PECAM-1 Is Required for Transendothelial Migration of Leukocytes

By William A. Muller,* Susan A. Weigl,* Xiaohui Deng,* and David M. Phillips‡

From the *Laboratory of Cellular Physiology and Immunology, The Rockefeller University; and ‡The Population Council, New York, New York 10021

Summary

Platelet/endothelial cell adhesion molecule 1 (PECAM-1; CD31) is crucial to the process of leukocyte transmigration through intercellular junctions of vascular endothelial cells. A monoclonal antibody to PECAM, or recombinant soluble PECAM, blocks transendothelial migration of monocytes by 70–90%. Pretreating either the monocytes or the endothelial junctions with antibody blocks transmigration. If the endothelium is first activated by cytokines, anti-PECAM antibody or soluble recombinant PECAM again block transmigration of both monocytes and neutrophils. Anti-PECAM does not block chemotaxis of either cell type. Light and electron microscopy reveal that leukocytes blocked in transmigration remain tightly bound to the apical surface of the endothelial cell, precisely over the intercellular junction. Thus, the process of leukocyte emigration can be dissected into three successive stages: rolling, mediated by the selectin class of adhesion molecules; tight adhesion, mediated by the leukocyte integrins and their endothelial cell counter-receptors; and now transmigration, which, based on these studies, requires PECAM-1.

Leukocytes leave the circulation by binding to the luminal surface of the vascular endothelium and then migrating between tightly apposed endothelial cells into the body tissues (1, 2). This process is constitutive in some situations. For example, monocytes (Mo)[1] exit the circulation at a low frequency to become tissue macrophages (3). Circulating lymphocytes enter lymph nodes by binding to and migrating across the specialized high endothelial venules (HEV) in lymphoid tissue (4, 5). This process involves recognition and binding of L-selectin to specialized, lymph node–restricted counter-receptors on the HEV (6, 7). During the process of inflammation leukocytes emigrate in large numbers into the inflammatory site by recognizing and binding to cytokine-induced cell adhesion molecules (CAMs) on the endothelium (8–10). A related process may occur during early atherogenesis when Mo (and lymphocytes) selectively emigrate into the subintima of affected arteries (11).

For >100 yr, it has been recognized that within the postcapillary venules of inflamed tissue, emigrating leukocytes leave the central stream of the circulation, roll more slowly along the vessel wall, then stop by adhering tightly to the endothelial

surface before squeezing between adjacent endothelial cells into the subendothelial tissue (12, 13). Much more recently, it has been appreciated that the rolling phenomenon is mediated by the selectin class of adhesion molecules, while the tight adhesion is mediated by the leukocyte β_2 integrins and their endothelial counter-receptors (14–16). We now report that platelet/endothelial cell adhesion molecule 1 (PECAM), a member of the Ig gene superfamily (17) concentrated in the junctions between endothelial cells (18) and expressed on the surfaces of Mo and neutrophils (PMN) (19–21), mediates adhesive interactions distal to the adhesion mediated by the β_2 integrins that are crucial for the transendothelial passage of Mo and PMN. Anti-PECAM mAb as well as soluble recombinant PECAM block transmigration of these leukocytes after the tight adhesion stage. Thus, leukocyte emigration is experimentally dissectable into three steps, each phase mediated primarily by a different family of adhesion molecules (selectins, integrins, Ig superfamily) and their counter-receptors.

Materials and Methods

Cells. Human umbilical vein endothelial cells (HEC) were isolated and cultured on collagen gels as described (18). In some experiments HEC were activated by culturing in the presence of recombinant human TNF-α (10 ng/ml in culture medium) for the times indicated in the figure legends. TNF-α induces the expression of endothelial cell adhesion molecules like E-selectin (22) and vascular

---

This paper is dedicated to the loving memory of Zanvil A. Cohn, who continues to be an inspiration to generations of scientists.

[1] *Abbreviations used in this paper:* EM, electron microscopy; HEC, human umbilical vein endothelial cells; Mo, monocytes; M199, medium 199; PECAM, platelet/endothelial cell adhesion molecule.

cell adhesion molecule 1 (VCAM-1) (23), which are not otherwise expressed by HEC in this culture system (24). PBMC and PMN were isolated from venous blood freshly drawn from healthy volunteers as described (24).

Antibodies. hec7 is a mouse mAb (IgG2a isotype) that binds to human PECAM-1 (18). mAb hec1 is an IgG2a raised in this laboratory from the same original fusion. It recognizes a junctionally restricted HEC integral membrane protein distinct from PECAM. Clones secreting both mAbs were adapted to growth in serum-free medium (Nutridoma NS; Boehringer Mannheim Biochemicals, Indianapolis, IN), and IgG was selectively precipitated by ammonium sulfate. IgG was redissolved, dialyzed in PBS, and sterilized by membrane filtration. Fab and F(ab')₂ fragments were produced by incubating with immobilized papain and pepsin, respectively (Pierce Chemical Co., Rockford, IL). Undigested antibody and Fc fragments were removed by passage over protein A–Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ). SDS-PAGE performed under nonreducing conditions verified that the fragments were properly cut. SDS-PAGE of overloaded samples demonstrated that all IgG and active fragments samples were pure; no extraneous bands were present on Coomassie blue stain.

Other mAbs used in this study, their antigens, and sources were: IB4 and 3C10, anti-CD18 and CD14, respectively (from Dr. Samuel Wright, The Rockefeller University); W6/32, anti–class I MHC (from American Type Culture Collection [ATCC], Rockville, MD); 9.3C9 (9.3F10), anti–class II MHC (HLA-DR and HLA-DQ) (ATCC HB 180; raised in this department); LB-2, anti-ICAM-1 (from Dr. Edward Clark, University of Washington, Seattle, WA); and 3G8, anti-FcγRIII (from Medarex, West Lebanon, NH). Rabbit anti-PECAM serum and preimmune serum were generated against PECAM-1 purified from platelets (25).

Recombinant Soluble PECAM-1. A cDNA encoding a form of PECAM containing the signal sequence and five and one-half of the six extracellular Ig loops was constructed from an existing full-length PECAM cDNA clone in the vector pCDM8 (26). This plasmid was cut with restriction enzymes HindIII and NheI. The 1,850-bp fragment encoding the external domain of PECAM was purified on an agarose gel and ligated to a gel-purified 4,056-bp fragment of pcDM8 previously digested with HindIII and XbaI, representing the entire vector minus the multiple cloning domain from the first XbaI site to the HindIII site. Since XbaI and NheI generate the same sticky ends, successfully annealed plasmids contain the fragment of DNA encoding truncated PECAM inserted in the pcDM8 in the proper orientation for expression. Unique BamHI sites in both PECAM and pcDM8 allowed verification of this by restriction digest of transformed *Escherichia coli* MC 1061/p3. Purified plasmid was obtained from these cultures on CsCl gradients by standard methods (27). COS-1 cells (ATCC) were transiently transfected by electroporation of 10⁷ cells in 0.5 ml DME with 20 μg plasmid in a gene pulser (Bio-Rad Laboratories, Richmond, CA) at 240 mV, 960 μF, 4-mm path length cuvettes. The electroporated cells were plated in DME + 10% FCS overnight, passed by trypsinization the next day to help remove dead cells, and replated in DME + 10% FCS. Expression of soluble PECAM was checked by immunoprecipitation from conditioned medium and immunofluorescence of COS cells on day 3.

Purification and Characterization of Recombinant Soluble PECAM. An immunoaffinity matrix was made by covalently coupling hec7 mAb to Affi-gel 10 (Bio-Rad Laboratories) at a concentration of 3.8 mg/ml, according to the manufacturer's directions. Conditioned medium was collected on day 5 from COS-1 cells transiently transfected with the construct described above, centrifuged free of cells, and passed over a 3-ml column of hec7-Affi-gel at 0.3 ml/min. The

column was washed with 10 vol of SA buffer, 8 vol of detergent buffer, and 20 vol of SA buffer (28). Soluble PECAM was eluted in 5 ml of 0.05 M diethylamine, pH 11.5, neutralized quickly with 1 M Tris, pH 8, and dialyzed overnight against PBS. The dialysate was concentrated using a Centricon® 30 microconcentrator (Amicon Corp., Danvers, MA). Purity was assessed by SDS-PAGE under reducing conditions followed by Coomassie blue or silver nitrate stain, which showed a single band migrating at 90 kD. The state of aggregation was assessed by running the material on a 4–15% polyacrylamide gel using nondenaturing buffer strips (Phast-gel® system; Pharmacia Fine Chemicals, Piscataway, NJ) for 154 average V-h along with nondenatured standards (Pharmacia Fine Chemicals) according to the manufacturer's directions, followed by silver staining. Protein concentration was determined using the BCA® assay (Pierce Chemical Co.). 40–60 μg of recombinant soluble PECAM was purified from ~350 ml of conditioned medium.

