In Vitro *Studies of Human Monocyte Migration Across Endothelium in Response to Leukotriene B_4 and f-Met-Leu-Phe*

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Relatively little is known about monocyte emigration from the vasculature or about the factors that regulate this process. In this study, a human *in vitro* model of a blood vessel wall was used for examination of monocyte transendothelial migration. Umbilical vein endothelial cells were grown to confluency on amnion connective tissue, and human monocytes were stimulated to cross the monolayer in response to the chemoattractants leukotriene B_4 or f-Met-Leu-Phe. The pattern and time course of monocyte migration were similar for the two chemotactic factors. In both cases, approximately 40–50% of the adherent monocytes extended single or multiple pseudopods into the apical endothelial surface. This indenting behavior was also observed in the absence of chemotactic factors. It was not affected by the medium (M199 or Gey's) or method of monocyte isolation. Neutrophils also displayed this behavior, but only about half as many neutrophils as monocytes indented the endothelial surface. The integrity of the endothelium remained intact as the monocytes traversed the monolayer. When the monocytes reached the basal surface of the endothelium, they frequently wedged themselves between the basal surface of the endothelium and its basal lamina. The monocytes then invaded the basal lamina and accumulated in the connective tissue. In response to both f-Met-Leu-Phe and leukotriene B_4, monocyte migration across the endothelium began as early as 10 minutes. The average rate of accumulation in the connective tissue peaked at 30 minutes; and by 60 minutes, 25–35% of the monocytes had traversed the monolayer. Approximately two to three times as many monocytes traversed the endothelium under conditions of chemotaxis as under conditions of chemokinesis or random migration. These studies provide the basis for understanding the process of monocyte migration out of the bloodstream and lay the foundation for the study of their differentiation into macrophages in the connective tissue. (Am J Pathol 1987, 127:157–167)

IN GENERAL, more neutrophils than monocytes initially accumulate at inflammatory lesions. However, within 24–48 hours, mononuclear cells usually predominate.[1-3] The kinetics of this cellular transition and the factors that regulate this progression are poorly understood. The process of white blood cell accumulation involves their migration across vascular endothelium, its underlying basement membrane, and the surrounding connective tissue. The route by which leukocytes traverse an endothelium remains controversial. Some studies have suggested that cells migrate through the endothelium, and other studies have suggested that cells traverse the intercellular space between endothelial cells.[4-11] Most studies of leukocyte transendothelial migration have focused on neutrophils.[4,5,12-14] The mechanism and time course of monocyte transendothelial migration remain largely unknown.

The effects of humoral mediators on endothelial–leukocyte interaction have been examined by a number of laboratories. Leukotriene B_4 (5 [5], 12 [R]-dihydroxy-6, 14-cis-8, 10-trans-eicosatetraenoic acid, LTB_4) has been shown to stimulate the transendothe-

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lial migration of human neutrophils *in vivo*[5,14,15] and *in vitro*.[16] However, the effect of this mediator on monocyte emigration remains to be elucidated. In this study, a human *in vitro* model of a vessel wall was produced by growing umbilical vein endothelial cells on amnion connective tissue. With this system, the direct effect of LTB_4 on the transendothelial migration of human monocytes was examined. The time sequence of this effect was determined and compared to that of f-Met-Leu-Phe, a synthetic analog of the major chemotactic factor produced by *Escherichia coli*.[17] The extensive cell–cell interaction between monocytes and endothelial cells is described.

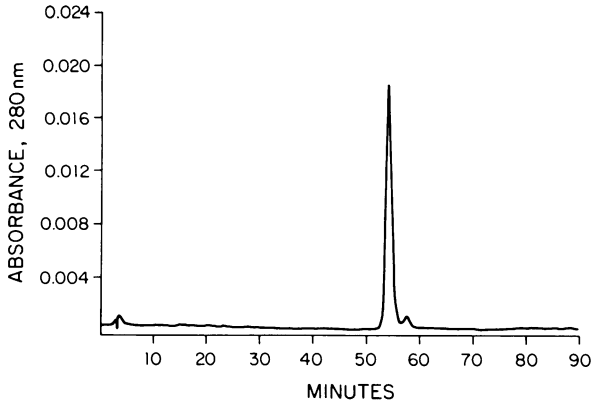

Figure 1—HPLC elution profile of purified LTB_4 used in these studies. Preparation and analysis are discussed in Materials and Methods.

