20 min. These in vivo data provide evidence for an early and transient role of P-selectin and PAF in inflammation, and experimental support for a multistep model of leukocyte adhesion to endothelium.

We thank Caroline Butcher for technical assistance.

This work was supported by grants from the National Health and Medical Research Council and National Heart Foundation of Australia. L. C. Dunlop is the recipient of a National Health and Medical Research Council Postgraduate Fellowship.

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Received for publication 23 July 1993 and in revised form 4 October 1993.
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