Divergent Effects of Platelet-Endothelial Cell Adhesion Molecule-1 and $\beta_3$ Integrin Blockade on Leukocyte Transmigration In Vivo

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Divergent Effects of Platelet-Endothelial Cell Adhesion Molecule-1 and $\beta_3$ Integrin Blockade on Leukocyte Transmigration In Vivo


The final stage in the migration of leukocytes to sites of inflammation involves movement of leukocytes through the endothelial cell layer and the perivascular basement membrane. Both platelet-endothelial cell adhesion molecule-1 (PECAM-1/CD31) and the integrin $\alpha_\beta_3$ have been implicated in this process, and in vitro studies have identified $\alpha_\beta_3$ as a heterotypic ligand for PECAM-1. In the present study we have addressed the roles of these molecules by investigating and comparing the effects of PECAM-1 (domain 1-specific, mAb 37) and $\beta_3$ integrins (mAbs 7E3 and F11) on leukocyte responses in the mesenteric microcirculation of anesthetized rats using intravital microscopy. The anti-PECAM-1 mAb suppressed leukocyte extravasation, but not leukocyte rolling or firm adhesion, elicited by IL-1$\beta$ in a dose-dependent manner (e.g., 67% inhibition at 10 mg/kg 37 Fab), but had no effect on FMLP-induced leukocyte responses. Analysis by electron microscopy suggested that this suppression was due to an inhibition of neutrophil migration through the endothelial cell barrier. By contrast, both anti-$\beta_3$ integrin mAbs, 7E3 F(ab')$_2$ (5 mg/kg) and F11 F(ab')$_2$ (5 mg/kg), selectively reduced leukocyte extravasation induced by FMLP (38 and 46%, respectively), but neither mAb had an effect on IL-1$\beta$-induced leukocyte responses. These findings indicate roles for both PECAM-1 and $\beta_3$ integrins in leukocyte extravasation, but do not support the concept that these molecules act as counter-receptors in mediating leukocyte transmigration. The Journal of Immunology, 2000, 165: 426–434.

Leukocyte recruitment is pivotal to the inflammatory process and is recognized to involve a number of distinct steps (1). Initially, leukocytes make contact with the vascular endothelium while still moving over its surface, a process described as tethering, leading to leukocyte rolling. Following further stimulation, possibly mediated via endothelial cell-bound chemotactants, leukocytes become stationary by firmly adhering to the endothelial cell surface. These initial leukocyte responses appear to be prerequisite for the final stage of leukocyte migration, emigration through the endothelium and the perivascular basement membrane. While our understanding of the molecular interactions that mediate leukocyte rolling and firm adhesion has greatly advanced (2, 3), relatively little is known about the mechanisms that mediate migration of leukocytes through vessel walls. In this context, a number of adhesion molecules have been implicated in the process of leukocyte extravasation (4, 5), including members of the $\beta_3$ integrin family, such as LFA-1 (\(\alpha_\beta_2\), CD11a/CD18) and Mac-1 (\(\alpha_\beta_2\), CD11b/CD18) (6–9), and members of the Ig superfamily, such as ICAM-1 and ICAM-2 (10, 11), platelet-endothelial cell adhesion molecule-1 (PECAM-1) (12, 13), and junctional adhesion molecule (14). Despite increased in vitro and in vivo evidence for the involvement of these molecules in leukocyte transendothelial cell migration, their relative importance in leukocyte emigration in vivo is still unclear.

Among the molecules listed above, PECAM-1 (CD31) is one of the very few that appears to specifically mediate leukocyte transmigration and has thus been the subject of much interest in the context of leukocyte emigration in the last 10 yr. PECAM-1 is a 130- to 135-kDa membrane glycoprotein, with an extracellular portion composed of six Ig-like conserved units of the C2 subclass, a single transmembrane portion, and a short cytoplasmic tail (15). It is expressed on the surface of platelets, most subsets of leukocytes, and endothelial cells, where it is concentrated at intercellular junctions (12). PECAM-1 has important signaling properties; it is capable of both initiating and responding to cellular activation, for which it is dependent on its cytoplasmic tail (16–19). Although PECAM-1 has been implicated in numerous biological functions, there is now ample evidence, from both in vitro and in vivo investigations, demonstrating an important role for this molecule in the control of leukocyte transmigration, a function for which its expression profile and binding/signaling properties are well suited (12, 20–23). Furthermore, recent studies with PECAM-1-deficient mice have shown a defect in neutrophil migration through the perivascular basement membrane (24), in agreement with previous findings using an anti-PECAM-1 Ab (23), although in the former study the overall number of recruited leukocytes was normal.

