An Anti–Platelet-Endothelial Cell Adhesion Molecule-1 Antibody Inhibits Leukocyte Extravasation from Mesenteric Microvessels In Vivo by Blocking the Passage through the Basement Membrane

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Summary

Platelet-endothelial cell adhesion molecule-1 (PECAM-1, CD31) plays an active role in the process of leukocyte migration through cultured endothelial cells in vitro and anti-PECAM-1 antibodies (Abs) inhibit accumulation of leukocytes into sites of inflammation in vivo. Despite the latter, it is still not clear at which stage of leukocyte emigration in vivo PECAM-1 is involved. To address this point directly, we studied the effect of an anti-PECAM-1 Ab, recognizing rat PECAM-1, on leukocyte responses within rat mesenteric microvessels using intravital microscopy. In mesenteric preparations activated by interleukin (IL)-1β, the anti-PECAM-1 Ab had no significant effect on the rolling or adhesion of leukocytes, but inhibited their migration into the surrounding extravascular tissue in a dose-dependent manner. Although in some vessel segments these leukocytes had come to a halt within the vascular lumen, often the leukocytes appeared to be trapped within the vessel wall. Analysis of these sections by electron microscopy revealed that the leukocytes had passed through endothelial cell junctions but not the basement membrane. In contrast to the effect of the Ab in mesenteric preparations treated with IL-1β, leukocyte extravasation induced by topical or intraperitoneal administration of the chemotactic peptide formyl-methionyl-leucyl-phenylalanine was not inhibited by the anti-PECAM-1 Ab. These results directly demonstrate a role for PECAM-1 in leukocyte extravasation in vivo and indicate that this involvement is selective for leukocyte extravasation elicited by certain inflammatory mediators. Further, our findings provide the first in vivo indication that PECAM-1 may have an important role in triggering the passage of leukocytes through the perivascular basement membrane.

The migration of leukocytes from the microvasculature into the tissue is an essential part of an inflammatory reaction. This response is mediated by the adhesive interaction of leukocytes with venular endothelial cells and appears to involve multiple sequential steps (1). The initial interaction appears to be that of a weak reversible adhesion between leukocytes and endothelial cells resulting in the rolling of leukocytes along the venular wall. There is now much in vitro and in vivo evidence demonstrating the importance of the selectin family of adhesion molecules in this phase of the response (2), although more recently, members of the integrin family have also been implicated in the phenomenon of leukocyte rolling (3–5). Rolling cells can then become activated by chemotactic stimuli expressed/ bound on the surface of endothelial cells or in solution at the site of inflammation. The interaction of activated leukocyte adhesion molecules, primarily members of the β_1 and β_2 integrins, with their endothelial cell ligands, such as intercellular adhesion molecule-1 (ICAM-1)[1] ICAM-2, or vascular cell adhesion molecule-1 (VCAM-1) enables rolling leukocytes to establish a firm adhesive bond with the vascular endothelium. Interestingly, the firm attachment of leukocytes to venular endothelial cells itself appears to trig-

[1]Abbreviations used in this paper: ICAM-1, intercellular adhesion molecule-1; PAF, platelet-activating factor; PECAM-1, platelet-endothelial cell adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1.

ger a secondary activation event within the leukocytes (6), and perhaps the endothelial cells, that may mediate the flattening of the leukocytes over the endothelium, enabling them to locate and penetrate interendothelial cell junctions. Despite the increasing number of in vivo studies investigating the molecular interactions involved in the rolling and firm adhesion of leukocytes, there is much less information regarding the in vivo mechanisms mediating the passage of leukocytes across the vessel wall.

Platelet-endothelial cell adhesion molecule-1 (PECAM-1), a member of the Ig superfamily, is a 130-kD glycoprotein expressed on the cell surface of platelets, leukocytes, and endothelial cells. The expression of PECAM-1 on cultured endothelial cells is concentrated at cell–cell junctions (7, 8), and there is now strong evidence demonstrating a role for this molecule in the passage of neutrophils and monocytes across resting and cytokine-activated endothelial monolayers in vitro (9). In three different in vivo models of acute inflammation, neutrophil accumulation into inflamed peritoneum of rats, neutrophil accumulation into airways after local deposition of IgG immune complexes in rat lungs, and neutrophil accumulation induced by intradermal TNF-α into human skin grafts transplanted onto immunodeficient mice, Vaporciyan et al. (10) found that an anti-PECAM-1 Ab inhibited the emigration of neutrophils. Similarly, an anti-murine PECAM-1 antibody inhibited thioglycollate-induced leukocyte accumulation into mouse peritoneum (11). Despite these findings, the precise role of PECAM-1 in leukocyte recruitment in vivo is still not fully understood. The aim of the present study was to investigate directly by intravital and electron microscopy the stage of leukocyte emigration at which PECAM-1 was involved, and thus determine more precisely the role of PECAM-1 in leukocyte extravasation in vivo.

Materials and Methods

Animals. Male Sprague-Dawley rats (250–300 g) were purchased from Harlan-Olac (Bicester, Oxfordshire, UK).

Materials. Pentobarbitone sodium (Sagatal, 60 mg/ml) was purchased from Rhone Merieux Ltd. (Harlow, Essex, UK). Hypnorm (0.315 mg/ml fentanyl citrate and 10 mg/ml fluanisone) was from Janssen Pharmaceutical Ltd. (Grove, UK). FMLP and rabbit IgG, purified from normal serum, were from Sigma Chemical Co. (Poole, Dorset, UK). Recombinant rat IL-1β was a gift from Dr. K. Vosbeck (Ciba-Geigy Ltd., Basel, Switzerland). The anti-PECAM-1 antibody was a rabbit polyclonal antibody generated by immunization with purified human PECAM-1. This Ab cross-reacts with rat PECAM-1 as previously described (10). Purified Ab from the preimmune serum was used as control Ab in some experiments. Both antibodies were prepared by protein G–Sepharose chromatography. A murine anti–rat MHC class I mAb from Harlan Sera-Lab (Crawley Down, Sussex, UK) was used as a second control antibody. All antibodies were assayed for endotoxin levels using a Limulus Amebocyte Lysate [QCL 1000] kit, from BioWhittaker Inc. (Walkersville, MD). The antibodies used in the study had endotoxin levels of <3.5 ng/ml. This meant that the rats were injected with <0.8 ng of endotoxin. Rats injected with the anti-PECAM-1 Ab were in-

jected with <0.2 ng of endotoxin and the rats injected with the purified rabbit IgG were injected with <0.8 ng of endotoxin.

