

Monocyte-selective Transendothelial Migration: Dissection of the Binding and Transmigration Phases by an In Vitro Assay

By William A. Muller and Susan A. Weigl

From the Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York, New York 10021

Summary

We describe a quantitative assay of transendothelial migration (TEM) that allows us to selectively study the interaction of monocytes with confluent human endothelial cell (HEC) monolayers. The HEC are grown on hydrated collagen gels; the monocytes need not be purified. 100% of monocytes transmigrated the monolayer within 1 h at 37°C and accumulated in the subendothelial collagen; TEM of lymphocytes was not detected within this time. Migration of neutrophils from the same donor was much slower and incomplete, with only 14% of PMN transmigrating in 2 h. This rapid TEM occurs in the absence of exogenous chemoattractants, and HEC in this system do not express cytokine-inducible leukocyte adhesion molecules. A slight modification of the TEM assay allowed us to separate binding to the apical HEC surface from TEM. We found that tight apical surface binding was the rate-limiting step for TEM. Two-thirds of this binding and TEM could be blocked by a monoclonal antibody against the leukocyte β_2 integrin chain CD18. This assay will allow us to dissect the mechanisms of both the binding and transmigration stages of diapedesis.

During the inflammatory response, specific cell adhesion molecules on the surfaces of the leukocytes (1, 2) and endothelial cells (1, 3, 4) mediate the initial loose contacts ("rolling") (5–7) and subsequent tight adhesion (1, 8) of leukocytes to the apical surface of the endothelium. During the subsequent process of transendothelial migration (TEM),¹ tight leukocyte-endothelial contacts are seen in vivo (9, 10) and in vitro (11, 12). However, much less is known about the molecules that mediate TEM, and most in vitro studies have focused on neutrophils (PMN) (12–15). Recently several in vitro studies of TEM by monocytes (Mo) have been published (11, 16–18) using human endothelial cells (HEC) cultured on three-dimensional matrices to model the blood vessel wall.

In this paper, we employ a quantitative in vitro assay of monocyte TEM across HEC cultured on hydrated collagen gels. We find that: (a) Mo selectively transmigrate HEC monolayers in the absence of endothelial activation or exogenous chemoattractants; (b) binding of Mo to the apical HEC surface can be distinguished from TEM per se, allowing dissection of the cell adhesion molecules important for each step;

and (c) tight binding to the apical surface, mediated in large part by the CD11/CD18 complex of the Mo, is the rate-limiting step in the otherwise rapid process of TEM by Mo.

Materials and Methods

Cell Culture. Isolation and culture of human umbilical vein endothelial cells on hydrated collagen gels (Vitrogen; Collagen Corporation, Palo Alto, CA) in 20% human serum in medium 199 was performed as previously described (19). For the quantitative assay of TEM, 50 μ l of collagen was allowed to gel in each well of a 96-well tissue culture plate (Becton Dickinson Labware, Lincoln Park, NJ), then coated with fibronectin, and seeded with HEC (19).

Preparation of soluble material extractable from the collagen gels under confluent HEC cultures ("infranate") was made by physically removing the HEC together with the underlying gel from the culture vessel, and centrifugation in a microfuge (14,000 rpm, 5 min, 4°C) to pellet cells and collapse the collagen gel. The supernate overlying this pellet was collected and used immediately or stored at $\leq 4^\circ\text{C}$. Control infranate was made from identical fibronectin-coated collagen gels incubated in complete culture media under the identical conditions, but without the addition of HEC.

Isolation and Labeling of PBMC and Neutrophils (PMN). Blood from healthy volunteers was drawn into a 1/10 volume of 2.7% disodium EDTA, diluted in an equal volume of HBSS, and layered over 12 ml of Ficoll/Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) in a 50-ml centrifuge tube. After centrifugation (2,200 rpm, 20 min, 25°C) the interface layer containing PBMC was collected, washed free of adherent platelets by washing with the over-

¹ Abbreviations used in this paper: DPBS, Dulbecco's PBS with Ca^{2+} and Mg^{2+} ; ELAM-1, endothelial-leukocyte adhesion molecule 1; HEC, human endothelial cells; ICAM-1, intercellular adhesion molecule 1; Mo, monocyte; MPO, myeloperoxidase; PECAM-1, platelet/endothelial cell adhesion molecule 1; TEM, transendothelial migration; VCAM-1, vascular cell adhesion molecule 1.

laying platelet-poor autologous plasma, then washed twice in cold HBSS.

In some experiments PMN were isolated along with PBMC from the same donor by using a double discontinuous gradient consisting of 10 ml Histopaque 1119 (Sigma Chemical Co., St. Louis, MO) under 10 ml of Ficoll/Hypaque. The blood was processed as described above. After removal of PBMC from the upper interface, PMN were collected from the cloudy layer above the erythrocyte (RBC) pellet. RBC were lysed by a 45-s pulse in hypotonic saline; the PMN were then washed in parallel with the PBMC and treated otherwise identically.

Labeling of cells with ^{111}In oxyquinoline (indium oxine; Amersham Clinical Products, Arlington Heights, IL) was performed as described (3) using PBMC resuspended to 10^7 cells/ml in HBSS. Labeling was carried out for 20 min on ice, followed by two washes in HBSS containing 0.1% HSA (to terminate labeling) and one wash in HBSS. Pilot experiments (not shown) demonstrated that this washing procedure removed all detectable radiolabel that was not cell associated. Spontaneous release of radiolabel over the time course of these experiments was negligible. Labeled, washed cells were resuspended in warm M199 or complete medium for use in the transmigration assay.

Isolation of Monocytes. Isolation of monocytes from mononuclear cell fractions of peripheral blood was performed on Percoll (Pharmacia Fine Chemicals) gradients as described (20).

Quantitative Assay of TEM. HEC grown to confluence on hydrated collagen gels in 96-well culture plates were washed three times in warm M199 immediately before addition of labeled leukocytes (see Fig. 1). In certain experiments the endothelial cells were preincubated for 20 min at 37°C with mAb directed against HEC antigens. When used, mAb directed against CD18 (IB4, the generous gift of Dr. Samuel Wright, The Rockefeller University) was added at the time of addition of leukocytes. Labeled cells were added in aliquots of $100\ \mu\text{l}$ in the presence or absence of human serum and placed in a CO_2 incubator at 37°C for the desired times. To stop the reaction, the culture vessel was removed from the incubator, the supernatant fluid removed by shaking over an absorbent mat, and the wells were filled ($\sim 400\ \mu\text{l}$) with warm 1 mM EGTA in HBSS. The trays were then sealed with acetate sheets (Plate Sealers; Dynatech, Alexandria, VA), inverted, and centrifuged (250 g, 5 min). The collagen gel apparently retained enough divalent cations to maintain the HEC monolayer. Leukocytes beneath the monolayer or in the process of migrating through it were retained (Figs. 1 and 2), whereas nonadherent leukocytes, and those adherent to the apical surface, were removed. To measure both binding and transmigration, the wells were filled with warm Dulbecco's PBS with Ca^{2+} and Mg^{2+} (DPBS).

