*Brief Definitive Report*

# Monoclonal Antibody to Murine PECAM-1 (CD31) Blocks Acute Inflammation In Vivo

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## Summary

A murine model of peritonitis was used to test the role of platelet/endothelial cell adhesion molecule 1 (PECAM-1/CD31) in acute inflammation. A monoclonal antibody (mAb) specific for murine PECAM-1 injected intravenously 4 h before the intraperitoneal injection of thioglycollate broth blocked leukocyte emigration into the peritoneal cavity for up to 48 h. This block was particularly evident for neutrophils. Control mAb, including one that bound to murine CD18 without blocking its function, failed to block emigration when used at the same or higher concentrations. The decreased emigration seen with the anti-PECAM-1 antibody was not due to neutropenia or neutrophil sequestration in the lung, spleen, or other organs; peripheral blood leukocyte counts were not diminished in these mice. In the mesenteric venules of the mice treated with anti-PECAM-1 mAb, leukocytes were frequently seen in association with the luminal surface of the vessel, but did not appear to emigrate. Thus, the requirement for PECAM-1 in the transendothelial migration of leukocytes previously seen in an in vitro model holds true in this in vivo model of acute inflammation.

Platelet/endothelial cell adhesion molecule 1 (PECAM-1/CD31) is a member of the Ig gene superfamily (1) concentrated at the intercellular junctions of cultured human endothelial cells (HEC) (2) and expressed on the surface of platelets (1) and most leukocytes. We have demonstrated that PECAM-1 is required for the migration of monocytes and neutrophils across resting and cytokine-activated HEC in a quantitative in vitro assay of transmigration (3).

Recent characterization of the murine homolog of PECAM-1 has afforded us the opportunity to test the role of this molecule in an in vivo model of inflammation. Cloning of the murine homolog of PECAM-1 (4) revealed a predicted amino acid sequence with 79% homology to human CD31. L cells transfected with murine PECAM-1 cDNA aggregated in a PECAM-1–dependent manner, similar to human PECAM-1 transfectants. A mAb raised in hamsters that recognizes the murine form of CD31 (5) blocked this aggregation (4). Furthermore, immunohistochemical studies using this antibody demonstrated that murine PECAM-1 had a tissue distribution similar to that of human PECAM-1 (5).

We used a murine model of acute peritonitis to test whether intravenously administered anti-murine PECAM-1 mAb would block acute inflammation. The results demonstrate that anti-PECAM-1 mAb inhibits emigration of neutrophils (PMN) to nearly background levels, establishing PECAM-1 as an important adhesion molecule in the inflammatory response.

## Materials and Methods

*Animals.* Female mice of the $CD2F_1$ strain weighing ~20 g were purchased from Charles River Laboratories (Boston, MA) and housed at The Rockefeller University Laboratory Animal Research Center. Female mice of the AKR/J strain were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed at the Boston University School of Medicine Laboratory Animal Science Center. All animal procedures had been approved by the Rockefeller University and Boston University School of Medicine IACUCs. Animals were handled according to guidelines set forth in the "Guide for the Care and Use of Laboratory Animals" and the "Animal Welfare Act." Mice were housed together in standard cages and allowed free access to mouse chow and water.

*Monoclonal Antibodies.* mAb 2H8 hamster anti–murine PECAM-1 was produced as described (5) and purified by HPLC using a semi-preparative column (ABx; J. T. Baker, Inc., Phillipsburg, NJ), loading in 25 mM 2-[N-Morpholino]ethanesulfonic acid (MES), pH 5.7, and eluting with a linear 10–60% gradient of 500 mM $NH_4SO_4$ plus 5 mM $KH_2PO_4$, pH 6.7, according to the manufacturer's recommendations. Purification of the 2H8 mAb under denaturing conditions resulted in an antibody preparation that was less effective in vivo. The 2H8 mAb was negative for endotoxin by the Limulus amebocyte lysate assay (Sigma Chemical Co., St. Louis, MO). The hamster anti–murine CD18 mAb 2E6 was originally raised at The Rockefeller University (6) and was donated by Endogen, Inc. (Boston, MA). Hybridoma lines producing mAb 5C6 (rat anti–murine CD11b) (7) were generously provided by Dr. Hugh Rosen (Merck Research Laboratories, Rahway, NJ). IgG was purified from cell supernatant as previously

described (3). Normal hamster IgG was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). All mAb were documented to recognize their specific antigens by flow cytometry and/or immunohistochemistry.