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3 Abbreviations used in this paper: PECAM, platelet-endothelial cell adhesion molecule; IAP, integrin-associated protein; 7E3, chimeric 7E3.
PECAM-1 is known to support both homotypic and heterotypic cellular interactions, although the relative importance of these molecular events in the regulation of leukocyte migration in vivo remains unclear. In vitro, Liao et al. (25) demonstrated that different domains of PECAM-1, supporting homotypic or heterotypic interactions, mediated leukocyte transendothelial cell migration or leukocyte interaction with extracellular matrix components, respectively. Abs that inhibit PECAM-1 homotypic interactions have also been shown to suppress leukocyte migration in vivo (26, 27). Possible roles for PECAM-1-dependent heterotypic interactions in leukocyte migration have not been addressed however. In this context, a number of putative PECAM-1 ligands have been reported, including heparan sulfate proteoglycans (28), an as yet to be characterized 120-kDa glycoprotein expressed on activated T lymphocytes (29), ADP-ribosyl cyclase (CD38) (30), and the integrin αβ3 (31, 32). Of these reported heterotypic ligands, the β3 integrin, αβ3, is perhaps the most intriguing, because, like PECAM-1, it has been implicated in leukocyte transmigration (33).

The integrin αβ3 shares a common subunit with the platelet molecule αIIbβ3 (GPIIb/IIIa), although its expression profile is very different. αβ3, while being expressed at low levels by platelets, is expressed at higher levels by smooth muscle cells, endothelial cells, monocytes, and neutrophils and at several million copies per cell by osteoclasts (34–36). In many cell types it is physically associated with another protein within the cell membrane, integrin-associated protein (IAP, CD47), which is known to modulate a number of its functions (37, 38). Neutralizing Abs recognizing αβ3 or IAP reduce leukocyte migration through endothelial cell monolayers in vitro (33, 39). To two independent groups have reported that PECAM-1 and αβ3 act as counter-receptors (31, 32), but other groups have challenged the validity of such an interaction (40). The aim of the present study was to compare the effects of blocking PECAM-1 or αβ3 on leukocyte migration in vivo. For this purpose we chose to investigate and compare the effects of Abs directed against PECAM-1 or αβ3 integrins in two rat models of inflammation, namely leukocyte migration through IL-1β- and FMLP-activated mesenteric venules, previously shown to be PECAM-1-dependent and independent reactions (23). We hypothesized that if the molecules PECAM-1 and αβ3 acted as counter-receptors in leukocyte transmigration, blocking Abs directed against these molecules would exhibit similar effects on leukocyte migration in models of inflammation in vivo. Our findings, however, demonstrate that blockades of PECAM-1 and αβ3 exert very different inhibitory effects on leukocyte migration in vivo, arguing against the possibility that these molecules act as counter-receptors in the regulation of leukocyte migration.

Materials and Methods

Animals
Male Sprague Dawley rats (200–250 g) were purchased from Harlan-Oiac (Bicester, U.K.).

Reagents
Reagents were purchased as follows: Hypnorm (fentanyl–fluanisone mixture) from Janssen-Cilag (High Wycombe, U.K.), sodium pentobartitone from Rhône Mérieux (Harlow, U.K.), osmium (VIII) oxide from Johnson Matthey PLC (Royston, U.K.), and sodium cacodylate from Agar Scientific (Stanstead, U.K.). All other reagents were purchased from Sigma-Aldrich (Poole, U.K.).

Monoclonal Abs
The anti-PECAM-1 mAb 37 (murine IgG1) has been developed, and its specificity for domain 1 of PECAM-1 and its cross-reactivity with rat cells have been confirmed, as recently reported (41). The nonbinding isotype-matched control mAb 125 (murine IgG1) was produced in a similar manner. These Abs were used either as whole Ab or Fab. Fab were prepared by papain digestion and were purified by protein A affinity chromatography (see Ref. 41 for detailed description). The anti-β3 integrin mAbs used were mAb 7E3 (murine IgG1) and mAb F11 (murine IgG1). 7E3 is an anti-human β3 integrin mAb that recognizes the platelet integrin, αIIbβ3, and the vitronectin receptor, αβ3, with equal affinity and has been shown to exhibit some cross-reactivity with rat cells (42, 43). Ab F11 is a specific function-blocking anti-β3 integrin mAb (44). These Abs were used either as whole Ab or Fab (45) for in vivo experiments. Fab(′)2 fragments were prepared by papain digestion as detailed previously (45). Either murine anti-rat MHC class I mAb (IgG1, Harlan Sera-lab, Sussex, U.K.) or mouse myeloma IgG1, MOPC-21 (Sigma-Aldrich), was used as control mAb. A chimeric version of 7E3 (c7E3), in which the constant regions of the parent murine Ab have been replaced with the constant regions of human κ and λ IgG chains, was used for flow cytometry studies, with a human LFA-3-human Fc chimera used as the control molecule (a gift from Biogen, Cambridge, MA).