After centrifugation the plastic sheets were removed with the trays still in the inverted orientation. The monolayers were washed three times with $200\text{-}\mu\text{l}$ volumes of warm HBSS (or DPBS to measure binding plus transmigration) and checked by inverted phase contrast microscopy. The contents of the well (HEC monolayer, collagen gel, and remaining leukocytes) were picked up with a cotton swab and transferred to a disposable tube. Quantitation of γ emissions was performed on an Auto Gamma Scintillation Spectrometer (Packard Instrument Co., Downers Grove, IL) on the ^{51}Cr channel. Data are expressed as the mean $\pm \sigma$ for six replicates.

Recovery of Transmigrated Mo from Subendothelial Collagen. For these experiments HEC were grown on 30-mm culture dishes. After transmigration had taken place, the dishes were washed three times with warm HBSS, then incubated in 10 mM EDTA in HBSS on a gyrotory shaker (90 rpm, 20 min, 37°C) to remove HEC. The dishes were then washed three times with DPBS and incubated

in 1 ml of collagenase (150 U/ml in M199; Worthington Biochemical Corp., Freehold, NJ) on a gyrotory shaker (90 rpm, 20 min, 37°C) to digest the collagen. The contents of the dish were collected, washed twice in cold HBSS, and analyzed for leukocyte markers by FACScan[®] (Becton Dickinson & Co., Mountain View, CA) (see below). mAbs included anti-Leu-M5 (anti-CD11c) and LeukoGate[™], a mixture of fluoresceinated anti-CD45 and PE-labeled anti-CD14 (all purchased from Becton Dickinson & Co.).

Chemotaxis Assay. Migration of leukocytes in response to a chemotactic gradient was assayed in a Boyden chamber (Neuroprobe, Inc., Cabin John, MD) by previously published methods (8, 21). Briefly, test chemoattractants diluted in 0.1% human serum albumin in M199 were placed in the lower wells of the device and 2.5×10^4 purified leukocytes suspended in $50\ \mu\text{l}$ of the same buffer were added to the upper wells. During the 2-h incubation at 37°C , leukocytes that had migrated through the $2\text{-}\mu\text{m}$ pores of a polycarbonate filter separating the wells became embedded in the $0.45\text{-}\mu\text{m}$ nitrocellulose filter immediately underlying it. The nitrocellulose filter was stained with hematoxylin, and migrated leukocytes in the center $1\ \text{mm}^2$ from each well were counted using a $10\times$ objective and a $10\times$ ocular with a ruled graticule. In most experiments all conditions were carried out in six replicate wells.

FACS[®] Analysis. Confluent HEC monolayers cultured by the usual method or stimulated for 15 h with recombinant TNF- α (10 ng/ml; Biogen, South San Francisco, CA) in complete medium were harvested nonenzymatically by incubation for 15 min in 10 mM EDTA. The collagen gel traps significant amounts of divalent cation-containing culture medium, so relatively high concentrations are necessary to remove the cells. The resuspended HEC were then washed twice in cold HBSS and incubated for 30 min at 4°C in mAb at $\sim 2\ \mu\text{g/ml}$ in round-bottomed culture wells. The mAb, corresponding antigen, and sources were as follows: H4/18 and E1/6, anti-endothelial leukocyte and vascular cell adhesion molecules (ELAM-1 and VCAM-1), respectively, were generously provided by Dr. Michael Bevilacqua. LB2, anti-intercellular adhesion molecule 1 (ICAM-1), was provided by Dr. Edward Clark. W6/32, anti-class I MHC, was obtained from American Type Culture Collection (Rockville, MD). 9.3C9, anti-MHC class II (HLA-DR and DQ) and hec7, anti-platelet/endothelial cell adhesion molecule 1 (PECAM-1), were raised in this department.

Cells were then washed twice in cold HBSS by centrifugation, incubated in fluoresceinated F(ab')₂ fragments of rabbit anti-mouse IgG (Dako, Santa Barbara, CA) for 30 min at 4°C , washed twice more, and analyzed on a FACScan[®] using Consort 30 software (Becton Dickinson & Co.).

Photomicroscopy. Staining of live HEC monolayers with silver nitrate was performed as described (19). Staining for endogenous myeloperoxidase (MPO) activity in HEC monolayers bearing transmigrated leukocytes was performed by the method of Graham and Karnovsky (22). To stain for MPO activity in PBMC suspensions, $25\ \mu\text{l}$ of sample was added to $25\ \mu\text{l}$ of a $2\times$ concentration of the same substrate. Sodium azide added to a replicate sample to inhibit MPO activity served as a negative (background) control.

For optimal optical resolution of samples on collagen gels, the gels were carefully removed from the culture vessels, spread on glass slides, coverslipped, and viewed from above the monolayer by conventional brightfield or phase contrast microscopy. Photomicrographs were taken on a Nikon Microphot photomicroscope.

Results

TEM Is Monocyte Selective. HEC grown on hydrated collagen gels in human serum without exogenous growth factors