*Immunohistochemistry.* Leukocytes were isolated from buffy coats of blood collected by cardiac puncture in heparinized syringes. RBC were lysed by resuspension of the buffy coat pellet in 9 vol of pyrogen-free distilled water for 60 s on ice. 1 vol of 10 X PBS was added, the suspension was centrifuged, and the pellet containing leukocytes was washed in HBSS. Frozen sections of mouse tissue, or cytospin preparations of isolated PBL or peritoneal exudate cells, were stained by indirect immunoperoxidase histochemistry as previously described (2, 5). Briefly, endogenous peroxidase activity was quenched by reaction with $H_2O_2$, the sections were incubated with primary mAb diluted in PBS/OVA for 30 min at room temperature, washed in PBS/OVA, then incubated in a 1:200 dilution of horseradish peroxidase–conjugated rabbit anti-hamster IgG (Accurate Scientific Corp., Westbury, NY) for 30 min at room temperature before washing and developing with diaminobenzidine-$H_2O_2$.

*Experimental Procedure.* The emigration of PMN into the mouse peritoneal cavity in response to intraperitoneal injection of thioglycollate broth is a well-established model of acute inflammation (8, 9). Mice were injected intravenously via the lateral tail vein with the various mAb diluted in Dulbecco's PBS (DPBS) to a final volume of ~100 μl. Some control mice received DPBS alone or an equivalent dose of normal hamster IgG. 4 h later, mice were injected intraperitoneally with 1 ml of 4% Brewer's thioglycollate broth (Difco, Detroit, MI). 24 h after the mAb injection, mice were killed by exposure to $CO_2$. Peritoneal cells were recovered into tubes on ice by lavage with 5 ml of divalent cation-free HBSS (GIBCO BRL, Gaithersburg, MD) using standard techniques (10). Heparinized blood was collected by cardiac puncture or from the retro-orbital sinus. Peritoneal cell counts and peripheral white blood cell counts were performed employing erythrocyte lysis kits (Unopette; Fisher Scientific, Pittsburgh, PA) on samples from each mouse using a hemacytometer. Differential counts were performed on Wright/Giemsa-stained cytospin preparations (for peritoneal cells) and peripheral blood smears.

The abdominal and thoracic cavities were inspected for signs of gross pathology. The mesenteric LN were harvested and fixed in 4% paraformaldehyde, 2% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4. The liver and spleen were excised intact and weighed. These organs, along with the majority of the small and large bowel, were fixed in neutral buffered formalin. Representative regions were embedded in paraffin or methyl methacrylate. Sections were cut and stained with hematoxylin and eosin.