Materials and Methods

Chemoattractants

Synthetic LTB_4 was obtained from Dr. W. Scott, Squibb Laboratories (Princeton, NJ) and from Bromol Laboratories (Philadelphia, Pa). LTB_4 obtained from Dr. Scott was further purified by high-pressure liquid chromatography (HPLC). Stock preparations were stored in ethanol at −20 C. The integrity of the solutions used in the biologic assays was assessed by HPLC on the day of the experiment. The material was chromatographed with the use of a reverse-phase C18 column (Supelco, Inc. Bellephonte, Pa) and eluted at 1 ml/min with methanol/0.01 M phosphoric acid (65/35, vol/vol, pH 5.0, with ammonium hydroxide). The column effluent was monitored for absorbance (280 nm). Recoveries (calculated from integrated ultraviolet absorbance) were routinely in excess of 75%, and purity was greater than 95%. A representative chromatogram of an aliquot containing 0.80 nmoles is shown in Figure 1.

The synthetic chemoattractant f-Met-Leu-Phe was obtained from Peninsula Laboratories.

Chemotactic Assay

The migration of monocytes in response to both chemoattractants was initially examined in a 48-well microassay apparatus (Neuroprobe, Bethesda, Md) according to the method of Falk et al.[18] Monocytes were stimulated to migrate across a 5-μ pore size polycarbonate filter (Nucleopore, Pleasanton, Calif) in response to 10^{-6}–10^{-9} M LTB_4 or f-Met-Leu-Phe. Each concentration was tested in replicates of three to eight as indicated in Tables 1 and 2. After a 60-minute incubation (98% humidity, 95% air–5% CO_2 atmosphere at 37 C), the nonmigrating cells were removed, and the cells that had traversed the filter were fixed and stained with Hemacolor (Harleco, Gibbstown, NJ). The cells in four random 100× fields (0.01-sq mm/field) were counted and averaged, and the standard error of the mean (SEM) was determined.

Preparation of Human Amnion

Human amnion was prepared according to a variation of the method of Foltz et al[19] and Furie et al.[20] The amnion which had been separated from the chorion laeve was stretched across the bottom of lexan cylinders and held in place with a Viton O-ring. The cylinders (inside diameter [I D], 6.2 mm) were washed in three changes of phosphate-buffered saline (PBS) containing penicillin (500 U/ml) and streptomycin (200 μg/ml). The monolayer of amnion epithelial cells that faced the outside of the cylinder was destroyed by incubating cylinders in 0.25 M NH_4OH (30 minutes, room temperature). The damaged epithelial cells were then removed by gentle abrasion of the amnion with a cotton-tipped swab. The cylinders were washed in three changes of PBS and stored at 4 C until used.

Table 1—Effect of LTB_4 on the Directed Migration of Human Monocytes*

LTB_4 concentration (M)	Cell number†
0‡	16.5 ± 1.6 (8)
10^{-6}	69.4 ± 2.3 (8)
10^{-7}	95.5 ± 3.3 (8)
10^{-8}	83.2 ± 3.0 (6)
10^{-9}	49.0 ± 1.7 (7)

*Monocytes suspended in Gey's (4×10^6/ml) were stimulated to traverse a polycarbonate filter for 60 minutes in response to various concentrations of LTB_4 according to the method of Falk et al.[18]

†Mean ± SEM number of cells which had accumulated in a 0.01-sq mm area. The number of replicate wells appear in parenthesis. The significance of the difference between control and experimental values was $P < 0.001$ for all concentrations of chemoattractant.