Intravital Microscopy. Animals were prepared for intravital microscopy as previously described (12). Briefly, rats were sedated with an intramuscular injection of Hypnorm (0.2 ml/rat) and injected intraperitoneally with either 5 ml of sterile saline, 5 ml of sterile saline containing 10 ng IL-1β, or 5 ml of sterile saline containing 220 ng of FMLP. Animals were pretreated, 15 min before IL-1β or FMLP administration, with either anti-PECAM-1 antibody (1 or 5 mg/kg), control rabbit IgG (in some experiments, purified from preimmune serum), or an anti-MHC class I mAb, injected via a lateral tail vein. 3.5 h later, the rats were resedated intramuscularly with Hypnorm (0.1 ml) and anesthetized intravenously with Sagatal (20 mg/kg). Anesthesia was maintained intravenously with 20 mg/kg/h of Sagatal. Animals were then placed on a heated stage (37°C) and a 1–2-cm midline abdominal incision was made to expose the small intestine. A segment of the intestine was carefully exteriorized and placed over a transparent circular plastic mount and pinned in place. The exposed tissue was continuously superfused with sterile Tyrode solution maintained at 37°C and gassed with 5% CO_2 in air. The whole preparation was mounted onto the stage of a Diaplan microscope (Leitz, Germany) and the mesenteric microcirculation was viewed using high magnification water-dipping objectives. A camera (model WV CL702 CCD; Panasonic), mounted on the microscope relayed the image onto a color monitor (Sony Trinitron) and the images were recorded using an S-VHS video cassette recorder (model HR-54700EK; JVC) and a color video printer (model CVP-M3E, Sony). To determine the effect of the antibodies on circulating leukocyte numbers, blood samples were taken from the rats before and 4 h after antibody treatment.

In each animal, leukocyte responses were measured in three to five randomly selected postcapillary venules ranging between 20 and 35 μm in diameter and of at least 400 μm in length. In each vessel, three parameters were measured: leukocyte rolling, adhesion, and extravasation, as previously described (12). Rolling leukocytes were defined as cells visibly moving along the endothelium. For each vessel, the number of rolling cells per minute passing a reference point was counted over a 4-min time period. Adherent leukocytes were defined as cells that remained stationary within the vascular lumen for a period of at least 30 s. Adherent cells were counted in at least four consecutive 100-μm vessel segments for every vessel. Leukocyte emigration from the microcirculation into the tissue was quantified by counting the number of cells that had emigrated out of the vessel up to 50, 50–100, and 100–150 μm away from the vessel wall in parallel with 100-μm vessel segments. At least four readings were taken for each vessel at different distances from the vessel wall.

In a second series of experiments designed to investigate the effect of the anti-PECAM-1 Ab on rapid leukocyte responses elicited by topical FMLP, rats were prepared for intravital microscopy as described above; basal readings of rolling, adhesion, and extravasation were recorded for a period of 15 min in one venule of 20–35 μm in diameter. The anti-PECAM-1 antibody or a control antibody, both at 5 mg/kg, was then administered intravenously; readings of the three leukocyte responses were taken for a further 15-min period. The exteriorized mesentery was then superfused with FMLP (final concentration of 10^{-7} M, in Tyrode solution maintained at 37°C) for up to 30 min and measurements of leukocyte rolling, adhesion, and extravasation were taken at different time points throughout the duration of the experiment.

Preparation of Mesenteric Tissues for Electron Microscopy. At the end of intravital microscopy studies, the animal was killed by a lethal
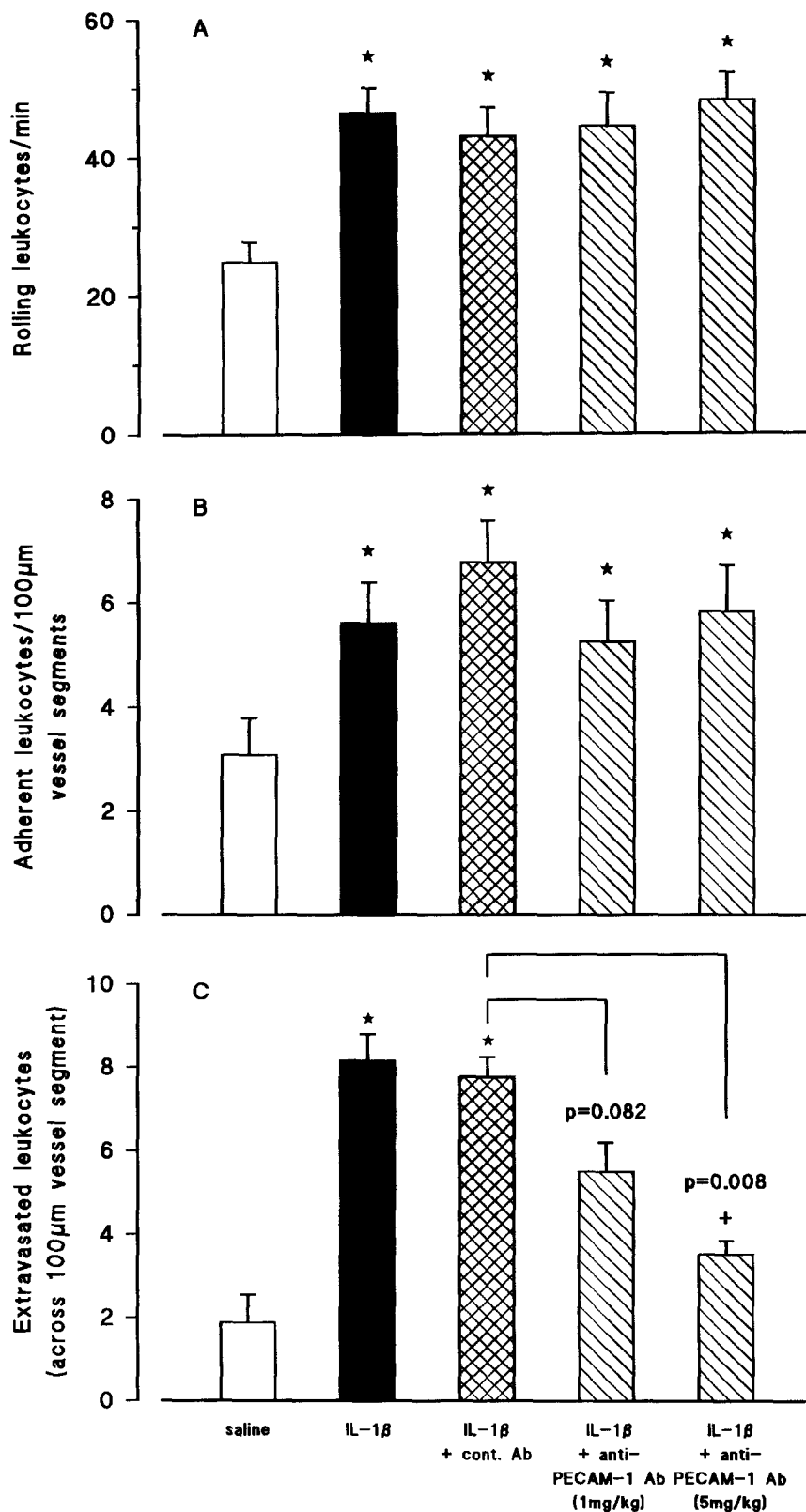
Figure 1. Effect of anti-PECAM-1 Ab on IL-1β–induced leukocyte responses within rat mesenteric microvessels. Rats were treated intraperitoneally with saline (□) or 10 ng IL-1β (■). In further groups of animals, rats were treated with a control Ab (rabbit IgG, 5 mg/kg i.v., ▨) or the anti-PECAM-1 Ab (1 or 5 mg/kg i.v., ▩), 15 min before the i.p. administration of IL-1β. 4 h later, the mesenteric tissue was exteriorized and the responses of leukocyte rolling (*A*), adhesion (*B*), and extravasation (*C*) quantified. Results are mean ± SEM for $n = 5$ animals. A significant difference between saline- and IL-1β- or IL-1β + control Ab-treated animals is shown by (⋆)$P < 0.05$. A significant difference between IL-1β + control Ab and IL-1β + anti-PECAM-1 Ab is shown by (+)$P < 0.01$.