## Results and Discussion

*mAb against Murine PECAM-1 Blocks Acute Inflammation.* To test whether PECAM-1 plays a critical role for acute inflammation in vivo, we administered the hamster anti-murine PECAM-1 mAb 2H8 (5), or appropriate positive or negative control mAb, intravenously, 4 h before the intraperitoneal injection of thioglycollate broth (Fig. 1). In this well-established model of acute peritonitis, intraperitoneal thioglycollate induces an influx of neutrophils into the peritoneal cavity within the first 2 h (8, 9). The degree of inflammation was measured by peritoneal lavage 20 h after thioglycollate injection. In seven out of seven experiments performed, the anti-PECAM-1 mAb prevented the emigration of leukocytes into the peritoneal cavity. Two representative experiments are shown in Fig. 1. In all cases, the effect of anti-PECAM-1 mAb was comparable with that of the positive control mAb 5C6 (anti-CD11b), which had previously been shown to block acute inflammation in this model (7). Production and purification of active Fab or $F(ab')_2$ fragments of 2H8 proved technically problematic. Therefore, in experiment 2 and other similar experiments, a hamster anti-mouse CD18 (2E6) served as a control, since it binds tightly to murine leukocytes, but is a relatively weak blocker of integrin function (6). The failure of this mAb to block emigration rules against nonspecific or Fc-mediated effects of bound mAb being responsible for the block seen with anti-PECAM-1 mAb. Immunostaining of peripheral blood from mice treated with 2E6 demonstrated that leukocytes still bound readily detectable levels of this mAb at the time of killing (data not shown).

Fig. 1 separately reports data on the PMN exudate and the total exudate cells (including lymphocytes and macrophages). The decrease in PMN accumulation produced by the anti-PECAM-1 mAb is particularly dramatic, since these

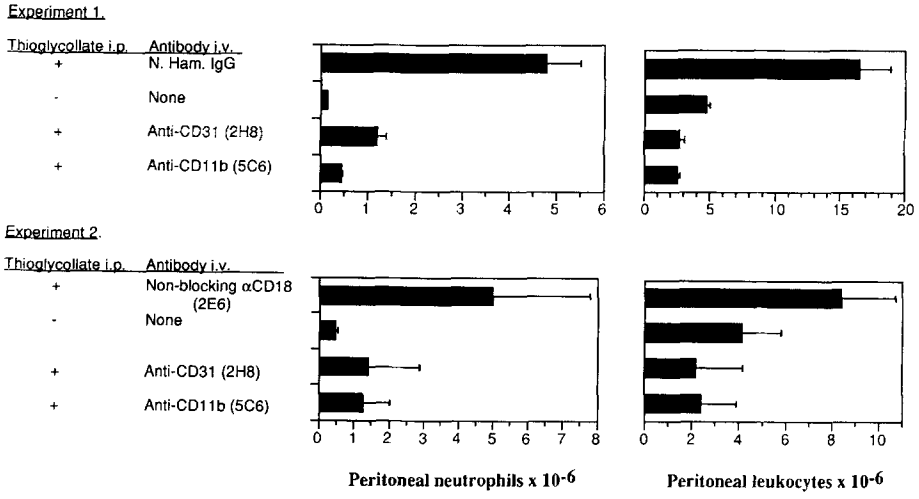



**Figure 1.** Anti-PECAM-1 mAb blocks leukocyte emigration in response to thioglycollate injection. Peritoneal cells were harvested at 24 h from mice that had received 250 μg of the indicated mAb, normal hamster IgG (*N. Ham. IgG*), or DPBS (*None*), intravenously, at time zero and thioglycollate or DPBS intraperitoneally at 4 h. Two experiments, representative of seven, are shown. AKR/J mice were used in experiment 1; $CD2F_1$ mice in experiment 2. Bars indicate mean ± SEM for each group (five mice per group).

cells are not normally resident in the peritoneal cavity. Additional experiments (not shown) revealed that significant suppression of inflammation by mAb 2H8 was achieved at doses as low as 50 μg/mouse (the lowest dose tested), and suppression was maintained for at least 48 h. This time course is similar to that described with anti-CD11b blockade (7) and significantly longer than the 4-h suppression seen in this model by blockade of L-selectin (9), or knockout of P-selectin (11).

*Anti-PECAM-1 mAb Appears to Arrest Emigration of Adherent Leukocytes.* Administration of these antibodies did not lower the circulating leukocyte count. In fact, at the time of killing, there was a granulocytosis in those mice that had received anti-PECAM-1 mAb (Table 1), consistent with the hypothesis that PMN were being recruited into the circulation, but blocked from emigrating to the site of inflammation. Histologic examination revealed no abnormal accumulations of leukocytes in the spleens or livers (data not shown). Absolute platelet counts were not performed, but no difference in platelet numbers or appearance on peripheral blood smears was seen among the experimental groups.