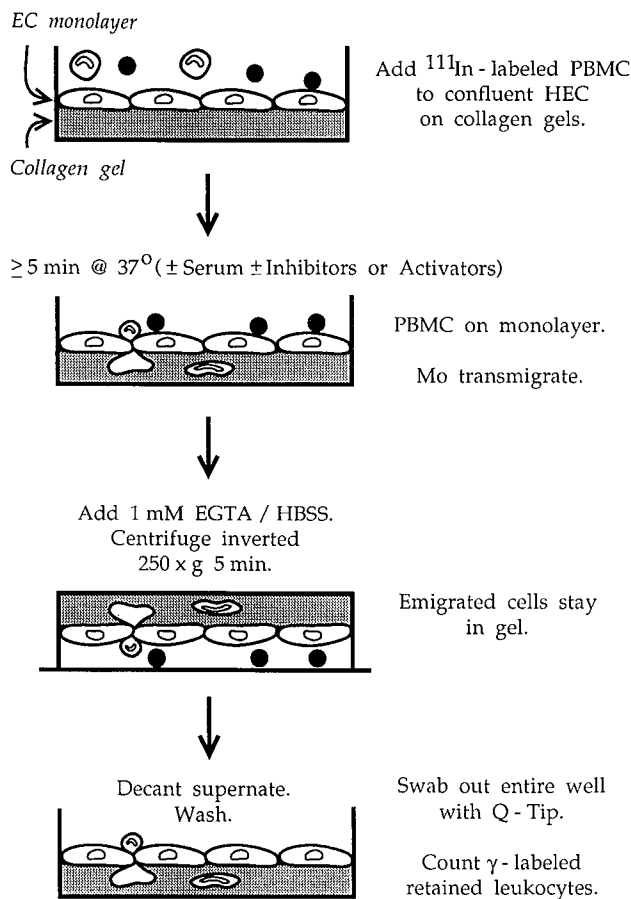


Figure 1. Schematic flow diagram of the quantitative transmigration assay (see text).

form stable, growth-arrested monolayers that resemble the vascular intima in vivo (11, 19). The cell borders stain with silver nitrate and selectively express PECAM-1 (CD31) (19, 23). Purified Mo were added to such confluent monolayer cultures at 37°C and observed on a warm stage by inverted

phase contrast video microscopy. Suddenly, and apparently stochastically over the course of the 45-min observation, individual, round Mo flattened somewhat, extended a pseudopod, and moved toward the nearest intercellular junction. They then migrated rapidly through the HEC monolayer. TEM was usually completed within 1 min of initial pseudopod extension. No permanent change in monolayer morphology was visible at the light microscope (data not shown) or ultrastructural level (11) as a result of TEM.

When a mixed population of PBMC were added to such monolayers at 37°C , most cells (later found to be lymphocytes) did not adhere, but a portion of the cells were observed to display the same microscopic behavior as the purified Mo. When such cultures were washed in the presence of DPBS (Fig. 1), a small subpopulation of leukocytes, subsequently found to be Mo, remained tightly bound to the apical HEC surface (Fig. 2 a). These leukocytes were round and could be seen when focusing above the plane of the monolayer. In addition, when the culture was stained with silver nitrate, silver was deposited on these apical leukocytes as well as at the intracellular junctions of HEC.

When elution was performed in EGTA no leukocytes were seen entirely on the monolayer surface. Rather, if we focused below the plane of the monolayer, spread mononuclear cells were seen, and no silver deposition was seen on these cells (Fig. 2 b). In these cultures, if we focused at the level of the HEC monolayer, we occasionally saw leukocytes caught in the process of transmigration with part of the cell between two tightly apposed HEC. In silver-stained cultures, that portion of the leukocyte under the HEC monolayer was free of silver, while the portion still on the apical surface was stained (Fig. 2 c). Since these partially transmigrated Mo remained with the HEC monolayer, they would be counted as transmigrated. Note that the HEC monolayer remained confluent and intact throughout the procedure.

The cells from the mixed PBMC population that transmigrated were virtually all Mo. When cultures were examined microscopically after the TEM assay, numerous leuko-

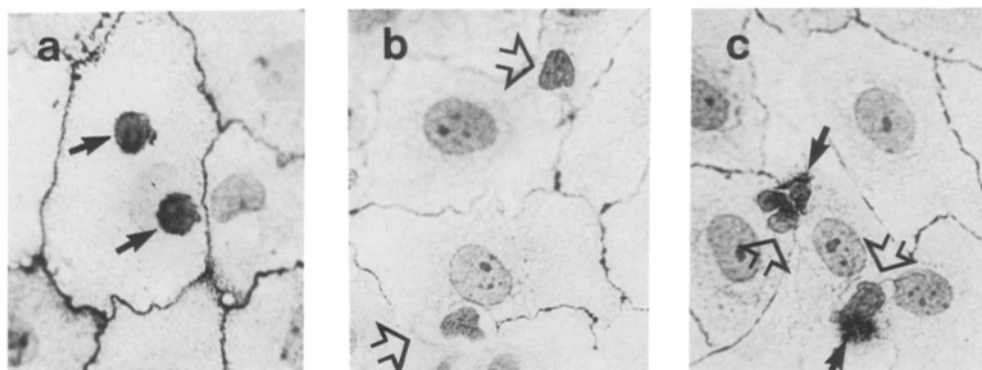


Figure 2. The TEM assay can distinguish Mo binding from transmigration. PBMC were subjected to the standard assay. Elution by inverted centrifugation was performed in the presence of divalent cations (a) or 1 mM EGTA (b and c). Monolayers were stained with AgNO_3 , then Wright-Giemsa as described (19), then retrieved onto glass slides for microscopy. (a) Two mononuclear cells (arrows) adherent to the apical surface of the monolayer. These cells were in a focal plane above the silver-stained junctions, and they themselves were covered with silver deposits. When inverted

centrifugation was performed in EGTA, no leukocytes were seen entirely on the surface. (b) Most mononuclear cells were in focal planes underneath the HEC monolayer (arrows) and bore no silver deposits. Focusing at the level of the monolayer occasionally revealed cells caught in the process of migrating (c). The portion of these leukocytes under the HEC monolayer was free of silver deposits (large arrows), while the portion still on the apical surface was decorated (small arrows) ($\times 200$).