overdose of sodium pentobarbitone and the exteriorized mesentery superfused with 2.5% glutaraldehyde (in 0.05 M sodium cacodylate buffer, pH 7.2) for 10 min. The area containing the vessels of interest was then removed and further fixed in 2.5%

glutaraldehyde overnight. After washing in cacodylate buffer, the mesenteric section was postfixed in 1% osmium tetroxide for 1 h, dehydrated in a series of solutions with increasing methanol concentrations in water, and transferred to propylene oxide, after which

the section was embedded horizontally in araldite. Smaller blocks containing the vessels of interest were cut out and mounted vertically for sectioning. Sections of vessels (1-μm-thick) were stained with alkaline toluidine blue and viewed by light microscopy. For electron microscopy, ultrathin sections were stained with uranyl acetate and lead citrate. Mesenteric sections from control Ab- and anti-PECAM-1–treated animals were prepared for electron microscopy in parallel.

Statistics. Data are expressed as the mean ± SEM for n animals and analyzed using the Mann-Whitney nonparametric test, or Student's t test when appropriate, for the comparison of two samples. P values of <0.05 were considered statistically significant.

Results

Effect of Anti-PECAM-1 Ab on Leukocyte Responses in IL-1β–activated Microvessels. In rats injected intraperitoneally with saline, there was a basal level of leukocyte rolling, ranging between 16 and 34 leukocytes per minute (25 ± 3.0, mean ± SEM, $n = 5$), a low level of leukocyte adhesion within the venules and a small number of emigrated leukocytes in the extravascular tissue (Fig. 1 and Fig. 2 A).

In animals treated with IL-1β (10 ng i.p. for 4 h), all three leukocyte responses measured were significantly increased (P <0.05, $n = 5$), with leukocyte rolling increasing to a range between 37 and 57 leukocytes per minute (Fig. 1 and Fig. 2 B). IL-1β increased the number of cells that had emigrated into the surrounding tissue at all three measurement points studied (Fig. 3).

Pretreatment of rats with control antibody (purified rabbit IgG, 5 mg/kg i.v.), had no significant effect on leukocyte responses in mesenteric preparations treated with IL-1β ($n = 5$, Fig. 1). Similarly, in two rats pretreated with antibody purified from preimmune serum (5 mg/kg i.v.), leukocyte responses elicited by IL-1β were not different from those obtained in untreated rats (data not shown). In contrast, anti-PECAM-1 Ab (1 or 5 mg/kg i.v.) had no significant effect on the leukocyte responses of rolling and adhesion, but dose dependently inhibited the number of leukocytes that had migrated into the tissue (0–50 μm from the venules) as compared with rats treated with IL-1β or IL-1β plus control Ab (39 and 72% inhibition at 1 and 5 mg/kg, respectively, Fig. 1 and Fig. 2 C). The leukocyte extravasation detected in mesenteric preparations activated with IL-1β in
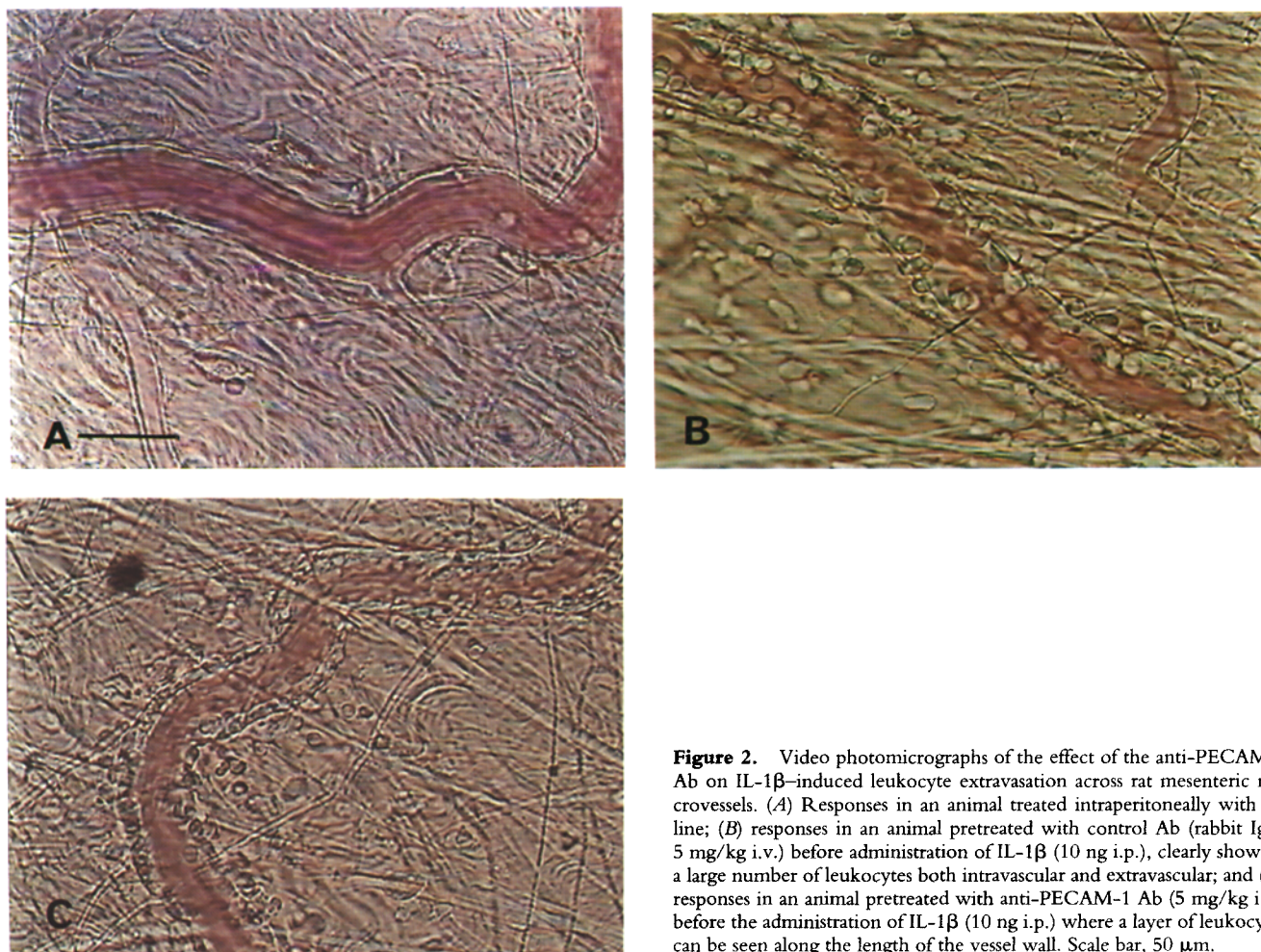