Flow cytometry
Blood was collected from untreated animals by cardiac puncture and was anti-coagulated with lithium heparin (10 IU/ml). Aliquots were incubated at 4°C with primary Ab (10 μg/ml) for 30 min, followed by FITC-conjugated goat anti-human Fc-specific IgG secondary Ab (Dako, Ely, U.K.) for 30 min. Erythrocytes were lysed using FACS lysing solution (Becton Dickinson U.K., Oxford, U.K.). Samples were analyzed using an EPICS XL flow cytometer (Beckman Coulter, High Wycombe, U.K.). Neutrophils were identified by characteristic forward and side scatter profiles.

Platelet aggregation
Male rats were treated by i.v. injection of anti-β3 Ab, 7E3 F(ab′)2, and blood was harvested 6, 24, or 48 h later. Blood was anti-coagulated with sodium citrate (0.32% final concentration), and platelet-rich plasma was prepared by centrifugation at 600 × g for 6 min. Once the platelet concentration had been adjusted to 2–3 × 1011 platelets/μl by addition of platelet-poor plasma, platelet aggregation in response to ADP (10 μM) was measured using a PAP-4 aggregometer (Biodata, Horsham, PA). Inhibition of ADP-induced platelet aggregation was calculated by comparison with control samples.

Intravital microscopy
Male rats were prepared for intravital microscopy as previously described (46). Briefly, following sedation with i.m. Hypnorm (0.1 ml), animals were anesthetized with i.v. sodium pentobartitone (30 mg/kg loading dose followed by 30 mg/kg/h). The animals were maintained at 37°C on a custom-built heated microscope stage. Following midline abdominal incision, the mesentery adjoining the terminal ileum was carefully arranged over a glass window in the microscope stage and pinned in position. The mesentery was kept warm and moist by continuous application of Tyrode’s balanced salt solution. Mesenteric venules (20–40 μm in diameter) were viewed on an upright fixed-stage microscope (Axioskop FS, Carl Zeiss, Welwyn Garden City, U.K.) fitted with water immersion objectives. Images were captured with a video camera (C8510-01, Hamamatsu Photonics U.K.) and storage by videocassette recorder (AG-MD830E, Panasonic U.K., Bracknell, U.K.). As the resolution of intravital microscopy does not allow definitive distinctions to be made between different subpopulations of leukocytes, all responses are quantified in terms of leukocyte behavior. Hence, rolling leukocytes were defined as those cells moving slower than the flowing erythrocytes, and rolling flux was quantified as the number of rolling cells moving past a fixed point on the venular wall per minute, averaged for 4–5 min. Firmly adherent leukocytes were defined as those that remained stationary for at least 30 s within a 100-μm segment of a venule. Extravasated leukocytes were defined as those in the perivascular tissue adjacent to, but remaining within a distance of 50 μm of, a 100-μm vessel segment under study. In some experiments the mesentery was excised at the end of the quantification to allow preparation for electron microscopy as detailed below.

In experiments studying IL-1β-induced leukocyte responses, male rats were sedated with i.m. Hypnorm, after which test Ab, control Ab, or vehicle was given by i.v. injection. Fifteen minutes later, recombiant rat IL-1β (10 ng in 5 ml of sterile saline; gift from Dr. K. Vosbeck, Ciba-Geigy, Basel, Switzerland) or vehicle was given by i.p. injection. Four hours later, the mesenteric tissue was exteriorized and prepared for intravital microscopy, as described above. In each animal multiple vessel segments from multiple vessels were quantified. In experiments studying FMLP-induced leukocyte responses, the mesenteric tissue was prepared for
study, and once a suitable vessel had been selected for observation, baseline readings were taken. The rats were then treated i.v. with test or control Ab, and after 15 min, FMLP (10⁻⁷ M final concentration) was applied topically to the mesenteric tissue in the superfusion buffer. Leukocyte responses within the chosen vessel were quantified for 1 h, during which the topical application of FMLP was maintained.