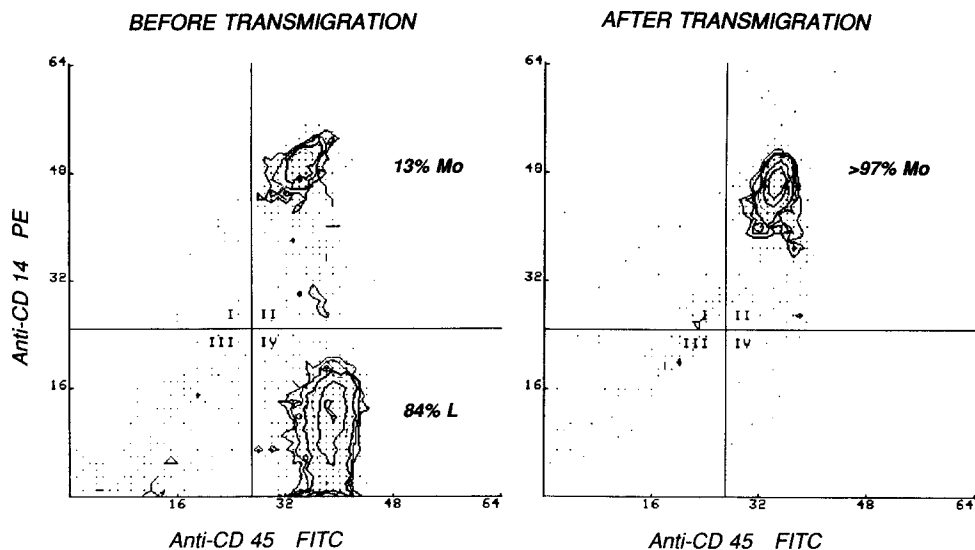


Figure 3. Transmigration is monocyte selective. PBMC were labeled with Leukogate™ (fluoresceinated anti-CD45 plus PE-labeled anti-CD14) then subjected to FACSscan® analysis (see Materials and Methods). Transmigrated leukocytes recovered from subendothelial collagen (*After Transmigration*) were compared with the starting population (*Before Transmigration*). The actual contour graphs are reproduced here. L, lymphocytes; Mo, monocytes.

cytes were seen in multiple focal planes in the subendothelial collagen gel. These cells had the morphologic characteristics of Mo, including multiple large pseudopods, kidney-shaped nuclei, and many phase-dense granules. When such cultures were stained with diaminobenzidine to mark endogenous myeloperoxidase activity, all of the transmigrated cells were peroxidase positive. Likewise, the apically adherent round mononuclear cells that resisted washing in DPBS but not EGTA (Fig. 2) were all peroxidase positive (not shown).

When transmigrated leukocytes were recovered from the subendothelial collagen and analyzed by FACS®, essentially all bore monocyte markers. Fig. 3 shows the results of a typ-

ical experiment. The starting population of PBMC in this experiment contained 87% lymphocytes (small cells expressing CD45 but not CD14) and 13% monocytes (expressing CD45 and CD14). More than 97% of the cells recovered after TEM bore CD14. In similar experiments (not shown), 98–99% of the recovered cells were positive for the monocyte marker CD11c.

TEM by Mo was rapid and complete (Fig. 4 *a*). Labeled Mo accumulated in the subendothelial collagen gel beginning at 5–10 min, and increased with time, reaching a maximum generally at 30–40 min, and always within 60 min. When the data were expressed as the percent of radiolabeled

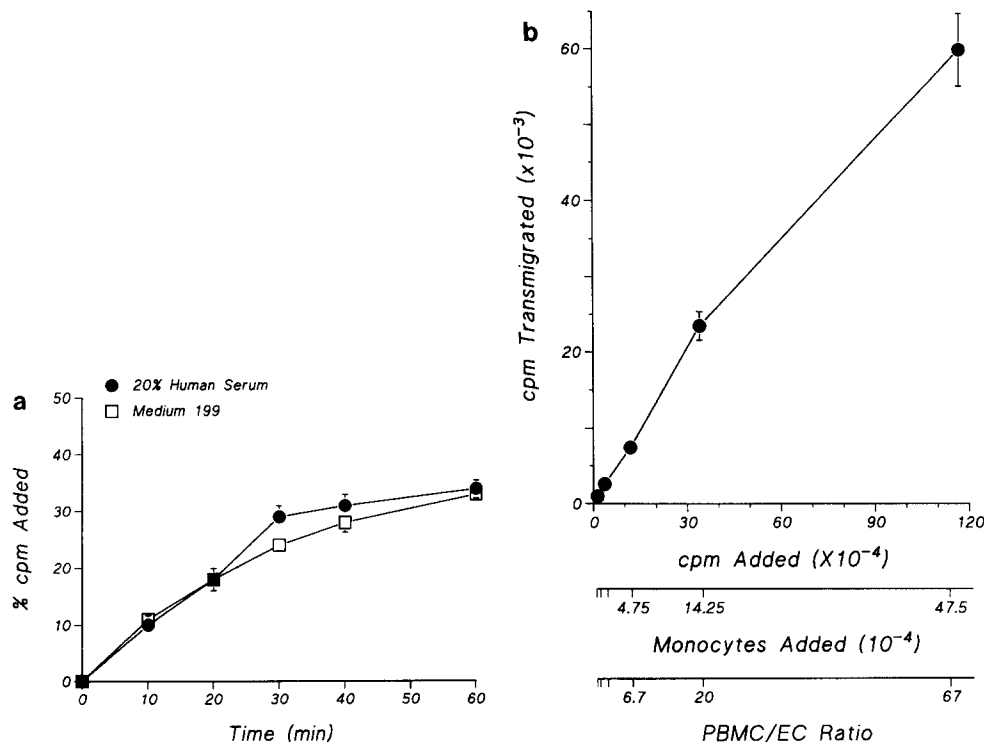


Figure 4. (a) Time course of TEM by monocytes. Labeled Mo were added to HEC monolayers in M199 (squares) or M199 + 20% human serum (standard HEC culture medium; circles) and subjected to the standard TEM assay for the times indicated. Data are expressed as the percentage of the added radiolabel remaining with the gel. In this experiment 32% of the labeled PBMC were Mo by morphologic and histochemical criteria. The data shown for each time point are the mean and SD of six replicate samples. (b) Dose-response curve for TEM. Measurements were made after TEM proceeded for 10 min.

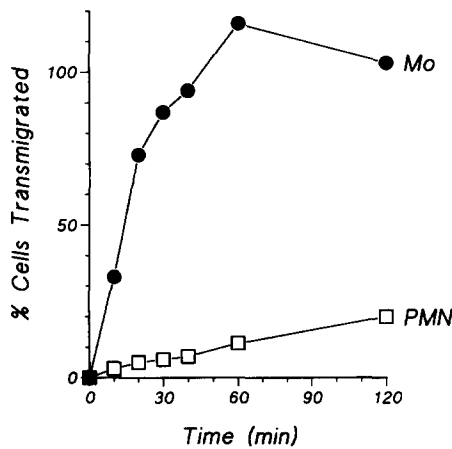


Figure 5. Monocytes transmigrate faster and to a greater extent than neutrophils. PBMC (circles) and PMN (squares) were isolated in a modified Ficoll/Hypaque gradient from the same donor, labeled, resuspended to the same cell density, and added to replicate HEC monolayers in the same culture tray. Transmigration at the indicated times was assessed by the usual method. Note that in this figure, the ordinate refers to the percent of PMN or of Mo that transmigrated. The figures for the Mo were derived by dividing the absolute percent of transmigrated PBMC by the percent of Mo in that population. The neutrophil population was >99% PMN by morphology and Wright's stain.