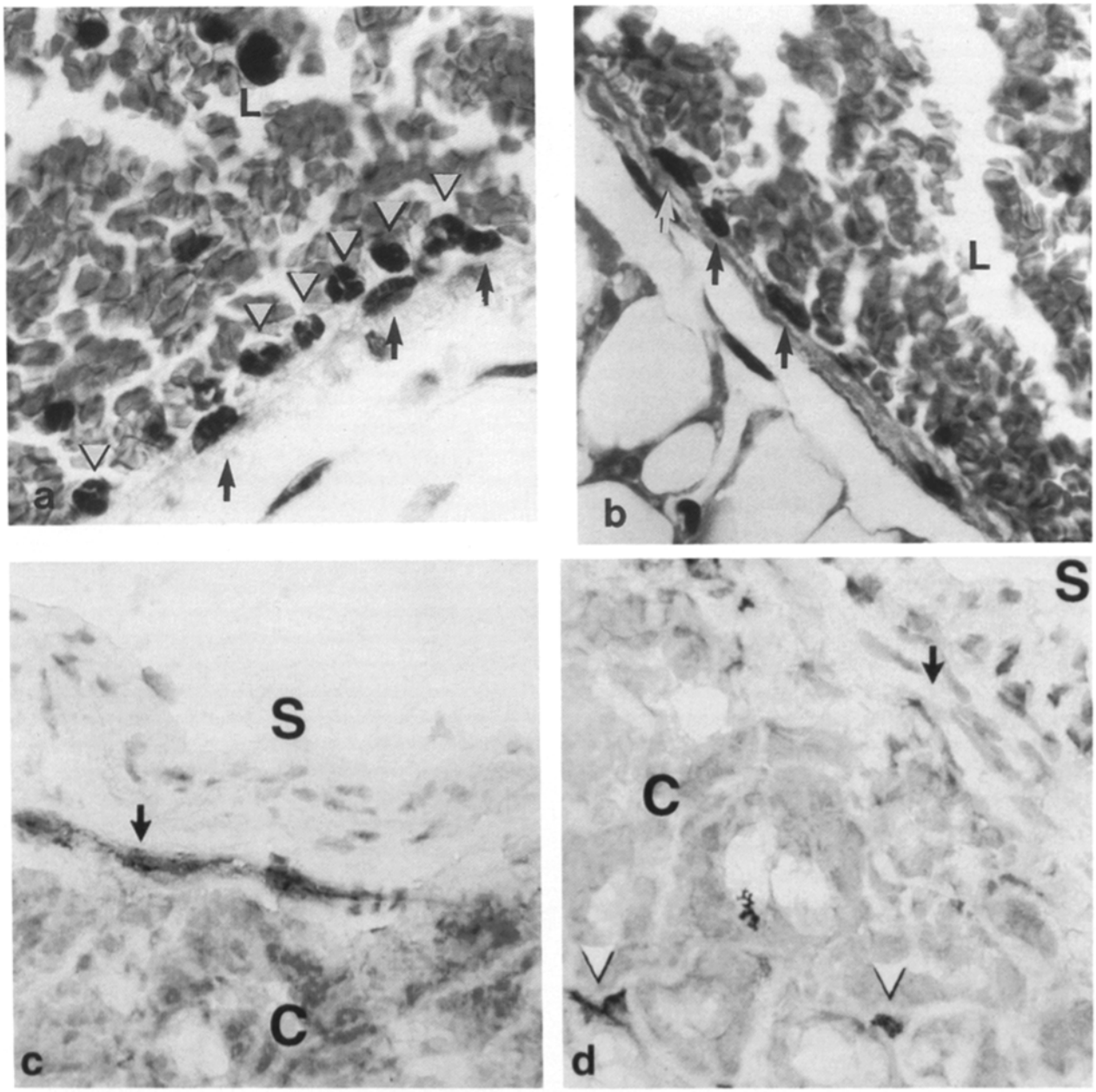
Histologic examination of the mesenteries of the mice receiving anti-PECAM-1 mAb showed increased numbers of intravascular PMN in the venules compared with controls. These PMN appeared to be in contact with the endothelial surface (Fig. 2), but were inhibited from migrating across the vascular wall, analogous to the block of transmigration observed in vitro (3). Fig. 2 *a* shows one of the more extreme examples of this phenomenon encountered; however, about 60% of random mesenteric venular profiles in anti-PECAM-1–treated mice appeared to bear adherent leukocytes (Fig. 2 *e*). This phenomenon was seen in postcapillary venules, as well as in slightly larger venules, which are also sites of emigration of PMN (12). Since the chances of finding a truly nonadherent leukocyte adjacent to the endothelium were less in these larger diameter venules, 10 random sections of such venules (50–150 μm diameter) were examined on coded slides from each mouse, and leukocytes in contact with the vessel wall or free in circulation were counted. Only rare leukocytes contacted the endothelium in control (no thioglycollate) animals or in animals receiving mAb other than 2H8 (Fig. 2, *b* and *e*). There was variability among venules, but overall, 34% of all leukocytes seen in the venules of the anti-PECAM-1–treated mice were in apparent contact with the endothelium. Immunohistologic examination of these mice demonstrated that anti-PECAM-1 mAb was still specifically retained by endothelium in vascular structures, including mesenteric venules. Since these vessels are found in fatty tissue and therefore are cut poorly by frozen section, this phenomenon is demonstrated on vessels within the bowel wall (Fig. 2, *c* and *d*). Residual 2H8 on circulating PMN could not be detected using immunoperoxidase techniques. However, this could be due to turnover of PMN during the 24 h and/or shedding of murine PECAM-1 from PMN membranes.