Electron microscopy

In selected experiments following the dynamic quantification of leukocyte responses the portion of mesentery containing the test vessels was excised and fixed in buffer containing 2.5% glutaraldehyde (50 mM sodium cacodylate, 4 mM HCl, and 0.18 mM CaCl₂). Samples were then postfixed in 1% osmium VIII oxide and, following dehydration in methanol, were embedded in araldite resin before sectioning. Thin sections (1 μm) were stained with toluidine blue to allow location of venules. Ultrathin sections (0.1 μm) were mounted on copper mesh grids and stained with uranyl acetate and lead citrate before viewing on a transmission electron microscope (Hitachi 7000, Hitachi U.K., Hayes, U.K.). The total number of leukocytes associated with each vessel was counted, and their positions, according to the following classification, were noted: A, within lumen of the venule; B, crossing the endothelium; C, between the endothelium and perivascular basement membrane; D, crossing the basement membrane; and E, outside the venule, but within 50 μm of it. For each venule, the fraction of leukocytes that had crossed the endothelium but were still inside the basement membrane was calculated according to the following equation C/(C + D + E). In each series of experiments, tissue samples from at least three animals were analyzed, and from each animal at least three vessels were studied in detail.

Peritoneal recruitment

Following sedation with i.m. Hypnorm, male rats were given test or control Ab (5 mg/kg) by i.v. injection. Fifteen minutes later, recombinant rat IL-1β (10 ng in 5 ml of sterile saline) or vehicle was given by i.p. injection. Four hours later, animals were humanely killed, and the peritoneal cavity was washed with normal saline containing 0.25% BSA and heparin (2 IU/ml). Total cell counts were performed using Kimura stain (47). Differential cell analysis was determined in exudate smears prepared in a cytocentrifuge (Cytospin-3, Shandon, Runcorn, U.K.) and stained with May-Grünwald/ Giemsa stains. A minimum of four animals were used for each group.

Statistical analysis

Data are presented as the mean ± SEM unless stated otherwise. Statistical significance was assessed using one- or two-way ANOVA or unpaired Student’s t test as appropriate. A p value <0.05 was considered significant. Analysis was performed using Prism 3.0 for Windows (GraphPad Software, San Diego, CA).

Results

A domain 1-specific PECAM-1-blocking Ab suppresses neutrophil transmigration through IL-1β-activated rat mesenteric venules at the level of the endothelium

In a previous study we demonstrated that an anti-PECAM-1 polyclonal Ab suppressed leukocyte migration through IL-1β-activated rat mesenteric venules by blocking the response at the level of the perivascular basement membrane (23), observations that suggested a novel in vivo role for PECAM-1 and have subsequently been supported by the findings of Duncan et al. in PECAM-1-deficient mice (24). However, as both in vitro and in vivo studies have identified domain 1 of PECAM-1 as a key component of the molecule in leukocyte transendothelial cell migration (25, 26), we sought to extend our earlier observations by studying the effect of a domain 1-specific anti-PECAM-1 mAb on IL-1β-activated leukocyte migration through rat mesenteric venules, as studied by intravital microscopy. Intraperitoneal administration of recombinant rat IL-1β (10 ng) 4 h before exteriorization of the mesenteric tissue resulted in a significant increase in leukocyte rolling flux (data not shown), leukocyte firm adhesion, and leukocyte transmigration compared with responses detected in rats treated with i.p. saline (Fig. 1). Intravenous pretreatment with domain 1-specific anti-PECAM-1 mAb 37 inhibited the cytokine-induced transmigration of leukocytes through the vessel wall in a dose-dependent manner (52 and 68% inhibition at 5 and 10 mg/kg, respectively; p < 0.05), but had no effect on leukocyte rolling flux (data not
shown) or leukocyte firm adhesion (Fig. 1A). A similar specific inhibitory effect of the Ab on leukocyte transmigration was detected when Fab of the Ab was used (67% inhibition at 10 mg/kg; \( p < 0.01 \); Fig. 1B). In contrast, an isotype-matched control Ab, mAb 125, used as a whole molecule or a Fab, had no effect on the leukocyte responses quantified (Fig. 1).