PBMC added, the plateau level always corresponded to the percent of monocytes in the population of PBMC added the HEC monolayers. That is, within 30–40 min, all of the monocytes had crossed the endothelial monolayer.

The time for half of the monocytes to migrate across the HEC monolayer was between 15 and 20 min in the course of experiments employing different leukocytes, serum donors, and HEC isolates. The rate and extent of TEM by Mo was the same whether PBMC were added to the HEC monolayers in complete culture medium or in serum-free M199 (Fig. 4 a). TEM in this system was linear out to 20–60 mononuclear cells added per HEC (Fig. 4 b) and corresponded to 5–15 Mo per HEC.

The PBMC fractions used in these experiments contained <1% neutrophils (PMN). PMN could transmigrate these monolayers, but TEM by PMN was both quantitatively and qualitatively different. When PMN isolated from the same donor on the same (modified) gradient were compared with PBMC in the TEM assay, all Mo had transmigrated within 1 h (Fig. 5), while only ~14% of the PMN had migrated by 2 h. Even when such assays were continued for up to 24 h, <30% of the added PMN transmigrated (not shown).

HEC Are Not Activated in This System. The rate and extent to which Mo migrated across HEC monolayers raised the possibility that our cultures were activated by exogenous LPS and/or cytokines. However, the HEC in this system did not express cytokine-inducible antigens and adhesion molecules. HEC grown in routine cultures were harvested nonenzymatically and assayed for surface expression of MHC antigens and cell adhesion molecules immunocytochemically by FACS[®]. Fig. 6 (broken line) shows that our standard HEC cultures expressed class I MHC antigen, basal levels

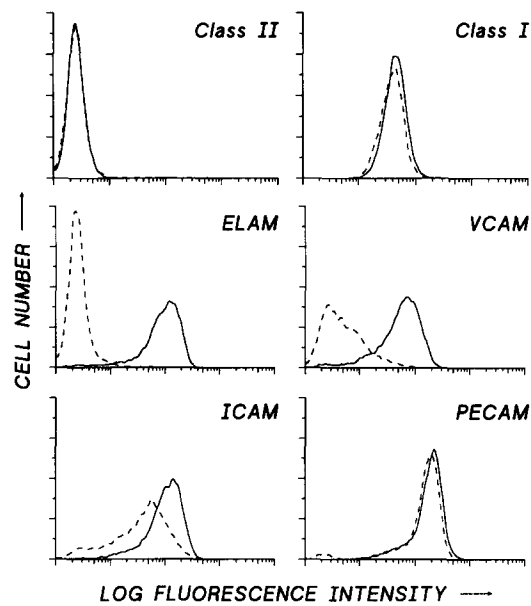


Figure 6. HEC in the TEM assay system are not activated. HEC monolayers grown under standard conditions (broken line) were nonenzymatically resuspended, incubated with mAb against the indicated cell surface molecules, and quantitative immunofluorescence analysis performed by FACSscan[®] as described in Materials and Methods. Cells exposed to a nonbinding mAb or no mAb had a modal fluorescence channel of ~3 in this experiment (not shown). An identical culture was exposed to TNF- α (10 ng/ml) for 15 h before processing as above (solid line). The cytokine-inducible adhesion molecules ELAM-1 and VCAM-1 are not expressed on our resting cultures, but can be induced by an appropriate cytokine. The mAbs used were 9.3C9 (anti-MHC class II, made in this department), W6/32 (anti-MHC class I, from American Type Culture Collection), H4/18 and E1/6 (anti-ELAM-1 and anti-VCAM-1, respectively, from Dr. Michael Bevilacqua, San Diego, CA), LB2 (anti-ICAM-1, from Dr. Edward Clark, Seattle, WA), and hec7 (anti-PECAM-1; reference 19).

of ICAM-1, and significant levels of PECAM-1 (CD31, [19, 23]), but did not express class II MHC antigen, ELAM-1, or VCAM-1. However, these cells were capable of responding appropriately to specific cytokines. In the experiment shown, incubation with TNF- α led to the synthesis and expression of ELAM-1 and VCAM-1, increased expression of ICAM-1, but no expression of MHC class II antigen, as has been reported for HEC grown under more traditional culture conditions (24, 25).

Role of Endogenous Soluble Chemoattractants in TEM. After passage through the HEC monolayer, the Mo continued to migrate into the subendothelial collagen gel (Fig. 2 b; reference 11), suggesting the presence of a chemoattractant. Indeed, a soluble, heat-labile factor was isolated from subendothelial collagen gels that could act as a classic chemoattractant in Boyden chamber “checkerboard” assays (Table 1). The production of this chemoattractant required the presence of HEC. Neither the culture medium nor material eluted from bare collagen gels incubated with or without medium were chemotactic (Table 2). The nature of this material is currently being investigated (see Discussion). It appears to be secreted or shed, as lower amounts of activity can be recovered from the conditioned media above the HEC monolayers. However, this

Table 1. Subendothelial Infranate Is Chemotactic for Monocytes

Concentration of infranate below membrane (% vol/vol)	Concentration of infranate above membrane (% vol/vol)			
	0	1	33	50
	<i>mean ± SD</i>			
0	23 ± 8*	25 ± 6	13 ± 2	16 ± 14
1	30 ± 15	54 ± 29	40 ± 6	21 ± 16
33	246 ± 53	394 ± 79	111 ± 22	67 ± 25
50	742 ± 137	645 ± 40	178 ± 50	107 ± 19

PBMC were isolated as usual and resuspended to a concentration of 2×10^5 Mo/ml in 0.1% HSA/M199. Aliquots were mixed with dilutions of infranate in 0.1% HSA/M199 to yield suspensions at 10^5 Mo/ml in the indicated final dilutions of infranate. 50 μ l (5×10^3 Mo) was added to the upper wells of the chemotaxis chamber in triplicate and incubated at 37°C for 2 h. The migrated cells in the center of each well were counted on the hematoxylin-stained membrane at 100 \times using a ruled graticule.

* Data shown are Mo migrated per mm².

chemoattractant is not monocyte specific, nor is it the only factor driving transmigration. The infranate was as good or better a chemoattractant for PMN as it was for Mo, in some experiments being as active as optimal concentrations of IL-8 or formyl-norleucyl-leucyl-phenylalanine 2 (fNLLP) (Table 2). Nevertheless, freshly isolated unactivated PMN did not bind or transmigrate our HEC monolayers at nearly the same rate

or to the same extent as Mo (Fig. 5). It seems, therefore, that other aspects of the leukocyte-endothelial interaction determine the rate and extent of TEM.