‡Gey's control.

Table 2—Effect of f-Met-Leu-Phe on the Directed Migration of Human Monocytes*

f-Met-Leu-Phe concentration (M)	Cell number†
0‡	22.0 ± 2.2 (3)
10^{-6}	43.1 ± 3.5 (4)
10^{-7}	87.9 ± 8.0 (4)
10^{-8}	88.1 ± 6.3 (4)
10^{-9}	43.6 ± 3.7 (3)

*Monocytes suspended in Gey's (2×10^6/ml) were stimulated to traverse a polycarbonate filter for 60 minutes in response to various concentrations of f-Met-Leu-Phe according to the method of Falk et al.[18]

†Mean ± SEM number of cells which had accumulated in a 0.01-sq mm area. The number of replicate wells appear in parenthesis. The significance of the difference between control and experimental values was $P < 0.001$ for all concentrations tested.

‡Gey's control.

Preparation of Endothelial Cell Cultures

Primary cultures of human umbilical vein endothelial cells were prepared by a modification of the method of Jaffe et al.[21] Endothelial cells were isolated in M199 (with Earle's salts, GIBCO, Grand Island, NY) containing 1 mg/ml collagenase (CLS, Cooper Biomedical,) at 37 C for 15 minutes. The endothelial cell suspension was removed, and the cord was washed with M199 for collection of the remaining endothelial cells. To reduce clumping and facilitate accurate counting of the endothelial cells, we added 0.01% trypsin for 10 minutes at 37 C. Cells were pelleted at 400*g* at 4 C for 10 minutes, washed, and resuspended at 1.5×10^5/ml in M199 containing 20% human serum (Flow Laboratories, McLean, Va) for plating on human amnion. Confluence of endothelial monolayers was confirmed by transendothelial electrical resistance measurements as well as silver staining according to Poole et al (Figure 2).[22]

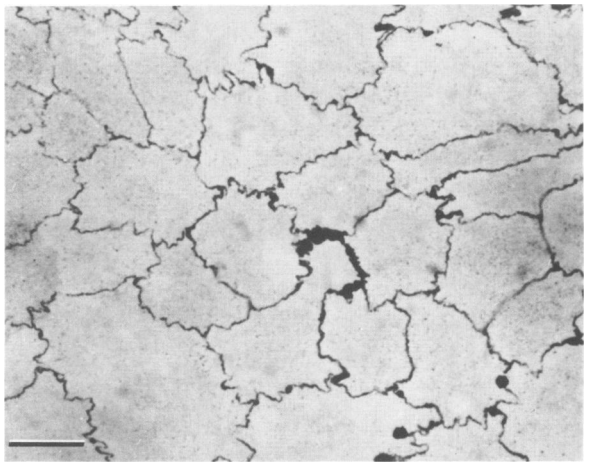

Figure 2—Light micrograph of a confluent monolayer of umbilical cord endothelial cells grown for 7 days on amnion connective tissue. The endothelium was stained with silver nitrate, and deposits of silver outline the borders of the endothelial cells. Bar = 19.2 μ.

Resistance Measurements

Transendothelial electrical resistance measurements were performed before all experiments. The cylinders were suspended in a modified Ussing chamber, and 10 μA of current was passed across the endothelial monolayers through Hg/HgCl electrodes connected to the chamber with 3M KCl-agar bridges. The voltage bridges were placed 1.0 mm above and below the monolayer. The voltage change was measured on a Keithley 191 digital multimeter (Keithley Instruments, Cleveland, Ohio), and Ohm's law was used for calculation of electrical resistance. The transendothelial electrical resistance of the monolayers was determined for each experiment by the use of the following formula: resistance of the endothelial monolayer = resistance of the (monolayer + amnion) − resistance of amnion alone. In these studies the resistance of the amnion alone averaged 15 Ohms × sq cm, and the resistance of the endothelial monolayers + the amnion averaged 23 Ohms × sq cm.