Figure 2. Video photomicrographs of the effect of the anti-PECAM-1 Ab on IL-1β–induced leukocyte extravasation across rat mesenteric microvessels. (*A*) Responses in an animal treated intraperitoneally with saline; (*B*) responses in an animal pretreated with control Ab (rabbit IgG, 5 mg/kg i.v.) before administration of IL-1β (10 ng i.p.), clearly showing a large number of leukocytes both intravascular and extravascular; and (*C*) responses in an animal pretreated with anti-PECAM-1 Ab (5 mg/kg i.v.) before the administration of IL-1β (10 ng i.p.) where a layer of leukocytes can be seen along the length of the vessel wall. Scale bar, 50 μm.
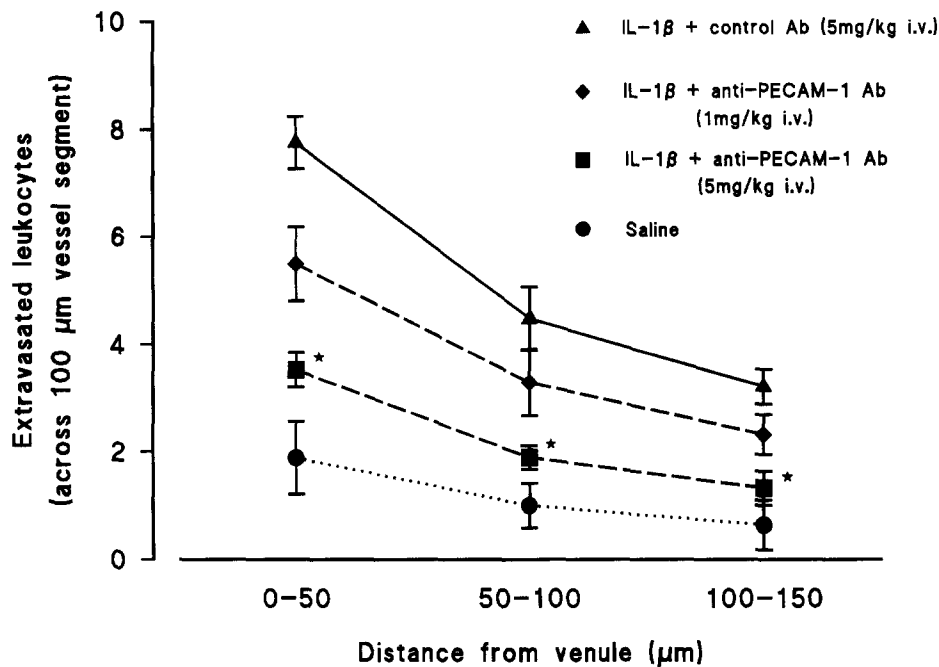
Figure 3. Effect of anti-PECAM-1 Ab on leukocyte extravasation induced by IL-1β. Animals were treated with control Ab (rabbit IgG, 5 mg/kg i.v.) or anti-PECAM-1 Ab (1 or 5 mg/kg i.v.) 15 min before the intraperitoneal administration of IL-1β. 4 h later, the mesenteric tissue was exteriorized and leukocyte extravasation, at distances of 0–50, 50–100, and 100–150 μm away from the vessel wall, across 100-μm vessel segments, quantified. Results are mean ± SEM for n = 5 animals. A significant difference between IL-1β + control Ab and IL-1β + anti-PECAM-1 Ab is shown by (*)P <0.05.

anti-PECAM-1 Ab (5 mg/kg)–treated rats was not statistically different from the small level of extravasation detected in rats injected intraperitoneally with saline. Further, the inhibitory effect of the anti-PECAM-1 Ab (5 mg/kg i.v.) on leukocyte extravasation was evident at all measurement points from the vessel wall (Fig. 3). An anti–rat MHC class I mAb, binding to both leukocytes and endothelial cells, had no effect on the leukocyte extravasation induced by IL-1β, i.e., the leukocyte extravasation responses were 8.2 ± 0.8 (n = 5 rats) and 7.2 ± 0.4 (n = 3 rats) for rats treated with IL-1β or IL-1β plus anti-MHC class I mAb (5 mg/kg i.v.), respectively. There was no significant difference between the circulating leukocyte numbers in animals pretreated with the control Abs or the anti-PECAM-1 Ab (data not shown).

In rats treated with the anti-PECAM-1 Ab, very frequently a layer of leukocytes was observed adjacent to the vessel wall (Fig. 2 C). Many of these leukocytes did not appear to be within the vascular lumen but appeared to have migrated out of the vessel, possibly coming to a halt within the vessel wall. These leukocytes could not be counted accurately. Further, it is important to note that these cells were not counted as adherent or extravasated, these parameters being limited to cells that were clearly intravascular or extravascular, respectively. Electron microscopy was used to localize these leukocytes.

Investigation of IL-1β–activated Mesenteric Tissues by Electron Microscopy. In control Ab-treated rats (5 mg/kg i.v.), in venules within sections taken from IL-1β–activated mesenteric tissues, leukocytes could be seen at different stages of their emigration from the vascular lumen to the extravascular tissue (Fig. 4 A). In addition to leukocytes apparently adherent to the luminal side of the endothelium,

the sections showed leukocytes that had crossed the endothelial cell layer to the abluminal side. These leukocytes had a rounded morphology and appeared to be in the process of migrating through the basement membrane. Electron micrographs of sections from IL-1β–activated mesenteric tissue, from rats pretreated with the anti-PECAM-1 Ab (5 mg/kg i.v.), also showed leukocytes that appeared to be in close contact with the luminal side of the endothelium (Fig. 4 B). However, in contrast to the sections from animals treated with the control Ab, there was a striking accumulation of leukocytes between the endothelium and the perivascular basement membrane (Fig. 4 B). The entrapment of leukocytes between the endothelium and the perivascular basement membrane is very clearly seen in Fig. 4 C. In 12 random vessel sections prepared from three control Ab (rabbit IgG)-pretreated rats (where a total of 439 leukocytes were counted), of the 270 leukocytes that had passed the endothelial cell junctions, 29.1 ± 7.1% were clearly sandwiched between the endothelium and the basement membrane. In contrast, in 15 vessel sections from four anti-PECAM-1 Ab-pretreated rats (where a total of 490 leukocytes were counted in all the sections), of the 377 leukocytes that had crossed the endothelial cell junctions, 57.5 ± 3.3% (P <0.05) were between the endothelium and the basement membrane (Fig. 5). These leukocytes, the majority of which had an elongated morphology, did not appear to be crossing the basement membrane so that in some instances they appeared in multiple layers within the vessel wall (Fig. 4 B). In these sections, very few leukocytes were seen in the extravascular tissue as compared with sections from control Ab-treated rats.