Distinguishable Binding from Transmigration. The fact that apically adherent mononuclear cells remained adherent when the HEC monolayers were eluted with DPBS (Fig. 2 a) allowed us to measure binding plus TEM. However, we found only occasional Mo adherent to the apical surface at early time points before TEM of the Mo population was complete (Fig. 2 a, and data not shown). The following experiment demonstrated that this method could detect a population of bound but not transmigrated Mo when one exists. "Infranate" inhibited TEM of Mo when applied to the apical surface of HEC, but strong binding was still observed. In the experiment shown in Fig. 7, control monolayers (no infranate) washed with DPBS after 20 min bore essentially the same number of Mo as those washed with EGTA. Infranate blocked TEM by 82%. However, when infranate-exposed monolayers were washed with DPBS, the Mo remained with the monolayer and recovered radioactivity was the same as for control (no infranate) monolayers.

Microscopic examination of such monolayers stained with DAB revealed that all of the cells remaining bound to the apical surface of the HEC monolayer were indeed Mo (not shown). Infranate was nontoxic and did not adversely affect the migratory ability of the Mo, since Mo continue to move into an infranate gradient for several hours (Table 1) and remain viable in the subendothelial collagen for weeks. We conclude that: (a) this assay is indeed capable of identifying a population of bound but nontransmigrated Mo when one

Table 2. Comparison of Chemotaxis by Mo, Neutrophils, and Nonadherent Mononuclear Cells

Exp.		IL-8 (4×10^{-10} M)	HSA (0.1%)	fNLLP			Infranate	Gel + HS extract		
				10^{-6} M	10^{-7} M	10^{-8} M		F.S.*	1:10	1:100
						<i>mean ± SD</i>				
1	PBMC		2.5 ± 2.8 [‡]	288 ± 67	189 ± 86	53.5 ± 13.5	332 ± 195	27 ± 2.7	3.5 ± 2.4	0.16 ± 0.4
2	PBMC		8.5 ± 5.7			49 ± 13.7	69 ± 23.8			
	NA [§]		ND			19 ± 15.5	1 ± 1			
	PMN		62.8 ± 15			342 ± 39	456 ± 39			
3	PBMC	40 ± 17.8	61 ± 54			250 ± 68.6				
	NA	3.3 ± 2.2	ND			4.8 ± 4.3				
	PMN	198 ± 53.8	14.5 ± 10			176 ± 22.4				

Chemotaxis assays were performed as described in Materials and Methods using freshly isolated mononuclear cells (PBMC), nonadherent mononuclear cells (NA) containing <1% peroxidase-positive cells, and neutrophils (PMN) from the same donor resuspending to 10^5 cells/ml. The monocyte-depleted mononuclear cells (NA) migrated poorly to the chemotactic stimuli, consistent with the visual observation that migrating cells in the PBMC fraction were all monocytes. Although the background and absolute number of cells migrating in each experiment varied somewhat, infranate was consistently chemotactic for both monocytes and PMN, whereas the concentrations of fNLLP and IL-8 used in these comparisons were selectively chemotactic for PMN.

* Full strength.

[‡] Data shown are cells migrated per mm².

[§] Nonadherent PBMC.

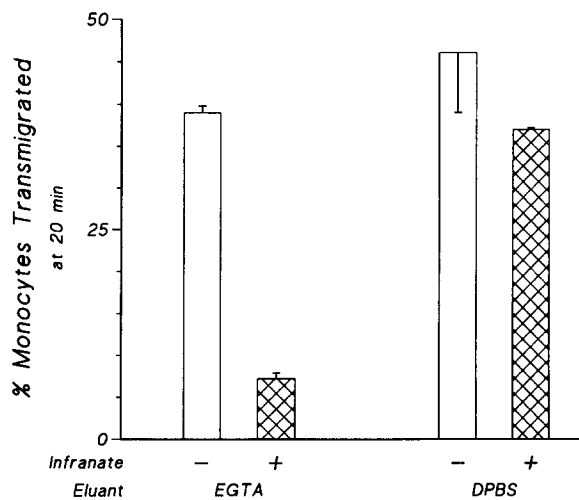


Figure 7. Infrinate inhibits transmigration but not binding of Mo to HEC. Equal numbers of PBMC were resuspended in HEC culture medium (-) or infrinate (+) and added to HEC monolayers for 20 min at 37°C. The inverted centrifugation was performed in the presence of divalent cations (DPBS) to measure bound plus transmigrated cells or in EGTA to measure TEM as usual. Infrinate inhibits TEM, so that when bound cells are washed with EGTA, they are eluted. However, when washed with DPBS they remain bound to the apical surface. These data represent one of five experiments with similar results.

exists, and (b) binding and transmigration are distinct events that can be temporally dissected by the apical application of chemoattractant.

We quantitated the time course for binding and transmigration using elution in the presence or absence of divalent cations. Fig. 8 shows a typical experiment. Neither the HEC monolayers nor the Mo were inherently "sticky" in this assay; low levels of binding were measured at the early time points. However, binding increased steadily with time. The curves for binding and TEM were virtually superimposable. Since elution in the presence of divalent cations measured binding plus TEM, this demonstrated that there was no detectable pool of adherent Mo waiting to migrate across the HEC monolayer. That is, once a Mo bound tightly enough to resist the 250-g displacement force of the washing step, it rapidly migrated through the EC monolayer so that the time course for these events on a population of cells could not be distinguished by this assay with its resolution time of 5 min.

Leukocyte β_2 Integrins Account for Most of the Tight Binding of Mo to HEC. In initial attempts to define the leukocyte and endothelial cell adhesion molecules responsible for TEM in this system, we focused on interactions that did not require HEC activation. mAb IB4 directed against the common β subunit of the leukocyte integrins (CD18) consistently blocked TEM of Mo by at least 67% (Fig. 9, and data not shown). Control mAb W6/32 against class I MHC had no effect (not shown).

Consistent with the idea that tight binding of Mo to the apical surface of HEC is the rate-limiting step for TEM, both binding and TEM were inhibitable to the same extent by mAb directed against CD18 (Fig. 9).