Preparation of Blood Cells

Blood from healthy donors was collected into sodium citrate containing ethylenediaminetetraacetic acid (EDTA; final concentrations, 0.38% and 0.5 mM, respectively). Monocytes were isolated either on gradients of Nycodenz Monocytes (Nycodenz; Nyegaard Diagnostica) or by differential adhesion.

Nycodenz

Anticoagulated cells were diluted with an equal volume of PBS containing 5 mM EDTA, then layered onto Ficoll-Hypaque (density, 1.077) and centrifuged at 400*g* for 45 minutes at 20 C. Cells from the mononuclear layer were collected and washed two times in PBS + 5 mM EDTA at 4 C. Platelets were removed by washing in autologous human serum according to Pawlowski.[23] Cells were washed twice in PBS + 5 mM EDTA, then were resuspended in calcium and magnesium-free Hanks' balanced salt solution (CMF Hanks') + 0.5 mM EDTA. Cells were layered onto 3 ml of Nycodenz and were centrifuged at 600*g* for 15 minutes at 22 C. Monocytes were collected, washed in CMF Hanks', and counted.

Differential Adhesion

Anticoagulated blood was centrifuged at 400*g* for 20 minutes at 20 C for separation of platelet-rich plasma from leukocytes and red blood cells (plasma spin). The layer of leukocytes was removed and di-

luted (2:1) with PBS containing 5 mM EDTA, then layered onto a cushion of Ficoll-Hypaque and centrifuged as described above. Mononuclear cells were washed twice in PBS + 5 mM EDTA, then washed in autologous serum.[23] Serum-washed cells were centrifuged at 400*g* for 10 minutes at 4 C, then washed twice in PBS + 5 mM EDTA and resuspended in M199 containing 20% autologous serum (M199-20). Autologous plasma generated in the plasma spin was centrifuged at 12,000*g* for 20 minutes for removal of the platelets. Platelet-poor plasma was then incubated with gelatin-coated plates, and a layer of fibronectin was deposited on the gelatin.[18] Coated plates were washed three times with PBS. Cells in M199-20 were added to the coated plates and were incubated for 20 minutes at 37 C. Lymphocytes, which do not adhere to fibronectin, were removed by washing with PBS. The adherent monocytes were released by incubating the cells for 20 minutes at 37 C in M199-20 diluted with an equal volume of PBS containing 10 mM EDTA. Cells were washed in PBS + 5 mM EDTA, then resuspended in CMF Hanks' for counting.

Just prior to the assay, cells isolated by either method were centrifuged at 400*g* for 10 minutes and resuspended in Gey's balanced salt solution containing 2% bovine serum albumin (Gey's, Hazelton Research products, Denver, Pa) or M199. Monocytes prepared by both methods were at least 95% pure as assessed by a positive reaction for nonspecific esterase,[25] morphologic study of Wright's stained cytospin preparations, and cell spreading on glass. Viability was greater than 98% as determined by trypan blue exclusion.

The granulocyte and red cell fraction was removed from the Ficoll-Hypaque gradient and diluted with an equal volume of 3% dextran T-500 in 0.85% sodium chloride. After gentle mixing, red cells were allowed to settle at unit gravity for 45 minutes. Neutrophils were collected from the supernatant fraction at 400*g* for 10 minutes at 4 C. Neutrophils were washed in CMF Hanks' and pelleted at 400*g* for 10 minutes. Contaminating red cells were eliminated by hypotonic lysis in 0.2% NaCl. Neutrophils were pelleted at 400*g*, and the cells were resuspended in CMF Hanks' for counting and viability determinations. Neutrophils prepared by this method were 98% pure with >98% viability.