Metabolic Labeling, Immunoprecipitation, and SDS-PAGE. These procedures were performed as previously described (18). Any variations unique to these experiments are described in the figure legends.

Transendothelial Migration Assay. A variation of the recently published method (24) was used. Briefly, purified PBMC or PMN were resuspended to 2 or 1 × 10⁶/ml, respectively, in warm medium 199 (M199) or complete culture medium (20% normal human serum in M199) and allowed to settle on confluent monolayers of HEC grown in 96-well trays on hydrated collagen gels. In most experiments anti-PECAM mAb, other anti-PECAM reagents, or control mAb were also added at the same time. Transendothelial migration was allowed to proceed at 37°C, usually for 1 h. The original procedure called for inverted centrifugation of the culture in the presence of EGTA to separate transmigrated cells from those merely bound to the apical HEC surface. To preserve maximum optical resolution, this step was replaced by three manual washes in 1 mM EGTA, followed by three manual washes in Dulbecco's PBS with Ca²⁺ and Mg²⁺ (DPBS). In some experiments the monolayers were then stained with AgNO₃ to visualize the intercellular junctions (18). The monolayers and remaining leukocytes were then fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. Before quantitative analysis the monolayers were stained with Wright-Giemsa.

Three separate methods were used to quantitate transendothelial migration. All three gave similar results when used by one or more observers to analyze the same experiment. The method described first gave somewhat better resolution; hence, most of the quantitative data presented in this paper are from experiments where this method was used. (*a*) IgG-coated sheep erythrocytes in cold M199 were added to the transmigration assay at the end of the experiment, just before the glutaraldehyde fixation step, along with PMA (or FMLP for PMN) to fully activate leukocyte Fcγ receptors (29). After incubation for 30 min at 4°C, nonbound erythrocytes were washed off, and the entire culture system consisting of the HEC monolayer, collagen gel, and all associated leukocytes was transferred intact to a glass slide and examined at 500× by Nomarski optics. Leukocytes were scored as apical if they were in the focal plane above the HEC monolayer and bound (rosetted) more than three Ig-coated sheep red cells. (*b*) Each well of the 96-well tray was centered in turn over the low-power objective of an inverted microscope, and the central field in each well was subsequently examined at 400× using Hoffmann modulation contrast optics (30) and the silver-stained HEC junctions to sharply define the apical surface of the monolayer. All leukocytes in focus on the apical surface were scored as nontransmigrated. This included those with a small pseudopod extending into the junction. Those leukocytes

in focus below the plane of the monolayer were scored as trans-migrated. (c) The entire culture system was removed from the well as for a, and random fields were examined at 500× under Nomarski interference optics, scoring apical and transmigrated leukocytes as for b. Data are expressed as percent of total cells viewed that had transmigrated; in most samples there were ~100 total leukocytes viewed in each field. The mean and standard deviation of at least six replicate cultures are presented. Figures show representative experiments; all experiments in this paper were repeated at least three, and usually more than six, times with similar results.

Quantitation of Scanning Electron Micrographs. After the transmigration assay carried out as described above, cultures were fixed in 2.5% electron microscopy (EM)-grade glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 (Polysciences, Warrington, PA). The fixed HEC monolayer, associated leukocytes, and collagen gel were physically removed from the 96-well tray and processed for scanning EM by critical point drying and gold coating as described (31). For quantitative evaluation, each specimen was centered in the beam at low-power (~20×), then Mo were counted in at least 10 random, nonoverlapping 1,000× fields (each field was ~10,323 μm^2) at or near the center. Apical Mo were observed as large, mostly spread cells with at least one pseudopod. Cells resembling lymphocytes were seen only rarely on the apical surface in the 1- and 3-h specimens. At 5 h apically adherent lymphocytes were observed at the same density (approximately two per field) in control and anti-PECAM-treated specimens, and were not counted. Data are expressed as mean ± standard deviation of apical Mo per 1,000× field.

Chemotaxis Assay. Hydrated collagen gels identical to those used for culture were impregnated with chemoattractant by incubation with 200 μl of chemoattractant solution overnight at 37°C in the tissue culture incubator. Control experiments using fluorescently labeled IgG demonstrated that equilibration occurred within 6 h ($t_{1/2}$ = 45 min) for both accumulation within and elution from the gel. Such gels were washed three times with M199. Leukocytes resuspended to 10^6/ml in M199 plus 0.1% human serum albumin with or without hec7 IgG or Fab were added to the wells (100 μl/well), and migration into the gels was allowed to proceed for 1 h. Cells that had not entered the gel were removed by inverted centrifugation in 20 mM EDTA. The collapsed gels were stained with Wright-Giemsa and leukocytes trapped within it were counted in the central 200× field of each well. Data are presented as the mean number of leukocytes ± standard deviation of six replicate wells.

Results

Anti-PECAM Antibodies Block Transendothelial Migration at the Intercellular Junction. The monocyte-selective in vitro transmigration assay we have recently reported (24) was used to compare transmigration of PBMC treated with anti-PECAM-1 mAb hec7 (18) or with isotype-matched control mAb. As previously found (24), all of the Mo in the control samples had transmigrated the HEC monolayer within 40–60 min, while lymphocytes remained unbound or loosely attached and were easily removed by the washes in 1 mM EGTA. The Mo treated with anti-PECAM mAb also resisted multiple washes in EGTA and remained with the HEC monolayer; our quantitative assay detected no differences in the number of ^{111}I-labeled Mo associated with the monolayer (not shown). However, the position of the Mo with respect to the en-
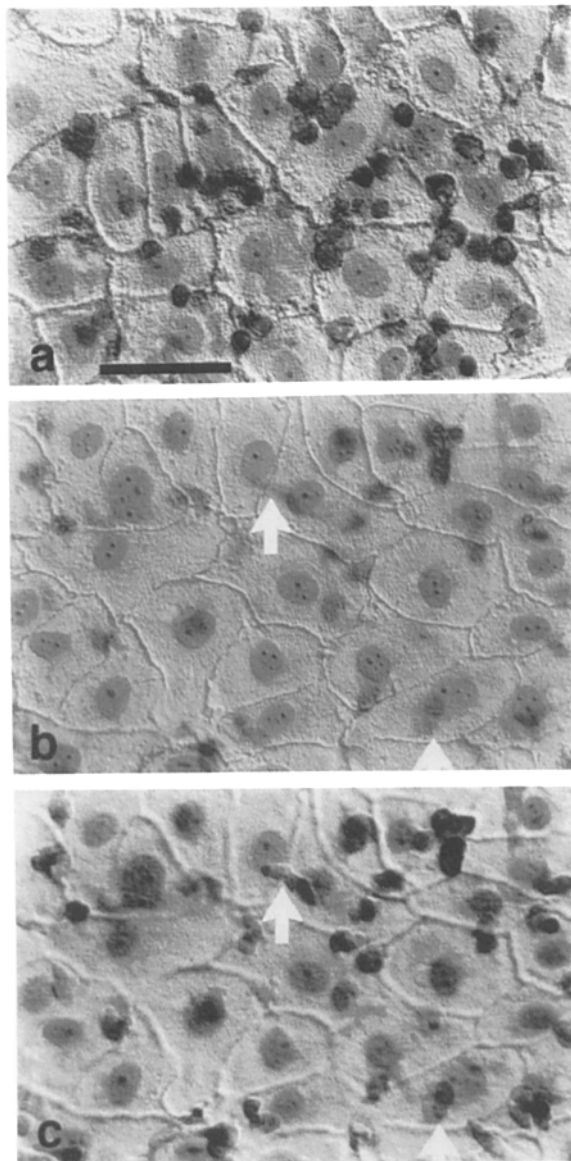
Figure 1. Anti-PECAM antibody blocks transendothelial passage of Mo. PBMC were incubated with hec7 anti-PECAM mAb (a) or isotype-matched control mAb (b and c), washed free of unbound antibody, and added to confluent HEC monolayers at 37°C. After 1 h, monolayers were washed three times in 1 mM EGTA in HBSS to remove loosely adherent leukocytes, stained with silver nitrate and Wright-Giemsa, then examined by Normarski interference optics. (a) In the hec7-treated sample, most Mo remain tightly attached to the HEC surface in association with the intercellular junctions, which stain black with silver nitrate. These Mo are all in a focal plane at the apical surface of the HEC monolayer. In contrast, control Mo have all transmigrated. No Mo are in focus in the plane of the apical surface of the monolayer (b), but they can all be visualized in various focal planes in the collagen gel below the monolayer (c). White arrows point to two such Mo in the corresponding photomicrographs (b and c). Bar = 200 μm.

dothelium was strikingly different. In contrast to the control Mo, which had clearly transmigrated (Fig. 1, b and c, and reference 24), most of the Mo treated with anti-PECAM antibodies remained bound to the apical surface of the HEC

monolayers, where they accumulated selectively at the intercellular junctions (Fig. 1 a).