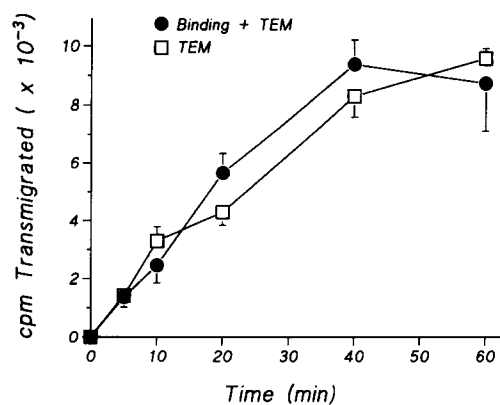


Figure 8. Tight binding of Mo to the HEC surface is followed closely by TEM. Labeled Mo were resuspended to 2×10^6 /ml in warm M199 and 100- μ l aliquots added to HEC monolayers at 37°C. At the indicated times, wells were filled with warm DPBS (circles) to measure binding plus TEM or 1 mM EGTA (squares) to measure TEM only. Plates were centrifuged inverted, washed, and quantitated as described in Materials and Methods. The mean and SD of six replicate samples are shown. No significant pool of bound but nonmigrated cells was apparent.

Discussion

Mo-selective Transmigration. We have developed an in vitro assay for TEM of leukocytes across human endothelium that is quantitative and reproducible. Since the assay is performed in 96-well culture plates (0.28 cm²/well) and a relatively small blood sample suffices, many variables can be analyzed at the same time with replicate samples. Mo transmigrate rapidly and completely in the absence of exogenous chemoattractants across HEC that do not express an activated phenotype of surface adhesion molecules.

Under the conditions described here, our assay is monocyte selective. None of the standard methods for separating monocytes from lymphocytes are as selective as the HEC monolayer. When starting with freshly isolated PBMC, virtually 100% of the transmigrated cells are Mo. Separation

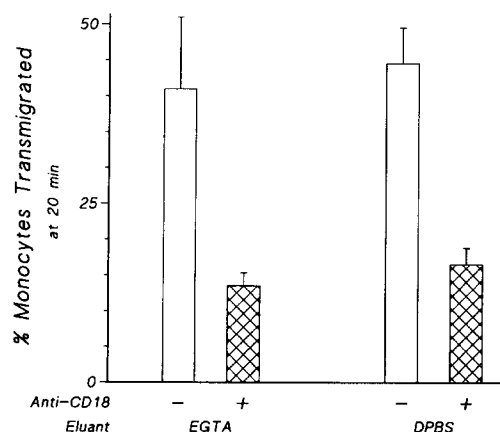


Figure 9. Antibody against CD18 inhibits Mo transmigration and binding to the same extent. PBMC were added to HEC monolayers in the presence or absence of mAb IB4 (anti-CD18) at 25 μ g/ml. After 20 min at 37°C, inverted centrifugation was carried out to measure TEM (EGTA) or binding plus TEM (DPBS).

of Mo from lymphocytes by adherence (26), Nycodenz (11), Percoll (20), and counter-current elutriation (18) results in populations of Mo reported to be up to only 90%, 85%, 90–95%, and 95% pure, respectively. Thus, we can study TEM by Mo without having to first separate them from the larger population of lymphocytes. Several groups have reported studies of TEM using purified leukocyte types and HEC grown on three-dimensional matrices (11, 16–18). However, these studies did not appreciate the virtually exclusive transmigration of Mo when starting with whole PBMC fractions.

This assay system may prove valuable in the construction of in vitro models of atherosclerosis and other pathologic conditions in which a relatively selective transmigration of Mo is seen. Transmigrated Mo remain viable in the subendothelial collagen for at least several weeks. Changes in Mo and endothelial cell physiology that follow TEM can be studied in this system. Soluble mediators of inflammation, particulate antigens or organisms, and other immune effector cells could be incorporated into the gel before culture of the HEC. Such a three-dimensional cellular culture system could be used to construct an in vitro model of cell-mediated immunity.

Tight Adhesion Involving β_2 Integrins Is Rate Limiting for TEM. Within minutes of tight adhesion of a Mo to the HEC monolayer, that Mo migrates across the monolayer. Thus, at a population level (Fig. 8), we cannot distinguish the time course for binding from that of transmigration. This implies that the rate-limiting step for TEM is the tight initial adhesion, and raises the question of which adhesion molecules are involved.

Since the HEC are not activated in this system, a likely candidate would be the Mo CD11/CD18 complex and its counter receptors on the HEC. There seems to be general agreement that under most conditions tested in vitro and in vivo the leukocyte β_2 integrin complex CD11/CD18 is largely required for TEM of PMN and Mo. Antibodies against CD18 block TEM of PMN (1, 6, 13) and of Mo (18) in published reports as well as in our system. Furthermore, PMN from patients with leukocyte adhesion deficiency (LAD) that lack the CD11/18 complex can bind to cytokine activated HEC in vitro, but do not transmigrate these monolayers (13). Efficient binding by the leukocyte integrins requires activation of these molecules (1, 8, 27), and it may be the time required for this activation that accounts for the delay in binding of an individual Mo in our system.

On the other hand, Mo and lymphocytes must use other molecules in addition to β_2 integrins in TEM, since these cells reach the site of chronic inflammation in LAD patients. In keeping with this, mAb IB4 never blocked TEM by Mo more than 75–80% in our assays, whereas it blocked TEM by PMN significantly better. The same results were obtained with the anti-CD18 mAb 60.3. Furthermore, in preliminary experiments, antibodies against CD18 blocked TEM significantly better than antibodies against ICAM-1, suggesting that Mo recognize a different counter receptor on HEC.

It was possible that the very low basal levels of VCAM-1

expressed on our unactivated endothelial cells were responsible for part of the adhesion and/or TEM through interaction with the β_1 integrin VLA-4 ($\alpha_4\beta_1$) on Mo. However, anti-VCAM-1 mAb 4B9 (generously provided by Dr. Roy Lobb, Biogen, Cambridge, MA) at concentrations up to 20 $\mu\text{g/ml}$ had no effect on TEM in our standard assay (data not shown).

Mo may be utilizing a set of yet undefined Mo-endothelial adhesion molecule(s) in the process of TEM. This assay makes an ideal system to search for such molecules, since mAbs, peptides, etc. can be tested in replicate samples for their ability to block TEM.

Binding of Mo to the Apical HEC Surface and TEM Are Physiologically Separate Events. The observation that subendothelial “infranate” blocked TEM without interfering with Mo binding (Fig. 7) demonstrated that these two processes were not obligatorily coupled. Tight adhesion was not sufficient to guarantee transmigration. Likewise, soluble chemotactic factor(s) in the infranate are capable of affecting Mo migration, but are not sufficient to explain TEM, since PMN migrated better than Mo to infranate in a Boyden chamber (Table 2), but transmigrated relatively poorly in this system (Fig. 5).