Effect of Anti-PECAM-1 Ab on Leukocyte Extravasation Induced by FMLP. As we have previously reported (12), in
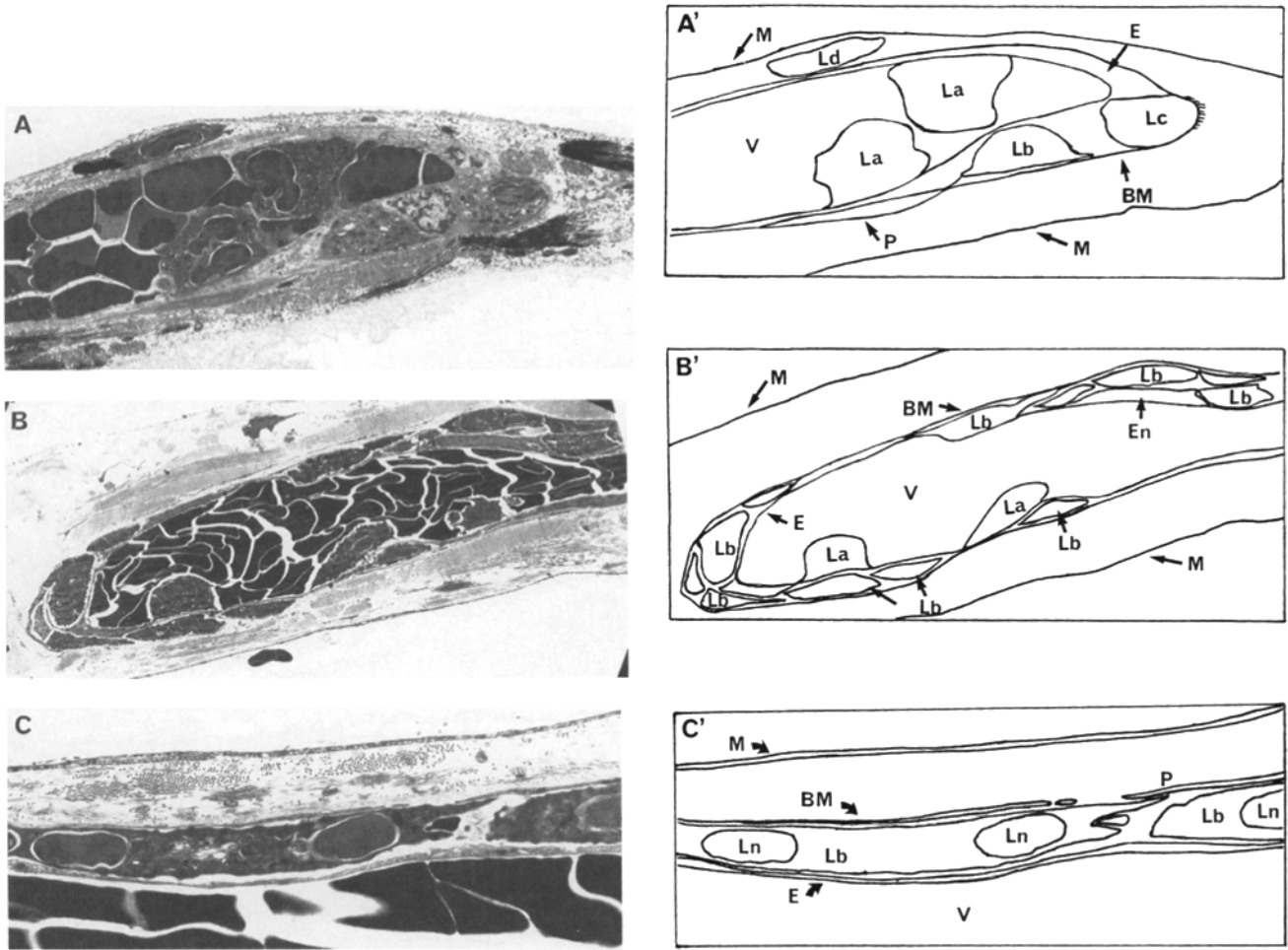
Figure 4. Electron micrographs and corresponding drawings of mesenteric venules from rats treated with (*A, A'*) control Ab (rabbit IgG, 5 mg/kg i.v.) plus IL-1β (10 ng i.p.) and (*B, B'*) anti–PECAM-1 Ab (5 mg/kg i.v.) plus IL-1β (10 ng i.p.). (*C*) Part of *B* at a higher magnification. In the section shown in *A*, leukocytes can be seen at different stages of emigration from the vascular lumen to the extravascular tissue, whereas in the section shown in *B* (more clearly seen in *C, C'*) there is a marked accumulation of leukocytes between the endothelium and the basement membrane. The drawings identify the vascular lumen (*V*), intravascular leukocytes (*La*), leukocytes between the endothelium and the basement membrane (*Lb*), a leukocyte crossing the basement membrane (*Lc*), extravascular leukocytes (*Ld*), leukocyte nuclei (*Ln*), the endothelial cell barrier (*E*), an endothelial cell nucleus (*En*), perivascular basement membrane (*BM*), a pericyte (*P*), and the mesothelium (*M*). (*A*) ×4,600, (*B*) ×2,800, and (*C*) ×10,500.
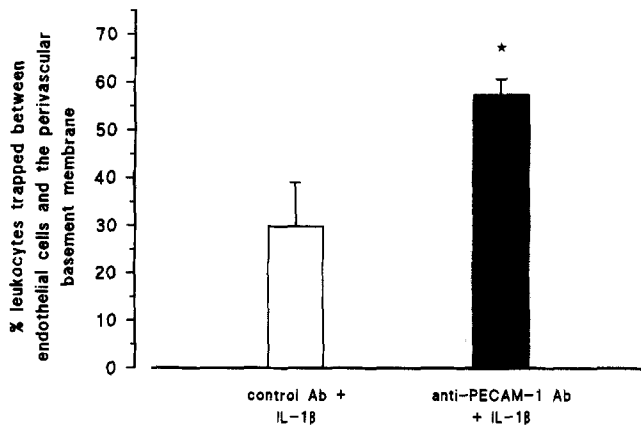

Figure 5. The effect of the anti–PECAM-1 Ab on the percentage of leukocytes trapped between venular endothelial cells and the perivascular basement membrane in IL-1β–treated mesenteric tissues, as determined by electron microscopy. Rats were treated with a control Ab (rabbit IgG,

animals treated intraperitoneally with chemotactic peptide FMLP (220 ng for 4 h), the leukocyte responses of adhesion and extravasation were greater than the levels detected in rats treated intraperitoneally with saline (results not shown). Pretreatment of rats with a control antibody (purified rabbit IgG, 5 mg/kg i.v.) or the anti–PECAM-1 Ab (5 mg/kg i.v.) had no effect on the FMLP-induced leukocyte adhesion (data not shown), or more importantly, FMLP-induced leukocyte extravasation (Fig. 6).

5 mg/kg i.v., *open column*) or the anti–PECAM-1 Ab (5 mg/kg i.v., *closed column*) 15 min before the i.p. administration of IL-1β. 4 h later, the mesenteric tissue was exteriorized and tissue sections prepared for electron microscopy. The graph represents the number of leukocytes observed between the venular endothelium and the perivascular basement membrane, quantified as the percentage of the total number of leukocytes that had passed the endothelial cell junctions. The results are from 12–15 sections prepared from *n* = 3–4 rats within each group. A significant difference is shown by (★)*P* <0.05.
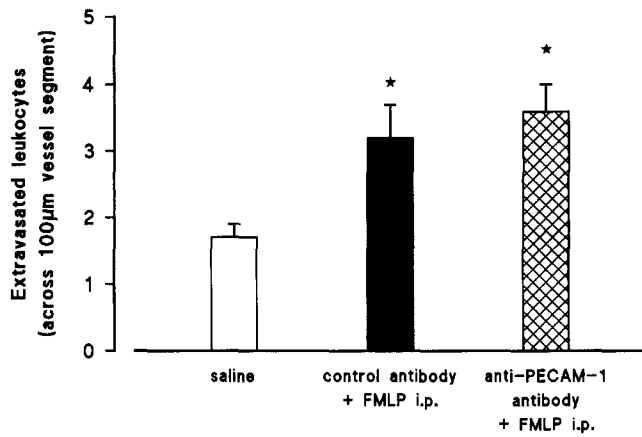
Figure 6. Effect of the anti-PECAM-1 Ab on leukocyte extravasation induced by i.p. FMLP. Rats were treated with saline (i.p., *open column*), a control Ab (rabbit IgG, 5 mg/kg i.v.) plus FMLP (220 ng i.p.; *closed column*), or the anti-PECAM-1 Ab (5 mg/kg i.v.) plus FMLP (220 ng i.p.; *crosshatched column*). 4 h later, the mesenteric tissue was exteriorized and leukocyte extravasation quantified. Results are mean ± SEM for $n = 4$ animals. A significant difference between saline- and IL-1β–treated rats is shown by (★)P <0.05.