Scanning EM of HEC monolayers exposed to control and hec7-treated PBMC for up to 5 h revealed dramatic differences. While only rare control Mo were observed on the HEC surface, (Fig. 2 a), numerous hec7-treated Mo remained attached to the apical surface of the monolayer (Fig. 2 b). Higher power views revealed that these Mo were well attached and mostly spread on the endothelial surface, where they were associated with intercellular junctions between apposing HEC. Most appeared to have at least a single pseudopod in the junction (Fig. 2 c).

Quantitation of the Inhibition of Transmigration. After 1 h at 37°C virtually all of the control Mo were clearly below the HEC, whereas <20% of anti-PECAM-treated Mo had transmigrated (Fig. 3 a). In >50 experiments using the quantitation methods described in Materials and Methods, hec7 inhibited Mo transmigration by 70–90%. The same results were obtained when the transmigration assay was carried out in the continuous presence of antibodies (Fig. 3). Since our previous results (18, 32) demonstrated that hec7 mAb does not disrupt junctions when added to HEC monolayers that have already achieved confluence, we presume that in this experiment, the effect of the mAb continuously present is solely on the Mo (Also see below).

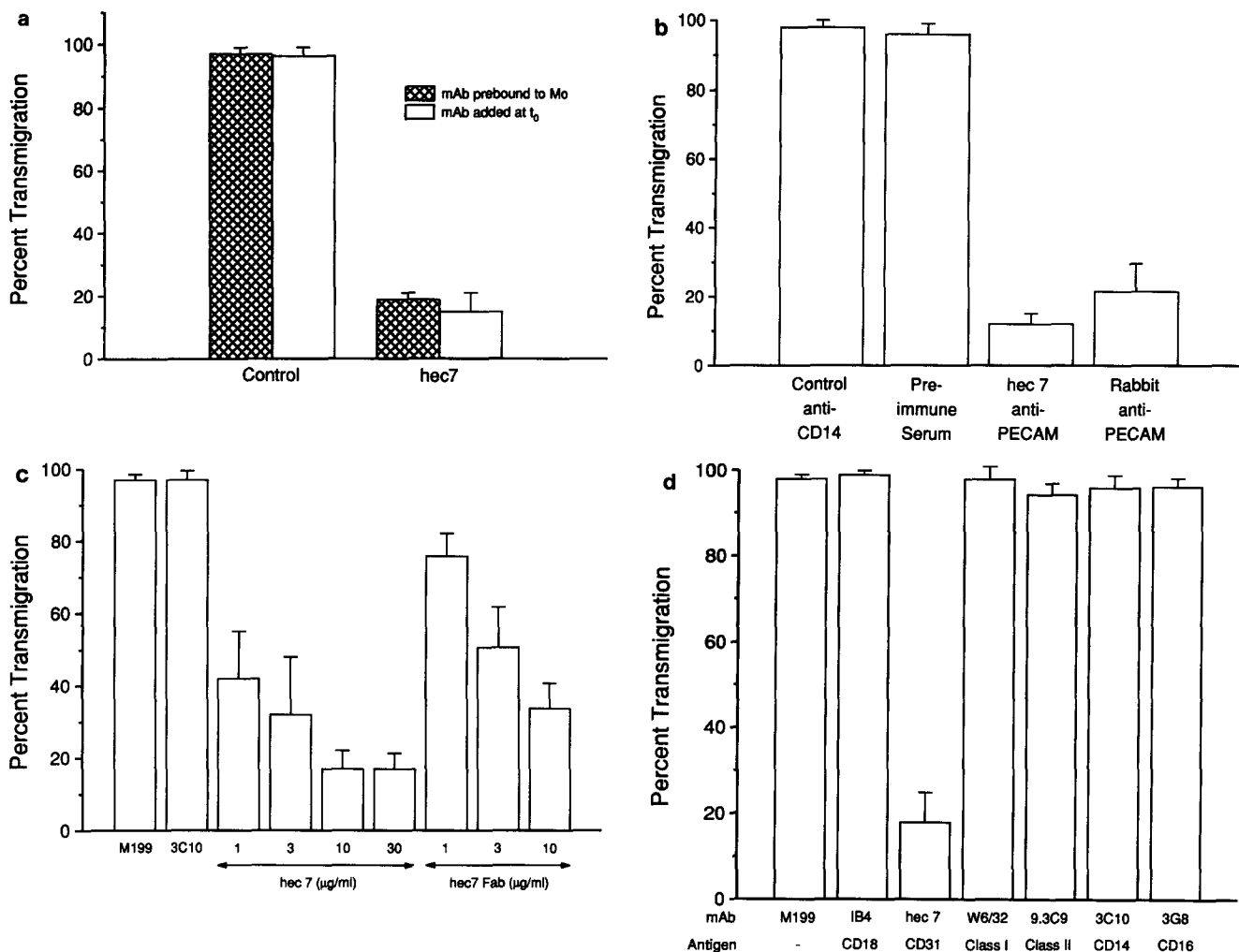Anti-PECAM mAb hec7 blocked transmigration significantly at doses as low as 1 μg/ml, with maximum effect seen at ~10 μg/ml. mAb hec7 at 10–20 μg/ml was as effective as a 1:100 dilution of rabbit anti-PECAM serum at blocking transmigration (Fig. 3 b). The effect of hec7 was not mediated via an effect on the Mo Fc receptor. Fab fragments of hec7 also demonstrated significant inhibitory activity in the 1–10 μg/ml range (Fig. 3 c). The blockade of transendothelial migration was specific for PECAM-1. Several other mAbs against Mo surface antigens used at the same concentration failed to affect transmigration (Fig. 3 d). mAb IB4 against CD18, the leukocyte β_2 integrin chain, blocked adhesion of Mo to the HEC by ~70%, as previously found (24); however, those Mo that remained associated with the monolayer in the presence of IB4 transmigrated apparently normally.

Anti-PECAM mAb Does Not Affect Chemotaxis. Inhibition of transmigration in this system was not due to the effect of the mAb on Mo chemotaxis. Mo added to gels impregnated with formylated peptides or with the chemoattractive soluble material extractable from the subendothelial collagen gels ("infranate'" [24]) migrate into the gels in a dose-dependent manner, similar to that seen in a more conventional Boyden chamber assay (24, 33). Incubation of the PBMC with mAb hec7 had no effect on this chemotaxis, nor did it have any effect on the low level of random migration into the gel in the absence of chemoattractant (Fig. 4). Similarly, migration of PMN to a variety of chemoattractants was unaffected by these concentrations of hec7 mAb (Fig. 4).

The Effect of Anti-PECAM mAb on Transmigration Is Long Lasting but Reversible. Inhibition of transmigration by hec7 was maintained in culture for at least 6 h in the continued
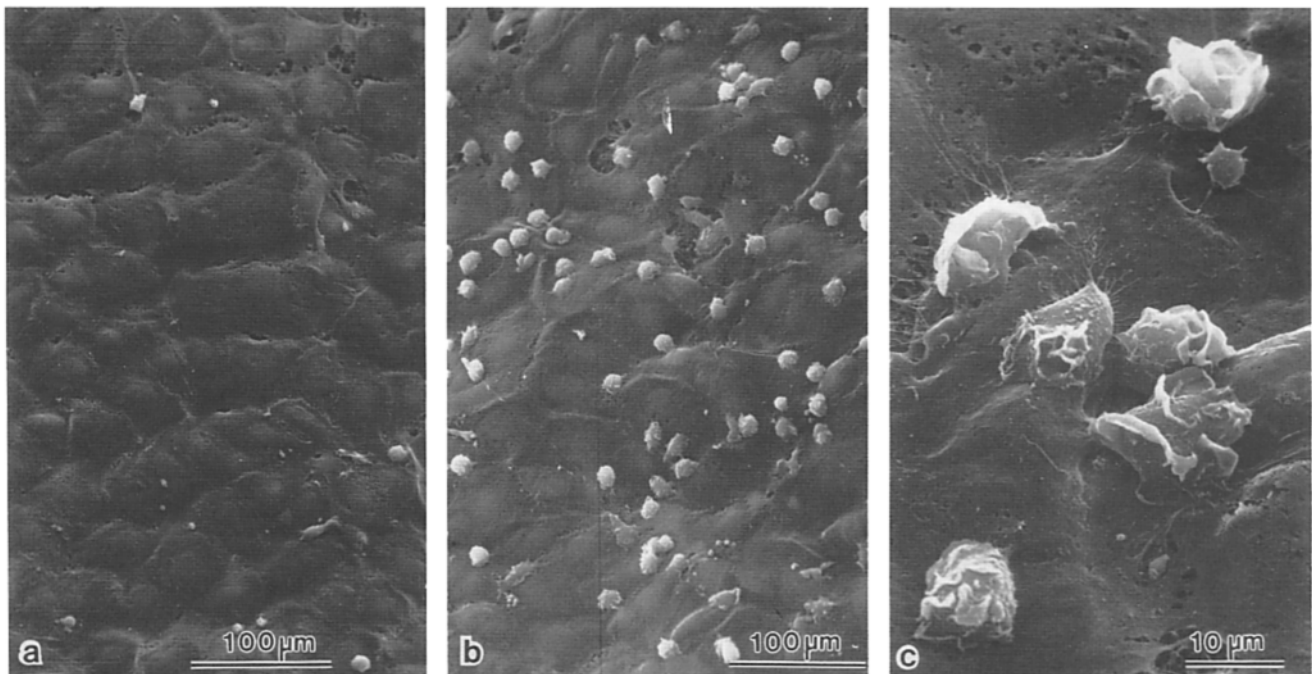
Figure 2. hec7 anti-PECAM mAb blocks migration of monocytes through the intercellular junction. PBMC were incubated for 1 h in complete medium containing hec7 or control mAb at 20 μg/ml, then washed in EGTA, fixed, and prepared for scanning EM. Control monolayers (a) were virtually devoid of apical Mo, whereas monolayers exposed to hec7-treated PBMC were carpeted with Mo (b), most of which appeared to be bound over the intercellular junctions. At higher magnification in tilted specimens (c), some of the hec7-treated Mo could be seen to be extending pseudopods into the junction, although the major portion of the cell remained on the apical surface.