*Anti-PECAM-1 mAb Blocks Emigration of Mononuclear Cells.* Significant numbers of mononuclear cells as well as PMN are recruited to the peritoneal cavity by 20 h after thioglycollate injection. Fig. 1 demonstrates that the increase in peritoneal exudate cells is not accounted for by the increase in PMN alone. Anti-PECAM-1 mAb 2H8 also blocked the influx of mononuclear cells into the peritoneum (Fig. 1, *right*). To determine if PECAM-1 blockade affected mononuclear cell trafficking, we examined the mesenteric LN draining the peritoneal cavity. The peritoneal cavity is drained by, among other lymphatic beds, the mesenteric plexus. Peritoneal mononuclear cells in the afferent lymphatics would enter draining LN via the subcapsular sinus. Examination of mesenteric nodes revealed large numbers of mononuclear cells entering via the subcapsular sinus in mice stimulated with thioglycollate, but relatively few in the subcapsular sinuses of mice given anti-PECAM-1 mAb before thioglycollate (Fig. 3). The paucity

**Table 1.** *Peripheral Blood Leukocyte Counts for Experiment in Fig. 1*

| | Thioglycollate | Antibody | White blood cells (cells/μl) | Differential (P/L/M) |
|---|---|---|---|---|
| Experiment 1 (AKR/J strain) | | | | |
| | + | Normal hamster IgG | 4,438 ± 169* | 36/59/5‡ |
| | – | None | 4,963 ± 699 | 19/75/6 |
| | + | Anti-PECAM (2H8) | 4,548 ± 242 | 62/35/3 |
| | + | Anti-CD11b (5C6) | 2,838 ± 234 | 20/65/15 |
| Experiment 2 ($CD2F_1$ strain) | | | | |
| | + | Anti-CD18 (2E6) | 9,640 ± 1,270 | 28/65/7 |
| | – | None | 12,260 ± 1,970 | 11/77/12 |
| | + | Anti-PECAM (2H8) | 16,060 ± 2,750 | 59/33/8 |
| | + | Anti-CD11b (5C6) | 11,580 ± 3,460 | 20/74/6 |