To investigate the effect of the anti-PECAM-1 Ab on rapid responses elicited by FMLP, additional experiments were carried out to test the effect of the antibody on changes in leukocyte responses after the topical administration of FMLP. In control rats, whereas topical administration of FMLP (final concentration of 10^{-7} M) had no significant effect on the number of rolling leukocytes (results not shown), it did cause a rapid increase in leukocyte adhesion (556% increase above basal levels at 30 min, $n = 5$ rats, P <0.05, Fig. 7 A) and leukocyte extravasation (395% increase above basal levels at 30 min, $n = 5$ rats, P <0.05, Fig. 7 B). The responses to topical FMLP were essentially complete within 30–45 min (Fig. 7 and data not shown). Pretreatment of rats with a control Ab (5 mg/kg i.v.), 15 min before the topical administration of FMLP, had no significant effect on FMLP-induced leukocyte responses (Fig. 7). Similarly, the anti-PECAM-1 Ab (5 mg/kg i.v.) had no significant effect on FMLP-induced leukocyte adhesion, and more importantly, had no significant effect on the leukocyte extravasation induced by the topical chemotactic peptide (Fig. 7).

Interestingly, in mesenteric preparations activated by IL-1β in control Ab-treated rats, the topical administration of FMLP resulted in a further increase in leukocyte adhesion (data not shown) and extravasation above the levels elicited by the cytokine (Fig. 8). In animals pretreated with the anti-PECAM-1 Ab, although the leukocyte extravasation induced by IL-1β was less than that observed in rats treated with the control Ab, the topical administration of FMLP stimulated the leukocytes that had come to a halt at the vessel wall to migrate into the extravascular tissue (Figs. 8 and 9). Thus it would appear that, in the presence of the anti-PECAM-1 Ab, FMLP could induce the migration of the arrested leukocytes through the basement membrane.
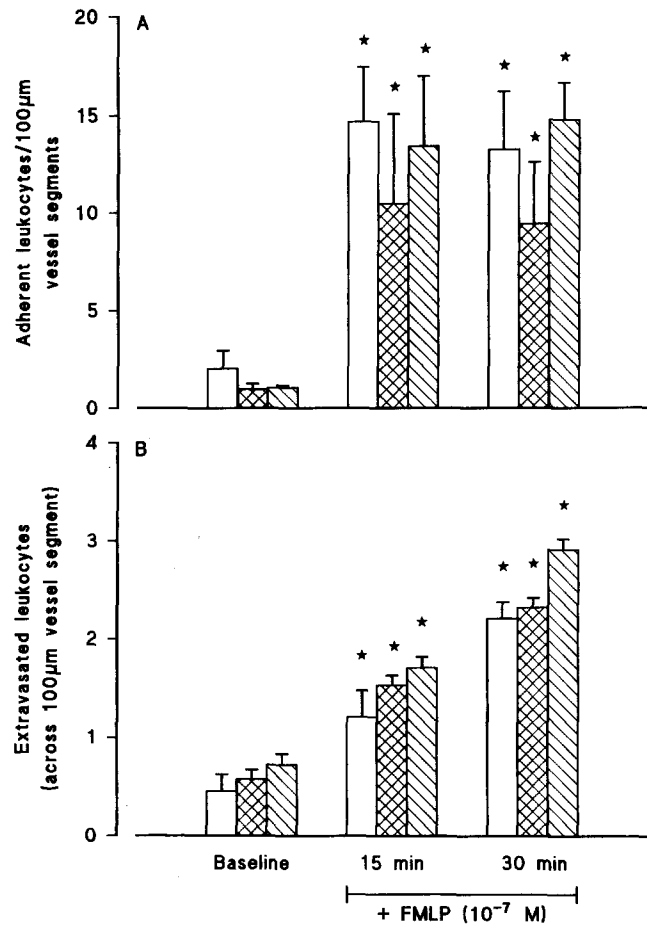

Figure 7. Effect of the anti-PECAM-1 Ab on leukocyte adhesion and extravasation induced by topical FMLP. Three groups of animals were used: untreated rats (□), rats treated with a control Ab (rabbit IgG, 5 mg/kg i.v., ▨), and rats treated with the anti-PECAM-1 Ab (5 mg/kg i.v., ▩). The animals were treated with the control Ab or the anti-PECAM-1 Ab and basal readings of leukocyte adhesion (A) and extravasation (B) recorded. 15 min later, FMLP (at a final concentration of 10^{-7} M) was applied topically to the preparation and readings taken at 15 and 30 min after application. Results are mean ± SEM for $n = 4$–5 rats. A significant difference between baseline values and FMLP-induced responses is shown by (★)P <0.05.

Discussion

Previous studies have shown that PECAM-1 mediates neutrophil and monocyte extravasation through endothelial cell monolayers in vitro (9) and anti-PECAM-1 Abs inhibit neutrophil and monocyte accumulation into sites of inflammation in vivo (10, 11). Despite the latter studies, the stage of emigration at which anti-PECAM-1 antibodies inhibit leukocyte accumulation and hence the precise role of PECAM-1, under dynamic conditions in vivo, remain unclear. The aim of the present study was to address this point, determining at which stage in the migration of leukocytes from the vascular lumen to the extravascular tissue PECAM-1 was involved. For this purpose, intravital microscopy was used to visualize directly the effect of an antibody recognizing rat PECAM-1 on leukocyte responses
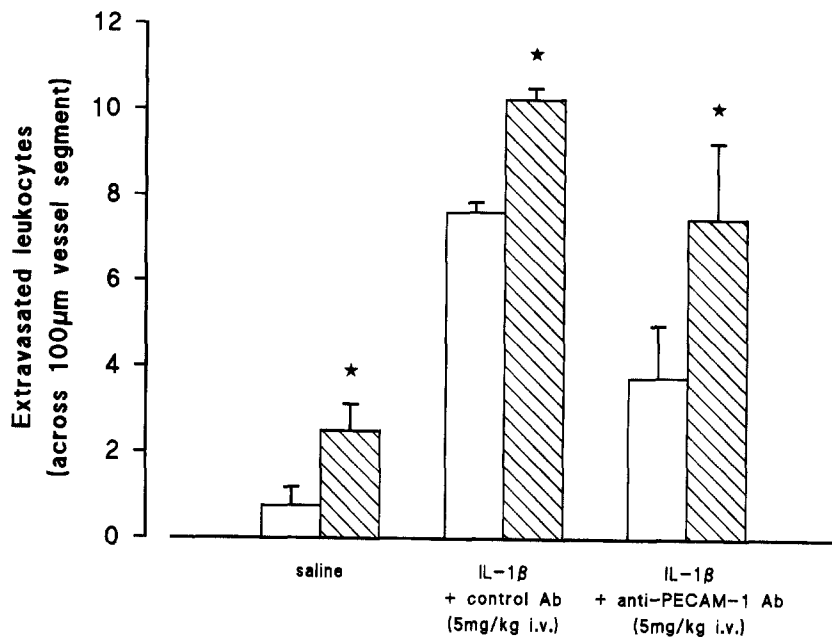
Figure 8. Effect of the anti-PECAM-1 Ab on leukocyte extravasation induced by FMLP in IL-1β–treated rats. Rats were pretreated with control Ab (rabbit IgG) or anti-PECAM-1 Ab before i.p. IL-1β. 4 h later, the mesenteric tissue was exteriorized and leukocyte extravasation 30 min after the topical administration of Tyrode (*open columns*) or FMLP (10^{-7} M; *dashed columns*) quantified. Responses are mean ± SEM for $n = 3$ animals. A significant difference between the leukocyte extravasation after topical Tyrode and FMLP is shown by (*)$P <0.05$.