Figure 3. Anti-PECAM mAb hec7 significantly blocks transendothelial migration of monocytes. (*a*) hec7 blocks whether prebound to Mo or added at the start of coculture. PBMC were incubated with hec7 (anti-PECAM) or control (anti-CD14) mAb at 20 μg/ml for 30 min on ice (*crosshatched bars*), washed free of unbound antibody, and added to HEC monolayers. Alternatively, equal numbers of PBMC were added to HEC in the presence of the same concentrations of mAb (*open bars*). (*b*) hec7 is as effective as polyclonal anti-PECAM at blocking transmigration. PBMC were added to HEC monolayers in the presence of 20 μg/ml hec7 or anti-CD14, or a 1:100 dilution of rabbit anti-PECAM serum or preimmune serum. (*c*) hec7 Fab as well as IgG significantly blocks transmigration at low concentrations. Equal numbers of PBMC were added to HEC monolayers in M199 or M199 supplemented with either anti-CD14 mAb 3C10 at 30 μg/ml, or hec7 IgG or Fab at the indicated concentrations. (*d*) Blockade of transmigration is specific for anti-PECAM mAb. Equal numbers of PBMC were added to HEC monolayers for 1 h in M199 containing the indicated mAb at 20 μg/ml. Only hec7 significantly decreased transmigration of Mo. In all of the above experiments, incubation proceeded at 37°C for 1 h, at which time cells were processed as in Materials and Methods, and the percentage of the cells remaining associated with the monolayer that had transmigrated was assessed. The data displayed are the mean ± standard deviation of six replicates for each experimental sample.

presence of the mAb (Fig. 5). Scanning EM of such an experiment revealed that control HEC monolayers bore an average of 0.4 ± 0.4 apical Mo per 1,000× field at all time points, while the monolayers of the hec7-treated specimen bore 15.8 ± 0.5, 16.0 ± 6.3, and 16.0 ± 6.4 apical Mo per 1,000× field at 1, 3, and 5 h, respectively. Upon removal of hec7 the blockade of transmigration was eliminated, and some Mo transmigration was detected after 30 min. (This contrasts with Fig. 3 *a*, where the effect of prebound hec7 lasted for at least 1 h. The most likely explanation is that in Fig. 5 the Mo were already tightly adherent at the HEC

junctions and trying to transmigrate when the block was removed. They may have been able to overcome a higher concentration of residual hec7 than the resting Mo in Fig. 3 *a* and other similar experiments.) By 2 h after return to control medium all Mo had transmigrated (Fig. 5). Thus, anti-PECAM mAb is not merely slowing down the transmigration process, and it is not permanently affecting the Mo's ability to recognize and respond to important signals required for transmigration.

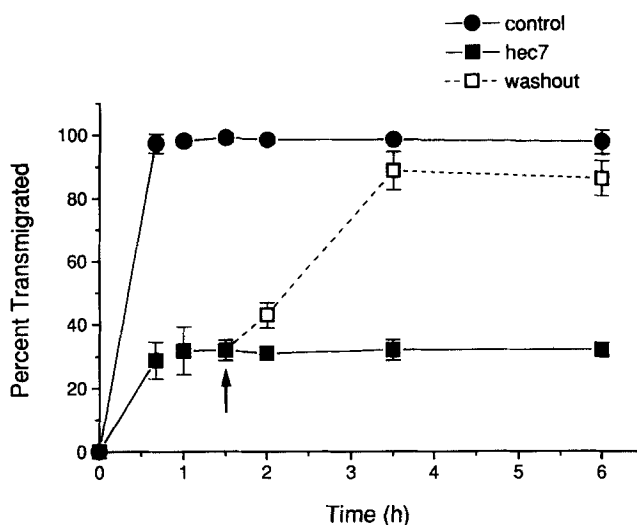*mAb Bound to PECAM in the HEC Junctions Blocks Mo Transmigration.* Greater than 85% of the PECAM on cul-
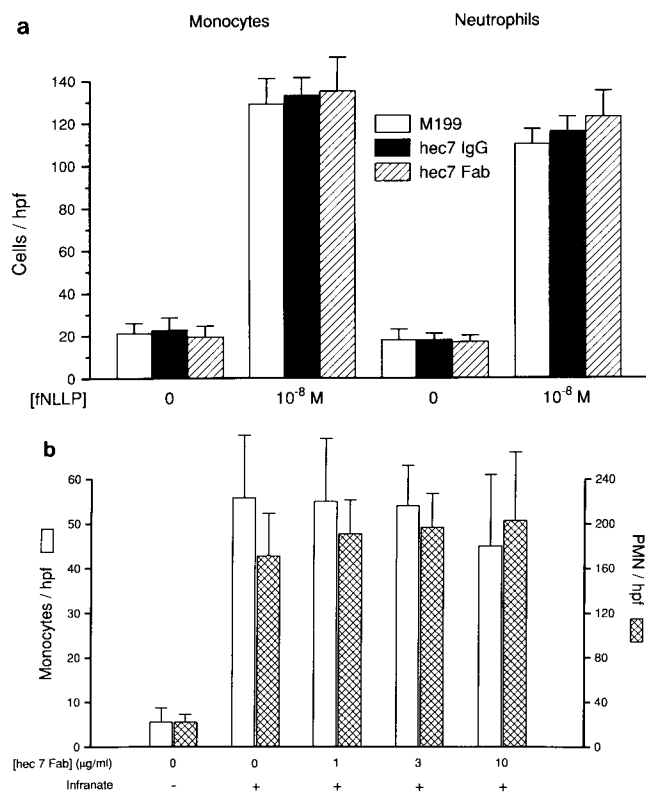
Figure 4. hec7 does not decrease chemotaxis of Mo or PMN. PBMC or PMN were isolated and resuspended in M199 + 0.1% HSA with or without hec7 IgG or Fab at 10 μg/ml (a) or hec7 Fab at the indicated concentrations (b). Leukocytes were then added to collagen gels impregnated with (a) M199 or formylnorleucyl-leucyl-phenylalanine (fNLLP), or (b) normal culture medium or infranate from confluent HEC cultures. After incubation for 1 h at 37°C, chemotaxis was quantitated as described in Materials and Methods.

Figure 5. Inhibition of transendothelial migration by hec7 anti-PECAM mAb is long lasting and reversible. PBMC were added to HEC monolayers in the presence of hec7 (*filled squares*) or control mAb W6/32 (*filled circles*) at 20 μg/ml in complete culture medium. At 1.5 h (*arrow*), one set of hec7-treated cultures was washed three times in M199 and returned to culture in control culture medium (*open squares*). Transmigration was assessed in the usual manner in six replicate cultures for each sample.

tured HEC is concentrated in the intercellular junctions (18). In this location it is poorly accessible to hec7 mAb applied to the apical surface of live, confluent monolayers. Indeed, hec7 and several other mAbs recognizing proteins on the HEC surface (including ICAM-1) failed to block transmigration when HEC were preincubated with these mAbs for 1 h at 37°C, then washed before addition of the PBMC (data not shown).

To study the role of PECAM in the HEC junctions, we devised a method to deliver hec7 to that site (see legend to Fig. 6). The addition of hec7 Fab fragments to subconfluent HEC increased the time required to achieve confluence relative to replicate control cultures, as expected (32, 34). However, once the monolayer became confluent, it was indistinguishable from control monolayers by phase microscopy (not shown). Indirect immunofluorescence microscopy performed on live monolayers localized hec7 Fab fragments to the junctions between adjacent HEC (Fig. 6 a).