* Peripheral white blood cell count. Data expressed as mean ± standard error of mean.
‡ Percentages of polymorphonuclear leukocytes (neutrophils)/lymphocytes/monocytes from ≥100 cells counted. Note that a normal mouse white blood cell differential count is dominated by lymphocytes. Mice receiving thioglycollate displayed increased percentages of myeloid forms, including immature forms, which are grouped with the neutrophils.




of mononuclear cells in the subcapsular sinus is a direct reflection of the block of mononuclear cell entry into the peritoneal cavity. These data are in agreement with those of Fig. 1, and selectively represent the mononuclear cell fraction. PMN do not recirculate into the draining lymphatics in general and were not seen in the subcapsular sinuses of the LN of these mice.

Furthermore, in some experiments, the thioglycollate-elicited peritoneal exudate cell count was lower for anti-PECAM-1–treated mice than for control mice not stimulated with thioglycollate (Fig. 1, *right*). This suggested that PECAM-1 may be required for the constitutive trafficking of mononuclear cells through the peritoneum, as well as for the thioglycollate-elicited emigration.

In an in vitro model using human leukocytes and endothelial cells, anti-PECAM-1 mAb or soluble recombinant PECAM-1 did not affect attachment, but blocked transmigration of monocytes and PMN (3), which remained tightly adherent to the apical surface over the intercellular junctions, the site at which endothelial PECAM-1 is concentrated (2). The function of PECAM-1 in transendothelial migration was distal to the selectin-mediated rolling and the $\beta_2$ integrin–mediated tight adhesion to the apical surface of the venular endothelium. The results of the present study (Fig. 2) suggest that PECAM-1 plays a similar role in vivo, since leukocytes blocked from entering the peritoneal cavity by anti-PECAM-1 mAb were apparently arrested on the luminal surface of the venular endothelium. We presume that the arrest on the en-

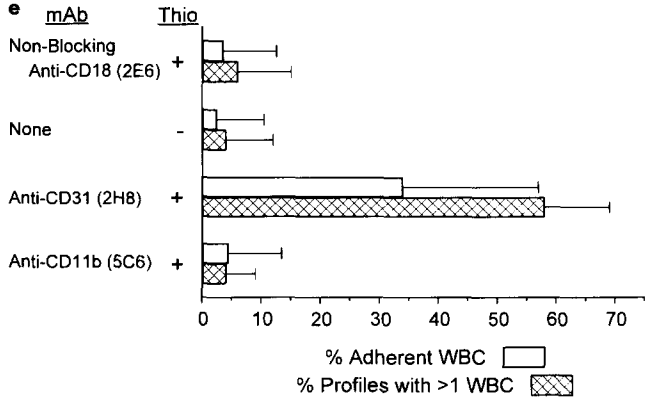



**Figure 2.** Mesenteric venules from anti-PECAM-1–treated mice show increased numbers of intraluminal leukocytes in apparent contact with the endothelium. Mice treated with anti-PECAM-1 mAb as in Fig. 1 revealed a high proportion of leukocytes (predominantly PMN, *arrowheads*) in random sections in contact with the endothelium (*a*), whereas mice treated with the nonblocking anti-CD18 mAb showed rare leukocytes on the endothelial surface (*b*), despite the fact that many had transmigrated. Immunoperoxidase staining of frozen sections of mesenteric tissue demonstrated that the anti-PECAM-1 mAb injected 24 h previously was still localized to endothelial cells in vascular structures (*arrow*, *c*), whereas anti-CD18 mAb (*d*) stained only occasional stellate cells adjacent to the crypts (*arrowheads*) and lamina propria (not shown) of the bowel, but not blood vessels (*arrow*). Quantitation of over 400 leukocytes in 10 random venular profiles from each of the five mice per group revealed that only the anti-PECAM-1 mAb group had significant numbers of leukocytes remaining in contact with the endothelium (*e*). Data are expressed as the percent total intravascular leukocytes apparently in contact with the venular wall (*open bars*) and as the number of profiles counted (out of 10 per mouse) that bore more than one adherent leukocyte. These data are converted to a percentage. The mean ± SD for groups of five mice is shown. (*a* and *b*) ×415; (*c* and *d*) ×260. Arrows in (*a*) and (*b*) point to endothelial nuclei. (*L*) Venular lumen; (*S*) small bowel serosal surface; (*C*) intestinal crypts.