Candidates for this chemotactic factor include monocyte chemotactic protein 1 (MCP-1; MCAF) and Monocyte colony-stimulating factor (M-CSF; CSF-1), both of which are produced by HEC and are relatively selective chemoattractants for monocytes. Two different rabbit antisera against MCP-1 (generously provided by Dr. Joost Oppenheim [NCI, Frederick, MD] and Dr. Anthony J. Valente [University of Texas, San Antonio, TX], respectively) and antiserum against CSF-1 (generously provided by Dr. Richard Stanley [Albert Einstein College of Medicine, New York, NY]) at titers as low as 1:100 failed to inhibit TEM in our system when added to the apical side of the HEC monolayers. Additionally, soluble recombinant forms of these molecules (provided by Drs. Oppenheim and Stanley), at concentrations that promoted chemotaxis of Mo in the Boyden chamber assay, did not affect TEM. These data (not shown) do not rule out the possibility that MCP-1 or CSF-1 are active components of the infranate, but that other chemoattractants also present mask the effect of inactivating them. Much additional work is required to identify the chemotactic factor(s) present in the infranate.

Based on indirect evidence, we speculate that tight binding preceding TEM occurs before insertion of a significant portion of the Mo pseudopod into the interendothelial junction. The adherent Mo that are blocked from transmigrating in the presence of infranate can be eluted with EGTA (Fig. 7). However, images such as those seen in Fig. 2c with Mo caught in the process of TEM were seen with EGTA elution. If such is the case, the pseudopod engagement in the junction may involve EGTA-resistant molecules different from those mediating apical adhesion. The assay we describe will allow us to dissect the role of individual cell adhesion molecules in the separate processes of binding and TEM.

We thank Amy Gruber for excellent technical assistance during the early phase of this work; Stuart Gezelter for assistance operating the FACScan®, Judy Adams for photography and artwork; and Drs. Ralph M. Steinman, Zanvil A. Cohn, and Samuel D. Wright for critical review of the manuscript.

This work was supported by a New Initiatives Grant from the Irvington Institute for Medical Research and a Pew Scholarship in the Biomedical Sciences to W. A. Muller.

Address correspondence to William A. Muller, Laboratory of Cellular Physiology and Immunology, The Rockefeller University, 1230 York Avenue, New York, NY 10021.

Received for publication 18 May 1992.

References

1. Springer, T.A. 1990. Adhesion receptors of the immune system. *Nature (Lond.)* 346:425.
2. Harlan, J.M. 1985. Leukocyte-endothelial interactions. *Blood* 65:513.
3. 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.
4. 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.
5. Atherton, A., and G.V.R. Born. 1972. Quantitative investigations of the adhesiveness of circulating polymorphonuclear leukocytes to blood vessel walls. *J. Physiol.* 222:447.
6. 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.
7. 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.
8. 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, α m β 2) on human neutrophils. *J. Exp. Med.* 173:1493.
9. Marchesi, V.T., and H.W. Florey. 1960. Electron micrographic observations on the emigration of leukocytes. *J. Exp. Physiol.* 45:343.
10. Marchesi, V.T. 1961. The site of leukocyte emigration during inflammation. *J. Exp. Physiol.* 46:115.
11. Pawlowski, N.A., G. Kaplan, E. Abraham, and Z.A. Cohn. 1988. The selective binding and transmigration of monocytes through the junctional complexes of human endothelium. *J. Exp. Med.* 168:1865.
12. Furie, M.B., B.L. Naprstek, and S.C. Silverstein. 1987. Migration of neutrophils across monolayers of cultured microvascular endothelial cells: an in vitro model of leukocyte extravasation. *J. Cell Sci.* 88:161.
13. Smith, C.W., R. Rothlein, B.J. Hughes, M.M. Mariscalco, 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.
14. Moser, R., B.S. Schleiffenbaum, P. Groscurth, and J. Fehr. 1989. Interleukin 1 and tumor necrosis factor stimulate human vascular endothelial cells to promote transendothelial neutrophil passage. *J. Clin. Invest.* 83:444.
15. Luscinskas, F.W., M.I. Cybulsky, J.M. Kiely, C.S. Peckins, V.M. Davis, and M.A. Gimbrone Jr. 1991. Cytokine-activated human endothelial monolayers support enhanced neutrophil transmigration via a mechanism involving both endothelial-leukocyte adhesion molecule-1 and intercellular adhesion molecule-1. *J. Immunol.* 146:1617.
16. Navab, M., G.P. Hough, L.W. Stevenson, D.C. Drinkwater, H. Laks, and A.M. Fogelman. 1988. Monocyte migration into the subendothelial space of a coculture of adult human aortic endothelial and smooth muscle cells. *J. Clin. Invest.* 82:1853.
17. Cushing, S.D., J.A. Berliner, A.J. Valente, M.C. Territo, M. Navab, F. Parhami, R. Gerrity, C.J. Schwartz, and A.M. Fogelman. 1990. Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. *Proc. Natl. Acad. Sci. USA* 87:5134.
18. 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.
19. 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.
20. Wright, S.D., and S.C. Silverstein. 1982. Tumor-promoting phorbol esters stimulate C3b and C3bi receptor-mediated phagocytosis in cultured human monocytes. *J. Exp. Med.* 156:1149.
21. 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.
22. Graham, R.C., Jr., and M.J. Karnovsky. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* 14:291.
23. 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.
24. Pober, J.S., M.A. Gimbrone, L.A. Lapierre, D.L. Mendrick, W. Fiers, R. Rothlein, and T.A. Springer. 1986. Overlapping patterns of activation of human endothelial cells by interleukin

- 1, tumor necrosis factor and immune interferon. *J. Immunol.* 137:1893.
25. Collins, T., L.A. Lapierre, W. Fiers, J.L. Strominger, and J.S. Pober. 1986. Recombinant human tumor necrosis factor increases mRNA levels and surface expression of HLA-A,B antigens in vascular endothelial cells and dermal fibroblasts in vitro. *Proc. Natl. Acad. Sci. USA.* 83:446.
26. Pawlowski, N.A., E.A. Abraham, S. Pontier, W.A. Scott, and Z.A. Cohn. 1985. Human monocyte-endothelial cell interaction in vitro. *Proc. Natl. Acad. Sci. USA.* 82:8208.
27. Albelda, S.M., and C.A. Buck. 1990. Integrins and other cell adhesion molecules. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 4:2868.