When untreated PBMC or PBMC in the presence of control mAb were added to such monolayers (Fig. 7, *hec 7 Fab*), transmigration was blocked. In the experiment shown in Fig. 7, as wells as in several similar experiments, the degree of
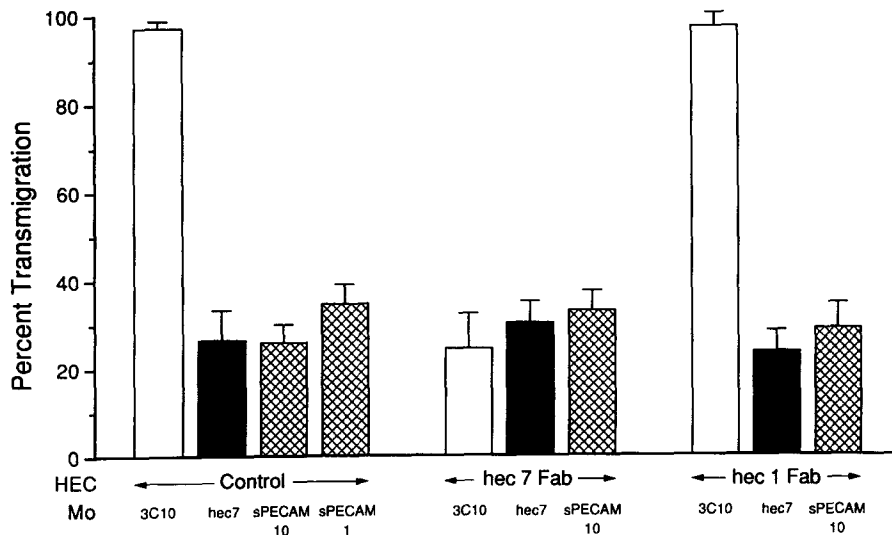
inhibition of transmigration was equivalent to what could be achieved by hec7 IgG added to the same donor's PBMCs on control monolayers (Fig. 7, *Control*). The effect of hec7 Fab was not merely a steric affect due to clogging the intercellular junction with antibody fragments. As a control for this, HEC monolayers were cultured in the presence of Fab fragments of mAb hec1, which recognizes a novel integral membrane protein of 130 kD that, like PECAM-1, is concentrated in the junctions of HEC, but unlike PECAM, is not expressed on leukocytes or platelets. Furthermore, hec1 is the same isotype as hec7, and Fab fragments of hec1 also accumulated in HEC intercellular junctions (Fig. 6 b). However, hec1 Fab pretreatment had no effect on transmigration of untreated Mo (Fig. 7, *hec 1 Fab*). Of note, blocking PECAM on Mo and HEC was not additive. No greater inhibition of transmigration was achieved when hec7 IgG was added to Mo exposed to hec7 Fab pretreated HEC (Fig. 7, *hec 7 Fab, filled bar*) than was achieved by adding this anti-PECAM reagent to either the Mo (Fig. 7, *Control* and *hec 1 Fab, filled bars*) or the HEC (Fig. 7, *hec 7 Fab, open bar*) alone.

The block of transmigration by hec7 Fab is not due to Fab leaking out of the HEC monolayer or subendothelial collagen and binding to Mo PECAM. In control experiments Fab-pretreated HEC cultures were processed in an identical manner to those used in the experiments of Fig. 7. Supernatant was collected from these cultures after 1 h at 37°C and used to resuspend freshly isolated PBMC, which were then subjected to the transmigration assay. Neither supernate from control or hec7 Fab-treated cultures inhibited transmigration of Mo (96.8 ± 2.6% and 97.6 ± 2.3% transmigration, respectively).

Recombinant Soluble PECAM Blocks Transmigration. A
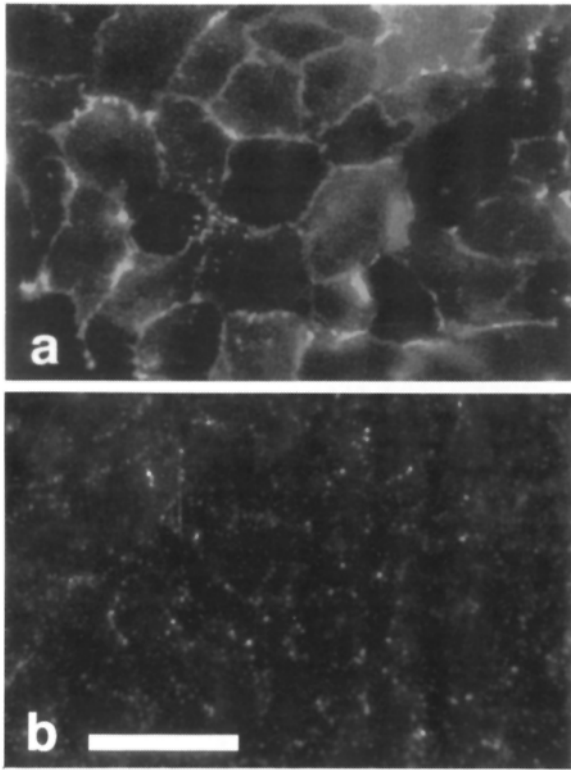
Figure 6. hec7 and hec1 Fab fragments bind to their antigens in the intercellular junctions. Normal culture medium supplemented with Fab fragments of hec7 (anti-PECAM) or control mAb hec1 at 20 μg/ml was added to subconfluent HEC and replaced with successive feedings as the cells came to confluence over 1 wk. The monolayers were washed extensively, then incubated successively in FITC-labeled rabbit anti–mouse IgG at 1:100 in culture medium (for hec7) or rabbit anti–mouse IgG at 1:400 followed by rhodamine-conjugated swine anti–rabbit IgG at 1:100 dilution (for hec1) before washing and formalin fixation. Phase contrast microscopy revealed typical intact, cobblestone monolayers (not shown) in both specimens. (*a*) Fluorescence image of HEC allowed to come to confluence in the presence of hec7 Fab demonstrates mAb fragments concentrated in the intercellular junction. (*b*) Fluorescence image of HEC that came to confluence in the presence of hec1 Fab shows hec1 antigen concentrated in the intercellular junction, but in a more patchy distribution than PECAM. Bar = 200 μm.

soluble recombinant form of PECAM-1 truncated in the middle of the sixth Ig domain significantly inhibits the transmigration of Mo at concentrations as low as 1 μg/ml when added to PBMC in our standard assay (Fig. 7, *Control* and *hec 1, crosshatched bars*). Concentrations of 10 μg/ml consistently inhibited as well as optimal concentrations of hec7 IgG in some experiments (not shown), blocking transmigration to 10% of control values. The presence of soluble PECAM in the incubation medium did not augment the inhibition of transmigration effected by hec7 Fab in the HEC junctions (Fig. 7, *hec 7 Fab, crosshatched bar*).

Blockade of transmigration by these concentrations of soluble PECAM predicts a K_d in the range of 10–100 nM. This unusually low effective concentration could be explained by the fact that the soluble PECAM in these experiments was aggregated. Analysis by nondenaturing gels revealed a ladder of species in multiples of ~90 kD, with most of the material running as apparent hexamers and octamers (not shown). While aggregation would arithmetically decrease the apparent molar concentration of this reagent, it would exponentially increase the apparent affinity if the monomers in the aggregates were still active.

Anti-PECAM mAb Blocks Transmigration of Mo across Cytokine-activated HEC Monolayers. The HEC used in the preceding studies did not express cytokine-inducible adhesion molecules like VCAM-1 or E-selectin, and had basal levels of intercellular adhesion molecule 1 (ICAM-1) (24). When such monolayers are induced by TNFα or IL-1 to express such adhesion molecules, transmigration of Mo is even more rapid than under basal conditions, with 100% of Mo entering the subendothelial collagen within 20–30 min (W. A. Muller, unpublished data). However, hec7 blocked transmigration across cytokine-activated HEC monolayers as well as it did on resting monolayers (Fig. 8).

Anti-PECAM Reagents Block Transmigration of Neutrophils. Neutrophils also bear surface PECAM (19–21), although hec7 does not bind to the form displayed by PMN as well as it binds HEC and Mo (W. A. Muller, unpublished observations). Preferential binding of individual anti-PECAM
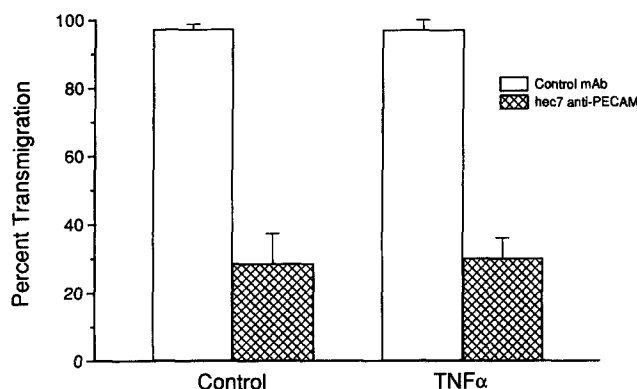

Figure 7. Transmigration can be blocked by anti-PECAM treatment of either Mo or the HEC junctions. HEC monolayers were cultured under standard conditions (*Control*) or in the presence of hec7 or hec1 Fab as described in the legend to Fig. 6. PBMC were resuspended in M199 containing control mAb 3C10 (*open bars*) or hec7 (*filled bars*), both at 20 μg/ml, or recombinant soluble PECAM (*sPECAM, crosshatched bars*) at 1 or 10 μg/ml, and transmigration proceeded for 1 h. hec7 blocked transmigration equivalently whether it was added to the Mo or the HEC, and there was no additional blockade when the two arms were combined (*hec 7 Fab/hec7*). Soluble recombinant PECAM blocked as well as hec7, and again, was not additive with hec7 Fab bound to the HEC (*hec 7 Fab/sPECAM*).