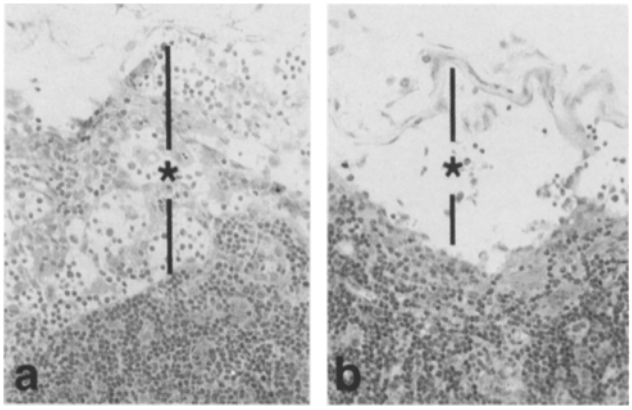



**Figure 3.** Relative paucity of mononuclear cells in the subcapsular sinus of the draining mesenteric nodes in anti-PECAM-1–treated mice. Mesenteric LN from thioglycolate-treated mice were fixed and sectioned as described in Materials and Methods. (*a*) The subcapsular sinus (*asterisk*, *vertical lines* define width of sinus) of a representative node from mice treated with normal hamster IgG. Note the large numbers of mononuclear cells within the sinus, indicating their recent arrival via afferent lymphatics. (*b*) The relative few mononuclear cells in the same area from a mouse treated with 2H8 anti-PECAM-1, correlating with the decreased total peritoneal cells recovered from peritoneal lavage. ×80.

dothelial surface is transient, since the vast majority of the luminal surface of the affected venules was free of leukocytes, and the granulocytosis was persistent. The block of emigration effected by anti-PECAM-1 mAb appears to involve a different mechanism than the block by anti-CD11b. In the latter case, no granulocytosis was seen in the peripheral blood, and, although PMN emigration into the peritoneum was inhibited, leukocytes were not seen in apparent contact with the venular wall. This is consistent with the proposed role of $\beta_2$ integrins in mediating the tight adhesion to the endothelial surface (13–15).

In our in vitro model, leukocyte transmigration could be blocked equally well by treating either the leukocytes or the endothelial cells with anti-PECAM-1 reagents. We do not know whether the 2H8 mAb is blocking leukocyte transmigration by blocking PECAM-1 on leukocytes, endothelium, or both.

Since anti-PECAM-1 mAb blocked emigration of mononuclear cells as well as PMN in vivo, PECAM-1 may mediate a function common to all leukocyte types that is necessary for the process of transendothelial migration. In addition, the inhibition was effected at a low dose of anti-PECAM-1 mAb (50 μg/mouse in certain experiments) and lasted up to 48 h after a single injection. These observations suggest that PECAM-1 may be a suitable molecule to target in anti-inflammatory therapy.

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*Note Added in Proof:* Vaporciyan et al. have recently reported experiments in which a rabbit anti-human PECAM-1 polyclonal antibody that cross-reacts with rat PECAM inhibited emigration of PMN for 4 h in three rat models of acute inflammation (16).

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