To determine the precise stage of leukocyte migration at which the domain 1-specific anti-PECAM-1 mAb was acting, mesenteric tissues from selected experiments, described above, were examined by transmission electron microscopy to evaluate the site of blockade. These experiments also demonstrated that the migrating leukocytes were predominantly neutrophils (\( \approx 85\% \)), as shown in Fig. 2, A and B. Interestingly, in contrast to our previous findings

The graph shows the number of neutrophils observed between the venular endothelium and the perivascular basement membrane, expressed as a percentage of the total number of neutrophils that had passed the endothelial cell junctions. The results are from eight randomly selected sections prepared from three vessel segments from multiple animals within each group.
with an anti-PECAM-1 polyclonal Ab (23), there was no evidence of neutrophil trapping between the endothelial cell layer and the perivascular basement membrane, in the IL-1β-stimulated vessel walls of animals treated with mAb 37 (Fig. 2C). These results suggest that the domain 1-specific anti-PECAM-1 mAb 37 blocks neutrophil transmigration by reducing their ability to penetrate the endothelial cell layer, as opposed to the basement membrane.

**Anti-β3 mAbs 7E3 and F11 do not affect leukocyte responses within IL-1β-activated mesenteric venules**

To compare the functional roles of PECAM-1 and β3 integrins in leukocyte migration in vivo, the aim of the following experiments was to extend the studies described above by investigating the effects of two different anti-β3 integrin Abs on IL-1β-induced leukocyte responses. The Abs used were mAbs F11 and 7E3.

mAb F11 is a murine anti-rat β3, mAb (44). Ab 7E3, however, is a murine anti-human β3 Ab, and although in vitro it has been shown to bind to rat aortic smooth muscle cells (42, 43), initial experiments were conducted to investigate the cross-reactivity of this Ab with rat molecules before its use in vivo. Platelet aggregation was measured ex vivo 6, 24, and 48 h after i.v. administration of 7E3 F(ab’)_2 (4 mg/kg). This treatment significantly reduced ADP-induced platelet aggregation both 6 and 24 h after Ab treatment (Fig. 3A). Additionally, 7E3 was found to bind to rat leukocytes, as shown by whole blood flow cytometry (Fig. 3B). Because leukocytes do not express αIIbβ3, these results suggest that 7E3 binds to rat leukocytes via an interaction with αIIbβ3, although the possibility of its binding to platelets adherent to leukocytes or to Mac-1 (7E3 has been reported to bind to an activated state of Mac-1 (48, 49)) cannot be excluded.

Having confirmed the cross-reactivity of 7E3 with rat molecules, the effects of Abs 7E3 and F11 on IL-1β-induced leukocyte migration were examined in vivo. For this purpose, mAb 7E3 F(ab’)_2 (5 mg/kg), mAb F11 IgG (10 mg/kg), or appropriate doses of control Abs were administered i.v. 15 min before the i.p. administration of IL-1β (10 ng/rat). Four hours later, the mesentery was exteriorized, and leukocyte responses were quantified as previously detailed. In contrast to our findings with anti-PECAM-1 Abs, the anti-β3 integrin Abs had no effect on leukocyte transmigration (Fig. 4). Abs 7E3 and F11 were also without an affect on leukocyte rolling flux and firm adhesion (results not shown). Similarly, neither Ab was able to inhibit the recruitment of leukocytes into the peritoneal cavity of rats 4 h after i.p. administration of IL-1β (data not shown).

Having observed differing effects of PECAM-1 or β3 integrin blockade on IL-1β-induced leukocyte responses, the studies were extended to a different model of leukocyte migration for further analysis of the roles of these molecules.

**FMLP-induced leukocyte transmigration is reduced by anti-β3 integrin, but not anti-PECAM-1, Abs**

In the following series of experiments the effects of anti-PECAM-1 mAb 37 and anti-β3 integrin mAbs 7E3 and F11 on leukocyte responses elicited by FMLP were examined. Topically administered FMLP (10^{-7} M) induced a time-dependent increase in leukocyte firm adhesion and transmigration over the 1-h test period. Significant levels of firmly adherent leukocytes were detected 10 min postapplication of the chemoattractant, with the response reaching a maximal level within 30 min of FMLP administration. As expected, the time course of leukocyte transmigration was slightly slower than that observed for firm adhesion, in that significant levels of extravascular leukocytes were first detected at around 20–45 min postapplication of FMLP, a response that continued to increase for the duration of the 1-h in vivo test period.

![Figure 4](http://www.jimmunol.org/)  
**FIGURE 4.** Effect of anti-β3 integrin Abs on IL-1β-induced leukocyte extravasation. Rats were pretreated with the test Abs i.v., 7E3 F(ab’)_2 (5 mg/kg; A) and F11 (10 mg/kg; B), or with the appropriate dose of a control Ab 15 min before the i.p. administration of IL-1β (10 ng). Four hours later the mesentery was prepared for intravital microscopy, and leukocyte transmigration was quantified. Data represent the mean ± SEM for five rats per group.