Figure 8. Anti-PECAM mAb hec7 blocks transmigration of monocytes across cytokine-activated HEC. HEC were activated by culturing in the presence of 10 ng/ml TNFα for 3 h to induce VCAM-1 and up-regulate ICAM-1. PBMC were added in the presence of hec7 or 3G8 mAb at 20 μg/ml, and transmigration was allowed to proceed for 1 h.

mAbs to certain types of cells has been previously documented (35). PMN do not migrate readily across resting HEC monolayers in this system (24), however, they do transmigrate TNFα-activated monolayers readily (W. A. Muller, unpublished observations). After 1 h in the presence of control antibody, 97% of the PMNs remaining with the TNFα-activated monolayer had migrated beneath it. hec7 blocked transmigration by ∼40%, consistent with its relatively weak binding to the neutrophil form of this molecule. However, a polyclonal rabbit antiserum against PECAM and soluble recombinant PECAM at 10 μg/ml blocked transmigration down to <20% of control levels (Fig. 9). hec7 had no effect on neutrophil movement in chemotaxis assays (Fig. 4). Thus, PECAM apparently plays a similar role in the transmigration of PMN as it does for Mo.
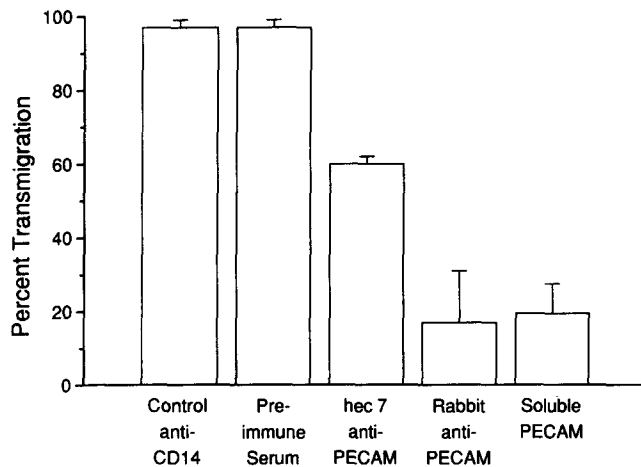
Figure 9. Anti-PECAM reagents block transmigration of neutrophils. HEC monolayers were cultured in the presence of 10 ng/ml TNF-α for 2 h to induce the expression of L-selectin as well as VCAM-1 and to enhance ICAM-1 expression. PMN were resuspended in warm M199 supplemented with 3C10 or hec7 at 20 μg/ml, preimmune serum or rabbit anti-PECAM serum at 1:100, or recombinant soluble PECAM at 10 μg/ml, and added to the activated HEC monolayers for 1 h.

Discussion

Anti-PECAM Reagents Block Transendothelial Migration. Our data demonstrate that PECAM-1 plays a crucial role in transendothelial migration of both Mo and PMN across activated and unactivated HEC monolayers. Interfering with PECAM function on either the leukocyte or the endothelial cell selectively blocks transmigration at an EGTA-resistant step after tight adhesion. By our original quantitative transmigration assay, which uses an inverted centrifugation in EGTA to strip adherent leukocytes off the apical surface of the monolayer (24), there was no decrease in the total numbers of leukocytes remaining associated with the monolayers after treatment with any anti-PECAM reagent. Thus, it would have appeared that anti-PECAM reagents had no effect on transmigration. It was only when the position of these leukocytes with respect to the endothelial monolayer was directly visualized by light and scanning EM that the block of transmigration was so graphically apparent.

Under control conditions, virtually 100% of the leukocytes transmigrate our HEC monolayers within 1 h. In contrast, only 10–30% of the leukocytes transmigrate in the presence of anti-PECAM reagents, and this blockade is maintained for at least 6 h in the presence of anti-PECAM reagents. Since all Mo and PMN express relatively high levels of PECAM (19), incomplete blockade is not due to absence of PECAM from these cells. Neither is it likely to be due to failure of our mAb, hec7, to interact with a different PECAM epitope. The same statistical results were obtained using a polyclonal anti-PECAM antiserum and a form of recombinant soluble PECAM comprising almost the entire extracellular domain of the molecule. It is possible that a subset of Mo uses a PECAM-independent pathway, or that incomplete block of transmigration is merely due to the imperfect nature of our in vitro assay.

The inhibitory effect of hec7 is not due to toxic effects on the leukocytes. The mAb does not inhibit chemotaxis of Mo or PMN to formylated tripeptides or to "infranate," which represents a relevant chemoattractant for transmigration in our standard assay (24). Thus, hec7 treatment does not interfere with either movement of leukocytes per se or with activation and function of leukocyte CD11/CD18, which is required for chemotaxis in these assays and is required for binding to HEC in this system (24). Moreover, the effect is reversible, with transmigration of Mo proceeding normally once anti-PECAM mAb is withdrawn.

The epitope of PECAM recognized by mAb hec7 is located in the first and/or second Ig domain. hec7 blocks transmigration, but not heterophilic aggregation, of PECAM transfectants, which appears to involve domain six (unpublished results). In preliminary experiments an anti-PECAM mAb against domain six that blocks heterophilic aggregation of transfectants did not block transmigration (not shown). Thus, there is specificity in the blockade of transmigration by hec7. Our results to date with the five and one-half domain soluble PECAM seem to confirm the results with the mAb. We are now repeating these initial experiments using more completely characterized forms of recombinant soluble PECAM-1.

There is some concern that initial interactions studied in vitro in static assay systems may not be relevant to inflammation in vivo where leukocyte adhesion to the endothelial surface must resist the shear forces of the flowing bloodstream. Indeed, selectins and integrins function differently under these conditions (36, 37). However, we are studying a step in emigration distal to the tight integrin-mediated adhesion, and once leukocytes are tightly bound via the β_2 integrins, leukocyte–endothelial adhesion appears to be refractory to shear stress (16). Therefore, our static transmigration system may be a relevant model for studying this phase of emigration in vitro.

The Stages of Leukocyte Emigration. The process of emigration can be divided into three stages (reviewed in references 4 and 8). First, leukocytes leave the circulatory stream, adhering loosely to the vessel wall as they roll along the endothelial surface. This adhesion is believed to be mediated by the selectin class of adhesion molecules and their specific carbohydrate ligands. Antibodies against leukocyte L-selectin can block this rolling in vivo (15) and purified forms of E- and P-selectin can induce this rolling behavior in in vitro models (14, 38). In the second stage the leukocyte-specific (β_2) integrins are activated on the rolling cells, resulting in cessation of rolling and tight adhesion to the endothelium (14, 16). The activation may be triggered by the selectin-mediated binding in an adhesion cascade (39). In addition, integrins can be activated by a variety of relevant chemoattractants and stimulants (IL-8 [40]; PAF, C5a, endotoxin, etc. [10, 41]) that they might be exposed to on the surface of activated endothelium. Antibodies against CD18, the β chain of this integrin class, block this tight adhesion (8–10). The third step is transendothelial migration: the extravasation of the leukocytes between tightly apposed endothelial cells. In many in vivo (15, 42) and in vitro systems (24, 43, 44) antibodies against leukocyte integrins and/or their endothelial counter-receptors appear to block transmigration, but may, in fact, block the earlier step of tight adhesion to endothelium. Our data certainly do not rule out a role for CD11/CD18 in the actual transmigration process itself (see below); however, they do demonstrate the requirement for PECAM-1.

The ability to arrest transmigration of tightly bound leukocytes defines the PECAM-1 mediated adhesion as a distinct event from the preceding phases of rolling and tight adhesion. The process of leukocyte emigration can, for the present time, be dissected into three distinct phases (rolling, sticking, transmigration) mediated by the actions of three distinct classes of adhesion molecules (selectins, integrins, PECAM [Ig superfamily]). This distinction is possible only because there are reagents that can block the adhesion molecules responsible for each step. There may well be other adhesion molecules awaiting discovery that mediate adhesion events in transendothelial migration intermediate or distal to these three.