within activated rat mesenteric microvessels in vivo. In addition, electron microscopy was used to determine the site of the inhibitory effect of the anti-PECAM-1 Ab. This Ab, which has been well characterized and shown to function as both intact IgG and as Fab fragments, has previously been shown to inhibit neutrophil accumulation into sites of acute inflammation in rat peritoneum and rat lungs, and into human skin grafts on immunodeficient mice (10).

In control rats, the i.p. injection of recombinant rat IL-1β, 4 h before the exteriorization of the mesenteric tissue, induced an inflammatory response that was associated with a significant increase in leukocyte rolling and adhesion within the mesenteric venules and a large number of emigrated leukocytes in the extravascular tissue. Although pretreatment of rats with an anti-PECAM-1 Ab, but not control Abs, had no effect on the IL-1β–induced leukocyte responses of rolling and adhesion, it significantly inhibited the leukocyte extravasation elicited by the cytokine. The effect of the anti-PECAM-1 Ab was dose dependent, resulting in 39 and 72% inhibition of extravasation at 1 and 5 mg/kg of the Ab, respectively. These results, showing a role for PECAM-1 in IL-1β–induced leukocyte extravasation by direct observation in vivo, provide a mechanistic explanation for the inhibitory effect of anti-PECAM-1 Abs on leukocyte extravasation into sites of inflammation (10, 11).

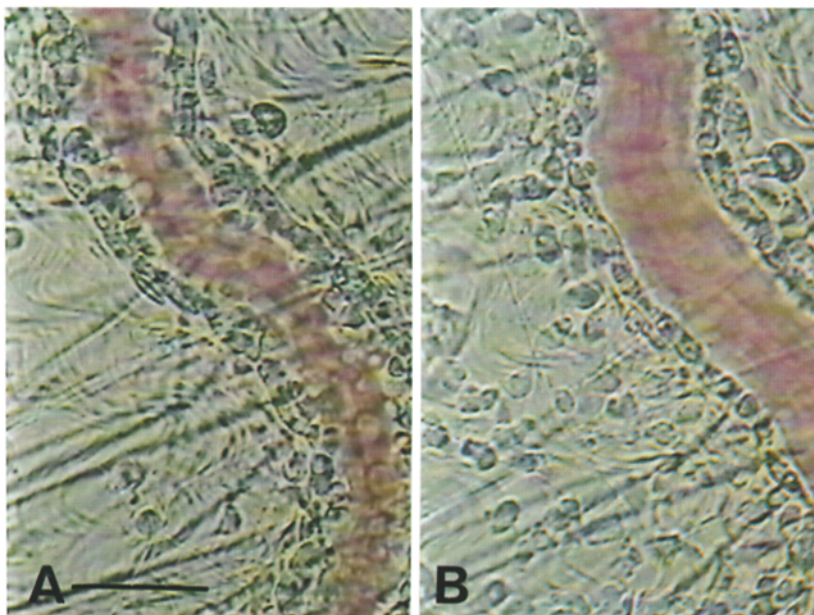In animals receiving the anti-PECAM-1 Ab, the inhibition

Figure 9. Video photomicrographs showing the effect of the anti-PECAM-1 Ab on leukocyte extravasation induced by FMLP in IL-1β–treated rats. For experimental details, see legend to Fig. 8. Briefly, rats were treated with the anti-PECAM-1 Ab before intraperitoneal IL-1β and 4 h later, the mesenteric tissue was exteriorized. The photos show responses before (*A*) and 30 min after (*B*) the topical administration of FMLP (10^{-7} M). Scale bar, 50 μm.

of leukocyte extravasation was associated with thickening of venular walls, which resembled a "wall of leukocytes" lining the length of vessel segments in the live microcirculation (Fig. 2 C). This finding is similar to the histological results of Bogen et al. (11) showing an increase in the number of leukocytes lining the length of mesenteric venular walls in anti-PECAM-1–treated mice. In the study of Bogen et al. (11), these leukocytes appeared retained within the vessels. In our study, in order to determine the precise site at which the leukocytes had come to a halt at the vessel wall, we used electron microscopy to analyze mesenteric tissues from anti-PECAM-1 Ab-treated rats. To our surprise, detailed analysis of numerous sections, obtained from several rats, indicated that within these animals the leukocytes had migrated through venular endothelial cell junctions, but not the perivascular basement membrane, and were thus trapped within the vessel wall. These leukocytes did not appear to be able to cross the basement membrane, resulting in the formation of multiple layers of leukocytes between the endothelial cell barrier and the perivascular basement membrane in some vessel segments.

Although early in vitro studies demonstrated a role for PECAM-1 in monocyte and neutrophil transendothelial cell migration through unstimulated and TNF-α–stimulated endothelial cell monolayers (9), recently, PECAM-1 has also been implicated in leukocyte interactions with components of the basement membrane (13). Liao et al. (13) showed that mAbs whose epitopes mapped to Ig domains 1–2 of the PECAM-1 molecule selectively blocked monocyte migration through unstimulated cultured endothelial cells in vitro, whereas mAbs recognizing domain 6 of the molecule inhibited the penetration of monocytes into the underlying collagen gel. Thus, the antibody used in our in vivo studies may be predominantly directed against Ig domain 6 of PECAM-1. Based on in vitro studies, in addition to PECAM-1, a number of other adhesion molecules have also been implicated in the process of IL-1–induced leukocyte extravasation (14). Although neutrophil migration through IL-1–activated endothelial cell monolayers appears to rely heavily on ICAM-1 (15), E-selectin, has also been implicated in this response (16). ICAM-1, E-selectin and VCAM-1 all appear to have roles in lymphocyte and eosinophil migration (17, 18). The relative contribution and precise role of these molecules in leukocyte extravasation in vivo remains to be determined. In addition to adhesion molecules, it is now clear that certain endothelial cell associated chemoattractants, such as platelet-activating factor (PAF) and IL-8, are also involved in the passage of neutrophils through IL-1–activated endothelial cells in vitro (19, 20). In support of these findings, we have recently shown that PAF receptor antagonists can selectively block the extravasation of leukocytes through IL-1–activated rat mesenteric microvessels in vivo (12). These observations can also explain the finding that pretreatment of ^{111}In–neutrophils with pertussis toxin to uncouple receptors, in addition to inhibiting the accumulation induced by chemoattractants such as FMLP, inhibited the accumulation of ^{111}In–neutrophils in response to intradermal IL-1 in rabbits (21).