Fig. 5 shows that in agreement with our previous findings (23), blockade of PECAM-1 had no effect on FMLP-induced leukocyte responses. In contrast, however, the anti-β3 integrin mAbs 7E3 and F11 selectively suppressed FMLP-induced leukocyte transmigration, while having no effect on leukocyte firm adhesion (Figs. 6 and 7). With respect to mAb 7E3, however, there was a trend toward increased leukocyte firm adhesion, although this did not achieve statistical significance. In these studies both Abs were used as F(ab’)_2, with 7E3 F(ab’)_2 and F11 F(ab’)_2 reducing FMLP-induced leukocyte transmigration at 60 min by 38% (p < 0.05) and 46% (p < 0.01), respectively. At the doses used, none of the Abs used in the present study had an effect on circulating leukocyte numbers (results not shown).

**Discussion**

PECAM-1 can mediate cellular adhesion via both homotypic (PECAM-1/PECAM-1) and heterotypic (PECAM-1/ligand) interactions. With respect to the latter, a number of PECAM-1 ligands have been reported, although to date the functional significance of such interactions has not been investigated in vivo models of leukocyte migration. Among the reported heterotypic ligands for PECAM-1 is the integrin αvβ3. In common with PECAM-1, in vitro studies have implicated αvβ3 in mechanisms of leukocyte...
motility and transmigration (27, 33, 50), but to our knowledge no studies have investigated the role of this integrin in leukocyte transmigration in vivo. The aim of the present study was to directly investigate and compare the roles of $\alpha_v \beta_3$ and PECAM-1 in leukocyte migration through stimulated rat mesenteric venules, as observed by intravital microscopy. Because we had no access to a selective $\alpha_v \beta_3$ inhibitor suitable for studies in rats, we analyzed the effect of an anti-PECAM-1 mAb in comparison with the effects of two anti-$\beta_3$ integrin mAbs. The results of this in vivo study confirm a role for PECAM-1 in leukocyte transmigration and provide the first demonstration that Abs against $\beta_3$ integrins can exert a specific effect on the migration of leukocytes through vessel walls. Of importance, however, our results demonstrate a clear difference in the inhibitory effects of the anti-PECAM-1 Ab and the anti-$\beta_3$ integrin Abs. Specifically, while PECAM-1 blockade suppressed IL-1$\beta$-induced leukocyte transmigration, anti-$\beta_3$ integrin Abs inhibited leukocyte transmigration induced not by IL-1$\beta$, but by the chemoattractant FMLP. These findings, while confirming roles for both PECAM-1 and $\beta_3$ integrins in leukocyte transmigration, suggest that PECAM-1 and $\alpha_v \beta_3$ do not necessarily act as counter-receptors to facilitate leukocyte migration in vivo.

PECAM-1-neutralizing Abs have proved effective at reducing leukocyte accumulation in a number of models, including leukocyte accumulation in inflamed rat peritoneum, rat lung, and murine peritoneum (21, 22). Although studies such as these have established a role for PECAM-1 in leukocyte recruitment to sites of inflammation in vivo, relatively few investigations have addressed the precise stage of leukocyte migration at which PECAM-1 is involved. In this context we have previously demonstrated that a polyclonal anti-PECAM-1 Ab inhibited leukocyte migration through IL-1$\beta$-activated rat mesenteric venules by suppressing the movement of leukocytes through vessel walls. Of importance, however, our results demonstrate a clear difference in the inhibitory effects of the anti-PECAM-1 Ab and the anti-$\beta_3$ integrin Abs. Specifically, while PECAM-1 blockade suppressed IL-1$\beta$-induced leukocyte transmigration, anti-$\beta_3$ integrin Abs inhibited leukocyte transmigration induced not by IL-1$\beta$, but by the chemoattractant FMLP. These findings, while confirming roles for both PECAM-1 and $\beta_3$ integrins in leukocyte transmigration, suggest that PECAM-1 and $\alpha_v \beta_3$ do not necessarily act as counter-receptors to facilitate leukocyte migration in vivo.