The Role(s) of PECAM in Transendothelial Migration. Previous studies on HEC and cell lines transfected with human PECAM have demonstrated that PECAM-1 can mediate both homophilic (25) and heterophilic (26) adhesion. Since anti-PECAM mAb hec7 can block transmigration when bound to either the leukocyte or the endothelial cell and these effects are not additive, the most straightforward explanation is that in this biological system leukocyte PECAM is engaging in a homophilic adhesion with PECAM on the HEC junction. Moreover, soluble recombinant PECAM blocks transmigration as well as anti-PECAM antibody when added to the leukocytes, as expected if leukocyte PECAM were binding homophilically to endothelial cell PECAM. However, other mechanisms such as bridging of the two cell types via PECAM binding to a soluble molecule, a heterophilic adhesion that depended upon functional PECAM on both interacting cells, or an indirect signaling role for PECAM that is blocked by our mAb are formally possible.

What is the critical function of PECAM in transmigration? The most obvious is that PECAM serves as a direct adhesion molecule binding the leukocyte tightly to the HEC during its passage through the junction (Fig. 10 *a*). Most of the endothelial PECAM is concentrated in the intercellular junctions, but ~15% is exposed to the apical surface (18). This suggests that an apical-basal gradient of PECAM could exist through the HEC junction. HEC are clearly capable of forming and actively maintaining apical-basal polarity of integral membrane proteins (28). An apical-basal gradient of adhesion molecule could act similarly to a surface-bound chemotactic gradient (haptotactic gradient) to produce directed migration of leukocytes through the junction (Fig. 10 *b*). PECAM also could act as an indirect adhesion molecule. Ligation of PECAM on a subset of T cells that express it has been shown to activate β_1 and β_2 integrins on those cells (45). Similarly, ligation of PECAM on the Mo and PMN
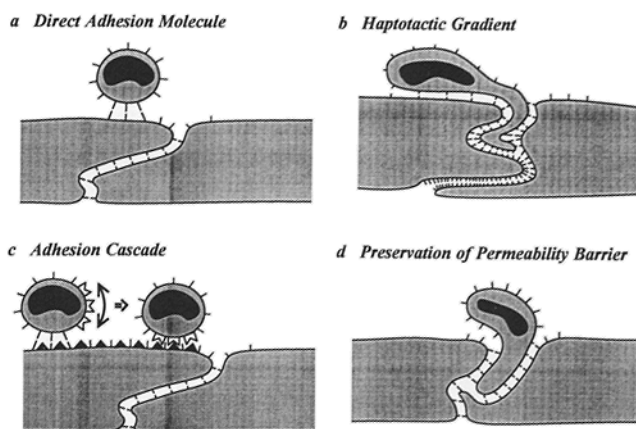
Figure 10. The possible roles of PECAM-1 in transendothelial migration of leukocytes. These schematic diagrams depict a Mo or PMN at the intercellular junction of human endothelial cells. Short black lines represent molecules of PECAM-1 on the leukocyte and endothelial cell that, when aligned, represent homophilic adhesion via PECAM. In *b*, the density of PECAM expressed in the junction increases from the luminal to the abluminal surface. In *c*, the filled triangles represent a ligand for a leukocyte adhesion molecule (▽), such as CD11/CD18, that is activated by PECAM-PECAM interactions. See text for details.

with anti-PECAM F(ab')$_2$ activates CD11/CD18 binding activity (M. E. Berman and W. A. Muller, manuscript in preparation). In the confines of the intercellular junction, where almost 10⁶ molecules of PECAM are concentrated on each HEC (46), homophilic interaction with leukocyte PECAM could serve to activate the β_2 integrin in such an "adhesion cascade" (Fig. 10 c). This mechanism could apply as well if there were an apical-basal gradient of PECAM. Finally, adhesion of leukocyte PECAM to endothelial PECAM during transmigration may help maintain the tight apposition of these cells during this process and help control the efflux of cells and soluble molecules during the inflammatory response (Fig. 10 d). The hypothesized role(s) of PECAM in transmigration are not mutually exclusive and are currently under investigation.

Pathophysiologic Significance. Mo transmigrate endothelium in vivo both constitutively and, in greater numbers, at sites of inflammation. In contrast, emigration of PMN is essentially restricted to sites of acute inflammation. These cells recapitulate this behavior in our in vitro assay (24); Mo transmigrate across both resting and cytokine-activated HEC, whereas PMN transmigrate only cytokine-activated HEC efficiently. This assay thus provides a good in vitro model of Mo and neutrophil emigration. Our data suggest that PECAM may play an important role that is common to the constitutive emigration of Mo and the elicited emigration of both Mo and PMN.

With respect to lymphocytes, a subpopulation of circulating naive CD8⁺ T cells bears PECAM (45), but we do not detect PECAM on more than a few percent of freshly isolated blood lymphocytes (W. A. Muller, unpublished observations). Furthermore, we do not detect transmigration of freshly isolated lymphocytes across our HEC monolayers (24). However, lymphocytes clearly enter sites of chronic inflammation in vivo. Since induction of PECAM has been reported on activated T cells (19), it is possible that when responding to an antigen challenge they do emigrate in a PECAM-dependent manner. This hypothesis is under investigation in our laboratory. PECAM-1 (CD31) has been identified on a variety of myeloid and lymphoid tumor cell lines (19, 20). It is possible that PECAM on these cells aids in their emigration from the bloodstream in the metastatic process.

Most of the well-known leukocyte–endothelial cell adhesion molecules have other roles in the immune response. For example, ICAM-1 and LFA-1 are important in T cell activation (8); CD3 (CD11b/CD18) is a receptor for a variety of ligands, including complement degradation products (47, 48), endotoxin (49), and bacterial wall components (50); and L-selectin is crucial for normal lymphocyte trafficking as well as the rolling behavior of PMN (4, 5). At present PECAM has no known function in the immune or inflammatory responses other than its role in transendothelial migration. If this proves to be the case, detrimental inflammatory reactions could be blocked with anti-PECAM reagents without adverse side effects on other aspects of the immune response.

References

1. Marchesi, V.T. 1961. The site of leukocyte emigration during inflammation. *J. Exp. Physiol.* 46:115.
2. Marchesi, V.T., and H.W. Florey. 1960. Electron micrographic observations on the emigration of leukocytes. *J. Exp. Physiol.* 45:343.
3. van Furth, R. 1986. Current view on the mononuclear phago-

cyte system. *Immunobiology.* 161:178.
4. Lasky, L.A. 1992. Selectins: Interpreters of cell-specific carbohydrate information during inflammation. *Science (Wash. DC).* 258:964.
5. Stoolman, L.M. 1989. Adhesion molecules controlling lymphocyte migration. *Cell.* 56:907.

6. Gallatin, W.M., I.L. Weissman, and E.C. Butcher. 1983. A cell-surface molecule involved in organ-specific homing of lymphocytes. Nature (Lond.). 304:30.

7. Gallatin, M., T.P. St. John, M. Siegelman, R. Reichert, E.C. Butcher, and I.L. Weissman. 1986. Lymphocyte homing receptors. Cell. 44:673.

8. Springer, T.A. 1990. Adhesion receptors of the immune system. Nature (Lond.). 346:425.

9. Osborn, L. 1990. Leukocyte adhesion to endothelium in inflammation. Cell. 62:3.

10. Pober, J.S., and R.S. Cotran. 1990. The role of endothelial cells in inflammation. Transplantation (Baltimore). 50:537.

11. Ross, R. 1986. The pathogenesis of atherosclerosis: an update. N. Engl. J. Med. 314:488.

12. Harlan, J.M. 1985. Leukocyte-endothelial interactions. Blood. 65:513.

13. Cohnheim, J. 1889. Lectures on General Pathology: A Handbook for Practitioners and Students. The New Sydenham Society, London, England.

14. Lawrence, M.B., and T.A. Springer. 1991. Leukocytes roll on a selectin at physiologic flow rates: Distinction from and prerequisite for adhesion through integrins. Cell. 65:859.

15. von Andrian, U.H., J.D. Chambers, L.M. McEvoy, R.F. Bargatze, K.-E. Arfors, and E.C. Butcher. 1991. Two-step model of leukocyte-endothelial cell interaction in inflammation: Distinct roles for LECAM-1 and the leukocyte beta 2 integrins in vivo. Proc. Natl. Acad. Sci. USA. 88:7538.

16. Smith, C.W., T.K. Kishimoto, O. Abbass, B. Hughes, R. Rothlein, L.V. McIntire, E. Butcher, and D.C. Anderson. 1991. Chemotactic factors regulate lectin adhesion molecule 1 (LECAM-1)-dependent neutrophil adhesion to cytokine-stimulated endothelial cells in vitro. J. Clin. Invest. 87:609.

17. Newman, P.J., M.C. Berndt, J. Gorski, G.C. White II, S. Lyman, C. Paddock, and W.A. Muller. 1990. PECAM-1 (CD31) cloning and relation to adhesion molecules of the immunoglobulin gene superfamily. Science (Wash. DC). 247:1219.