In these experiments, pertussis toxin may have inhibited the neutrophil accumulation induced by IL-1 by uncoupling the leukocyte PAF receptors. Interestingly, whereas PAF receptor antagonists and the anti-PECAM-1 Ab both inhibited IL-1β–induced leukocyte extravasation, there appeared to be one important difference in the profile of their inhibitory effects. In rats pretreated with PAF receptor antagonists, the leukocytes appeared to remain within the vascular lumen, whereas in animals pretreated with the anti-PECAM-1 Ab, the leukocytes appeared trapped in the vessel wall.

The in vitro studies of Liao et al. (13) and our in vivo results implicate a functional role for PECAM-1 in the passage of leukocytes through the basement membrane. However, the mechanism by which this occurs is unclear. A possible explanation is that an important role of PECAM-1 in vivo is as a molecule triggering leukocyte activation. It may be that the ligation of PECAM-1, via domain 6 of the Ig molecule, is involved in the local activation of leukocytes at endothelial cell junctions, resulting in the stimulation of other mechanisms that enable the leukocytes to interact with and penetrate the basement membrane. This may involve both adhesive interaction with basement membrane components and proteolytic degradation by surface-expressed enzymes on the leukocytes. Indeed, there is now much in vitro evidence indicating that PECAM-1, as well as acting as an adhesion molecule, can act as a receptor capable of triggering leukocyte activation. In this context, PECAM-1–dependent interactions can activate both β_1 and β_2 integrins on leukocytes or integrin-expressing COS cells (22–25). A PECAM-1–dependent activation of β_1 and β_2 integrins may facilitate both the passage of leukocytes through endothelial cell junctions and their interaction with basement membrane components. In contrast to the well-established role of β_2 integrins in neutrophil migration in vivo (1, 14), there is as yet no in vivo evidence for the involvement of β_1 integrins in this response. However, in vitro studies have shown that β_1 integrins can be induced on the surface of transmigrating neutrophils (26), and that β_1 integrins can mediate the interaction of neutrophils with components of the basement membrane such as laminin (27, 28). In addition to endothelial cells and extracellular matrix proteins, pericytes may also play a role in regulating the passage of leukocytes through the vessel wall. In most of the tissue sections analyzed by electron microscopy, pericytes could be seen in the vessel wall embedded within the basement membrane (Fig. 4). Hence, an alternative explanation of our results is that PECAM-1 may be involved in the interaction of leukocytes with microvascular pericytes.

Although the cascade of intermolecular interactions mediating the passage of leukocytes through IL-1–activated endothelial cells is yet to be fully understood, sequential activation of leukocytes may be critically important in this process. Hughes et al. (29) have presented evidence showing that increments in chemotactic stimuli are required to bring CD11b/CD18 to the surface of neutrophils (from granules), and that this newly mobilized CD11b/CD18 is necessary for adherence-dependent neutrophil migration.

This concept of a stepwise increase in neutrophil activation agrees well with our observations indicating roles for both PAF and PECAM-1 in leukocyte extravasation. A possible explanation of our findings may involve a sequence of events such as the following: (a) stimulation of endothelial cells by IL-1 results in the upregulation or activation of selectins and generation of PAF which remains predominantly endothelial cell associated; (b) tethering of leukocytes to the endothelium by selectins allows an interaction between the endothelial cell–associated PAF and the leukocyte's cell surface PAF receptors; (c) stimulation of leukocytes by PAF triggers a partial activation of leukocyte β_2 integrins stimulating the firm adhesion and transendothelial cell migration of the leukocytes, a response involving ICAM-1, PECAM-1 (domains 1–2), as well as other induced endothelial cell adhesion molecules; and (d) at the endothelial cell junctions, the additional interaction of the leukocytes with endothelial cell PECAM-1 (through domain 6) triggers further activation of leukocyte β_1 and β_2 integrins and release of granular proteases aiding the passage of leukocytes across the perivascular basement membrane. Hence, in animals treated with PAF receptor antagonists, leukocyte extravasation may have been blocked by inhibiting the passage of leukocytes into the interendothelial cell junctions, thus forcing the leukocytes to remain within the vasculature. In contrast, in animals treated with the anti-PECAM-1 Ab (possibly predominantly directed against Ig domain 6), once the leukocytes had entered the endothelial cell junctions, the Ab may have prevented the PECAM-1–dependent activation of leukocyte integrins that mediates and indeed propagates the passage of leukocytes across the basement membrane, thus maintaining the leukocytes within the vessel wall. This proposal is currently under further investigation.

The above proposal is further supported by our findings that both PAF receptor antagonists (12) and the anti-PECAM-1 Ab had no effect on the rapid leukocyte extravasation induced by the chemotactic peptide FMLP. One clear difference between the responses elicited by the cytokine and the chemotactic formyl peptide is that presumably the extravasation induced by FMLP is as a result of direct leukocyte activation and is not dependent on endothelial cell stimulation. Thus, leukocyte activation via endothelial cell–associated PAF or PECAM-1 is not required for the FMLP-induced leukocyte extravasation. It is important to note that FMLP was able to induce the rapid migration through the basement membrane of the leukocytes held up by anti-PECAM-1 Ab in the IL-1β–treated mesentery (Fig. 8). These results, which clearly show that in the same animal the anti-PECAM-1 Ab inhibits the extravasation induced by IL-1β but not FMLP, further demonstrate that (a) the inhibitory effect of the anti-PECAM-1 Ab on leukocyte extravasation was specific to the response induced by certain stimuli including IL-1β, and (b) the antibody did not have a nonspecific inhibitory effect on the leukocytes. It has also been shown that anti-PECAM-1 Abs and soluble PECAM-1 do not affect chemoattractant-induced neutrophil or monocyte chemotaxis in vitro (9, 10). Finally, in agreement with our previous findings (21), our present studies with FMLP and IL-1 also indicated the apparent difference in the potency of these stimuli in eliciting neutrophil accumulation in vivo. Even though FMLP had to be used at an ~20-fold-greater dose than IL-1β in order to induce leukocyte extravasation, the level of FMLP-induced response was smaller than that observed with the cytokine, suggesting that the difference in the profile of effects seen with the anti-PECAM-1 Ab on the two stimuli is unlikely to be due to the difference in their respective doses.

Full details of the mechanisms mediating the passage of leukocytes across venular walls are still unknown. However, our results provide the first direct evidence for a role for PECAM-1 in leukocyte extravasation across venules activated by IL-1β, but not FMLP, in vivo. These findings demonstrate a differential requirement for PECAM-1 in the recruitment of leukocytes in response to different inflammatory stimuli and strongly implicate PECAM-1 in the passage of leukocytes across the basement membrane in vivo.

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