FIGURE 5. Effect of anti-PECAM-1 mAb 37 on FMLP-induced leukocyte responses within rat mesenteric venules. Rats were prepared for intravital microscopy, and basal readings of leukocyte firm adhesion (A) and transmigration (B) were taken. The animals were then treated with an i.v. dose of mAb 125 (control mAb) or mAb 37 (anti-PECAM-1 mAb), both at 5 mg/kg, and further readings of leukocyte responses were recorded (0 min). Fifteen minutes later, FMLP (at a final concentration of $10^{-7}$ M) was applied topically to the preparation, and further readings of leukocyte responses were recorded at the indicated time points during a 60-min in vivo test period. Data represent the mean $\pm$ SEM for four rats per group.

FIGURE 6. Effect of anti-$\beta_3$ integrin mAb 7E3 on FMLP-induced leukocyte responses within rat mesenteric venules. Rats were prepared for intravital microscopy, and basal readings of leukocyte firm adhesion (A) and transmigration (B) were recorded. The animals were then treated with an i.v. dose of a control Ab or 7E3 F(ab')2 (anti-$\beta_3$ integrin mAb), both at 5 mg/kg, and further readings of leukocyte responses were recorded (0 min). Fifteen minutes later FMLP (at a final concentration of $10^{-7}$ M) was applied topically to the preparation, and further readings of leukocyte responses were recorded at the indicated time points during a 60-min in vivo test period. Data represent the mean $\pm$ SEM for four rats per group. *, $p < 0.05$. The Journal of Immunology
PECAM-1 homotypic interactions and their ability to suppress cutaneous leukocyte migration in the human skin/SCID mouse model, it does not rule out a functional role for other PECAM-1-dependent mechanisms in leukocyte migration in vivo. One important point may explain the difference between the in vivo results obtained with mAb 37 in our study and those conducted using human skin/SCID mice. In the in vivo model used in the latter investigation, only transplanted human endothelial cell PECAM-1 is susceptible to Ab blockade by mAb 37, because mAb 37 does not cross-react with murine PECAM-1 on infiltrating leukocytes. In contrast, in the present study as mAb 37 cross-reacts with rat PECAM-1, the Ab can bind to both endothelial cell and leukocyte PECAM-1 molecules, thus enhancing its potential to suppress PECAM-1-mediated adhesion events and, hence, PECAM-1-dependent leukocyte transmigration.

The present study as well as using a combination of intravital and electron microscopy to demonstrate that an anti-domain-1 PECAM-1 mAb (37) can specifically reduce the ability of neutrophils to penetrate the endothelium also reaffirms that the requirement for PECAM-1 in leukocyte transmigration is stimulus specific (23, 52). In agreement with our previous observations (23), PECAM-1 blockade had no effect on rapid leukocyte migration through rat mesenteric venules induced by the chemottractant FMLP. This finding supports our previous explanation that leukocyte chemotactants, such as FMLP, may stimulate leukocyte migration by directly acting on leukocytes to activate their integrins and perhaps mobilize their granular proteases. Such a chemotactant-induced response may bypass the need to activate endothelial cells and hence trigger leukocyte transmigration by PECAM-1-independent mechanisms (23).

The integrin α3β1 is expressed by endothelial cells, neutrophils, monocytes, and, to a lesser degree, platelets (34, 36). Neutralizing Abs against it and its membrane-associated protein, CD47 (IAP), have proven effective at reducing leukocyte transmigration in vitro (39, 39). Because α3β1 has been proposed as a ligand for PECAM-1 (31, 32), we examined its role in leukocyte migration in vivo. Hence, we investigated the effects of two anti-β3 integrin mAbs, namely 7E3 and F11. mAb 7E3 is a murine anti-human α3 integrin Ab (36). Before its use in our rat models of inflammation, we first examined the ability of this Ab to cross-react with rat molecules. In this context, 7E3 was found to inhibit rat platelet aggregation ex vivo and to bind to rat neutrophils, as determined by flow cytometry. F11 is a specific anti- rat β3 integrin mAb (44).