18. Muller, W.A., C.M. Ratti, S.L. McDonnell, and Z.A. Cohn. 1989. A human endothelial cell-restricted, externally disposed plasmalemmal protein enriched in intercellular junctions. J. Exp. Med. 170:399.

19. Ohto, H., H. Maeda, Y. Shibata, R.-F. Chen, Y. Ozaki, M. Higashihara, A. Takeuchi, and H. Tohyama. 1985. A novel leukocyte differentiation antigen: two monoclonal antibodies TM2 and TM3 define a 120-kd molecule present on neutrophils, monocytes, platelets, and activated lymphoblasts. Blood. 66:873.

20. Goyert, S.M., E.M. Ferrero, S.V. Seremetis, R.J. Winchester, J. Silver, and A.C. Mattison. 1986. Biochemistry and expression of myelomonocytic antigens. J. Immunol. 137:3909.

21. Stockinger, H., S.J. Gadd, R. Eher, O. Majdic, W. Schreiber, W. Kasinrerk, B. Strass, E. Schnabl, and W. Knapp. 1990. Molecular characterization and functional analysis of the leukocyte surface protein CD31. J. Immunol. 145:3889.

22. Bevilacqua, M.P., J.S. Pober, D.L. Mendrick, R.S. Cotran, and M.A. Gimbrone, Jr. 1987. Identification of an inducible endothelial-leukocyte adhesion molecule. Proc. Natl. Acad. Sci. USA. 84:9238.

23. Osborn, L., C. Hession, R. Tizard, C. Vassallo, S. Luhowskyj, G. Chi-Rosso, and R. Lobb. 1989. Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. Cell. 59:1203.

24. Muller, W.A., and S. Weigl. 1992. Monocyte-selective transendothelial migration: dissection of the binding and transmigration phases by an in vitro assay. J. Exp. Med. 176:819.

25. Albelda, S.M., W.A. Muller, C.A. Buck, and P.J. Newman. 1991. Molecular and cellular properties of PECAM-1 (endoCAM/CD31): A novel vascular cell adhesion molecule. J. Cell. Biol. 114:1059.

26. Muller, W.A., M.E. Berman, P.J. Newman, H.M. Delisser, and S.M. Albelda. 1992. A heterophilic adhesion mechanism for platelet/endothelial cell adhesion molecule 1 (CD31). J. Exp. Med. 175:1401.

27. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

28. Muller, W.A., and M.A. Gimbrone. 1986. Plasmalemmal proteins of cultured vascular endothelial cells exhibit apical-basal polarity: analysis by surface-selective iodination. J. Cell Biol. 103:2389.

29. Wright, S.D. 1986. Methods for the study of receptor-mediated phagocytosis. Methods Enzymol. 132:204.

30. Hoffman, R. 1977. The modulation contrast microscope: principles and performance. J. Microsc. (Oxf.). 110:205.

31. Phillips, D.M., and A.S. Bourinbaiar. 1992. Mechanism of HIV spread from lymphocytes to epithelia. Virology. 186:261.

32. Muller, W.A. 1992. PECAM-1: an adhesion molecule at the junctions of endothelial cells. In Mononuclear Phagocytes. Biology of Monocytes and Macrophages. R. van Furth, editor. Kluwer Academic Publishers, Norwell, MA. 138–148.

33. Deuel, T.F., R.M. Senior, J.S. Huang, and G.L. Griffin. 1982. Chemotaxis of monocytes and neutrophils to platelet-derived growth factor. J. Clin. Invest. 69:1046.

34. Albelda, S., P.D. Oliver, L.H. Romer, and C.A. Buck. 1990. EndoCAM: a novel endothelial cell-cell adhesion molecule. J. Cell Biol. 110:1227.

35. Ashman, L.K., G.W. Aylett, A.C. Cambareri, and S.R. Cole. 1991. Different epitopes of the CD31 antigen identified by monoclonal antibodies: Cell type-specific patterns of expression. Tissue Antigens. 38:199.

36. Spertini, O., F.W. Luscinskas, G.S. Kansas, J.M. Munro, J.D. Griffin, M.A. Gimbrone, Jr., and T.F. Tedder. 1991. Leukocyte adhesion molecule-1 (LAM-1, L-selectin) interacts with an inducible endothelial cell ligand to support leukocyte adhesion. J. Immunol. 147:2565.

37. Spertini, O., F.W. Luscinskas, M.A. Gimbrone, Jr., and T.F. Tedder. 1992. Monocyte attachment to activated human vascular endothelium in vitro is mediated by leukocyte adhesion molecule-1 (L-selectin) under nonstatic conditions. J. Exp. Med. 175:1789.

38. Ley, K., P. Gaehtgens, C. Fennie, M.S. Singer, L.A. Lasky, and S.D. Rosen. 1991. Lectin-like cell adhesion molecule 1 mediates leukocyte rolling in mesenteric venules in vivo. Blood. 77:2553.

39. Lo, S.K., S. Lee, R.A. Ramos, R. Lobb, M. Rosa, G. Chi-Rosso, and S.D. Wright. 1991. Endothelial-leukocyte adhesion molecule 1 stimulates the adhesive activity of leukocyte integrin CD3 (CD11b/CD18, Mac-1, $\alpha_m\beta_2$) on human neutrophils. J. Exp. Med. 173:1493.

40. Detmers, P.A., S.K. Lo, E. Olsen-Egbert, A. Walz, M. Baggiolini, and Z.A. Cohn. 1990. Neutrophil-activating protein 1/interleukin 8 stimulates the binding activity of the leukocyte adhesion receptor CD11b/CD18 on human neutrophils. J. Exp. Med. 171:1155.

41. Tonnesen, M.G., D.C. Anderson, T.A. Springer, A. Knedler, N. Avdo, and P.M. Henson. 1989. Adherence of neutrophils to cultured human microvascular endothelial cells: stimulation

by chemotactic peptides and lipid mediators and dependence upon the Mac-1, LFA-1, p150,95 glycoprotein family. *J. Clin. Invest.* 83:637.

42. Arfors, K.-E., C. Lundberg, L. Lindbom, K. Lundberg, P.G. Beatty, and J.M. Harlan. 1987. A monoclonal antibody to the membrane glycoprotein complex CD18 inhibits polymorphonuclear leukocyte accumulation and plasma leakage in vivo. *Blood.* 69:338.

43. Smith, C.W., R. Rothlein, B.J. Hughes, M.M. Mariscalso, H.E. Rudloff, F.C. Schmalstieg, and D.C. Anderson. 1988. Recognition of an endothelial determinant for CD18-dependent human neutrophil adherence and transendothelial migration. *J. Clin. Invest.* 82:1746.

44. Hakkert, B.C., J.M. Rentenaar, W.G. Van Aken, D. Roos, and J.A. van Mourik. 1990. A three-dimensional model system to study the interactions between human leukocytes and endothelial cells. *Eur. J. Immunol.* 20:2775.

45. Tanaka, Y., S.M. Albelda, K.J. Horgan, G.A. Van Seventer, Y. Shimizu, W. Newman, J. Hallam, P.J. Newman, C.A. Buck, and S. Shaw. 1992. CD31 expressed on distinctive T cell subsets is a preferential amplifier of beta1 integrin-mediated adhesion. *J. Exp. Med.* 176:245.

46. Newman, P.J., and S.M. Albelda. 1992. Cellular and molec-

ular aspects of PECAM-1. *Nouv. Rev. Fr. Hematol.* 34:9.

47. Carlo, J.R., S. Ruddy, E.J. Studer, and D.H. Conrad. 1979. Complement receptor binding of C3b-coated cells treated with C3b inactivator, beta 1H globulin and trypsin. *J. Immunol.* 123:523.

48. Wright, S.D., P.E. Rao, W.C. Van Voorhis, L.S. Craigmyle, K. Iida, M.A. Talle, E.F. Westberg, G. Goldstein, and S.C. Silverstein. 1983. Identification of the C3bi receptor of human monocytes and macrophages by using monoclonal antibodies. *Proc. Natl. Acad. Sci. USA.* 80:5699.

49. Dana, N., B. Styrt, J.D. Griffin, R.F. Todd, III, M.S. Klempner, and M.A. Arnaout. 1986. Two functional domains in the phagocyte membrane glycoprotein Mo1 identified with monoclonal antibodies. *J. Immunol.* 137:3259.

50. Relman, D., E. Tuomanen, S. Falkow, D.T. Golenbock, K. Saukkonen, and S.D. Wright. 1990. Recognition of a bacterial adhesin by an integrin: macrophage CD3 ($\alpha_m\beta_2$, CD11b/CD18) binds filamentous hemagglutinin of *Bordetella pertussis*. *Cell.* 61:1375.

51. Lampugnani, M.G., M. Resnati, M. Raiteri, R. Pigott, A. Piscacane, G. Houen, L.P. Ruco, and E. Dejana. 1992. A novel endothelial-specific membrane protein is a marker of cell-cell contacts. *J. Cell Biol.* 118:1511.