In contrast to our findings with the anti-PECAM-1 mAb, anti-β3 integrin Abs had no effect on leukocyte migration through IL-1β-activated rat mesenteric venules, but selectively suppressed the transmigration phase of FMLP-induced leukocyte emigration. The mechanism by which β3 integrins may be regulating FMLP-induced leukocyte migration in vivo is at present unclear, although a number of possible mechanisms can be proposed. The β3 integrin, α3β1, has been implicated in a number of cellular responses intimately associated with the process of leukocyte transmigration. Specifically, it has been shown to regulate leukocyte motility on endothelial cell-associated adhesion molecules, such as VCAM-1 and ICAM-1, by modulating the functions of leukocyte integrins such as α5β1 and αLβ2 (LFA-1) (33, 50). Such αLβ2-mediated down-regulation of integrin-dependent firm adhesion may provide a mechanism by which firmly adherent leukocytes become dead-herent, allowing them to engage in the process of transmigration (33). With respect to the transmigration phase, there is some evidence suggesting that α5β1 may have a regulatory role on the activity of leukocyte proteases (53), and through its ability to interact with extracellular components, such as vitronectin and fibronectin, it may mediate leukocyte migration across and through
the perivascular basement membrane (54, 55). Hence, in our experimental system, FMLP, through its ability to directly stimulate neutrophils, may initiate a cascade of integrin activation events that induces β2 integrin-mediated firm adhesion followed by αβ2-mediated leukocyte deathadhension, triggering the transition from firm adhesion to extravasation. Furthermore, it is unclear at present whether αβ3 is involved in leukocyte migration through the endothelium or the perivascular basement membrane, an aspect of leukocyte extravasation that is currently under investigation in our laboratory. The lack of effect of the αβ3 integrin mAbs on IL-1β-induced leukocyte transmigration may suggest that in response to an endothelial cell-activating factor, the activation of adhesion pathways involving molecules such as PECAM-1 overrides the αβ3-dependent mechanisms. If the results obtained with the anti-αβ3Abs were predominantly due to suppression of αβ3, then they would suggest that while PECAM-1 and αβ3 are both involved in leukocyte transmigration in vivo, these molecules do not necessarily act as counter-receptors in the process of leukocyte emigration. However, although our observations may support a role for αβ3 in leukocyte transmigration, the possible role of alternative ligands with which the Abs 7E3 and F11 could interact needs to be considered.

As indicated above, both 7E3 and F11 bind to rat αmβ2 (present study and Ref. 44), and αmβ2 blockade on platelets could contribute to our in vivo observations. Because both platelets and neutrophils can bind to fibrinogen, via αmβ2, and Mac-1, respectively, platelets adherent to the endothelial surface may facilitate the adhesion of neutrophils and their subsequent recruitment, using fibrinogen as a bridging molecule. Not only has this bridging phenomenon been shown in vitro under flow conditions (56), but additionally, 7E3 has been shown to inhibit binding of cell surface Mac-1 to fibrinogen (57). However, if platelet αmβ2 were contributing to leukocyte recruitment in our model, one or both anti-αβ3 Abs would be expected to reduce firm adhesion as well as transmigration, which they did not. Although the present results do not rule out a role for αmβ2, it seems unlikely that the inhibitory effect of the anti-β2 integrin Abs is as a result of inhibiting platelet αmβ2; it is more likely due to a blockade of αβ3 on leukocytes. This is supported by two lines of evidence. First, ααβ2 neutralizing Abs can inhibit leukocyte transmigration in vitro (33), and second, a recent study has shown that even in the absence of platelets, the β3 integrin-blocking Ab fragment, c7E3 Fab (aabciximab, ReoPro), can reduce leukocyte accumulation in an ex vivo model of myocardial ischemia (58).

In addition to binding the β2 integrins (αmβ2 and αβ3), 7E3 has been shown to bind to an activated form of the β2 integrin, Mac-1 (48, 49), although its affinity for Mac-1 has been estimated to be 20- to 50-fold lower than that for αmβ2 (57). Mac-1 is present on neutrophils and monocytes and has been shown to be involved in leukocyte transmigration (6, 8). Although it is possible that the reduction in leukocyte transmigration seen in 7E3-treated animals in the present study is the result of inhibition of this β2 integrin, two lines of evidence argue against this. First, if inhibition of Mac-1 were a significant confounding factor, then suppression of leukocyte firm adhesion would also have been detected. Anti-Mac-1 mAbs have been shown in vitro to reduce FMLP-induced leukocyte adhesion under both static and flow conditions (59, 60). In the present study 7E3 had no effect on FMLP-induced firm adhesion. Second, an additional anti-β2 integrin mAb (F11), which to our knowledge does not bind Mac-1, produced results identical to those for 7E3.

This study is the first to demonstrate roles for both PECAM-1 and β2 integrins, most likely αβ3, in leukocyte transmigration in vivo. The findings further demonstrate that the roles of these molecules in leukocyte migration are divergent with respect to stimulus specificity, suggesting differential requirements for PECAM-1 and β2 integrins in the regulation of leukocyte transmigration in response to different inflammatory mediators.

References