Transendothelial Migration

Endothelial monolayers grown on the amnion connective tissue were washed in Gey's prior to each experiment. The lexan cylinders were suspended in 24-well tissue culture dishes (Falcon). Monocytes were isolated by differential adhesion, except where indicated. These cells were placed in the upper compartment (formed by the cylinder) and were stimulated to cross the monolayer in response to a chemoattractant placed in the lower compartment (formed by the tissue culture well). All migration studies were performed at 37 C in a 95% air–5% CO_2 atmosphere. At the end of the experiment the endothelial monolayers were washed in Gey's, fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3), postfixed in osmium tetroxide in 0.1 M phosphate buffer (pH 7.3), dehydrated, and embedded in Epon 812.

Quantitation of Leukocyte Migration

For each cylinder, three cross-sections (1 μ thick and approximately 4 mm in length) of the endothelial monolayer were cut at random intervals of greater than 30 μ, stained with toluidine blue, and examined by light microscopy. The cells that had adhered to and migrated across the monolayer were counted in each cross-section and were expressed as the average number of cells per millimeter endothelium per cylinder. Each experimental group represents the averaged data ± SEM from four cylinders. Statistical differences between groups were determined by the Student *t* test.

Results

Concentration of LTB_4 for Chemotaxis

A 48-well chemotactic filter assay was used to determine the concentration of LTB_4 which stimulated the maximum number of monocytes to undergo directed migration. Monocytes suspended in Gey's were stimulated to cross a polycarbonate filter in response to various concentrations of LTB_4 (10^{-6}–10^{-9} M). While all concentrations of LTB_4 tested significantly increased monocyte migration above control levels, the optimal monocyte response occurred with LTB_4 at 10^{-7} M. LTB_4 at 10^{-8} M was slightly less effective (Table 1). Similarly, all concentrations of f-Met-Leu-Phe significantly increased monocyte migration, with the maximal response occurring at 10^{-7} and 10^{-8} M (Table 2). These optimal concentrations remained the same regardless of the monocyte concentration used in the chemotactic assay (data not shown).

Growth of Endothelial Cells

Primary cultures of endothelial cells grown on the stromal surface of the amnion became confluent between 6 and 7 days after plating (Figure 2). Endothe-

lial cell cultures were determined to be free of contaminating cell types by immunofluorescent localization of Factor VIII antigen (not shown). Ultrastructural examination of cultured endothelial cells revealed areas of close contact between adjacent cells. By 7 days in culture, the confluent monolayer of endothelial cells formed a basal lamina (Figures 5 and 6) on the connective tissue surface.

The net transendothelial electrical resistance of monolayers was typically less than 20 Ohms × sq cm. Monolayers with a resistance of less than 3 Ohms × sq cm were generally found to be subconfluent and were not used in these studies.

Time Course of Monocyte Migration

The transendothelial migration of monocytes in response to both chemoattractants was measured as a function of time. Adherence to and migration across the endothelium were determined with the light microscope on 1 μ sections (Figure 3). In response to LTB_4 (Table 3), monocyte (1.8×10^6 cells/ml) adherence to the endothelium was observed by 10 minutes and reached maximal levels at 30 minutes. Monocyte migration across the endothelium began as early as 10 minutes, and the number of cells that had accumulated in the connective tissue increased approximately 3.5-fold by 30 minutes and 5.6-fold by 60 minutes. By 1 hour, the average rate of accumulation had begun to decline (1.0/min at 30 minutes versus 0.8/min at 60 minutes). At 60 minutes, approximately 37% of the monocytes placed in the upper chamber had traversed the monolayer.

Monocyte adherence to the endothelial cell surface occurred even in the absence of a LTB_4 (Table 3). Such adherence was comparable to that observed in the presence of the chemoattractant. Monocytes were also capable of transendothelial migration under conditions of random migration. The amount of emigration, however, was significantly less than that observed under conditions of chemotaxis.

Table 3—Time Course of Monocyte Transendothelial Migration in Response to LTB_4*

	Minutes	Adherence/mm†	Traversion/mm†
LTB_4			
	10	19.3 ± 1.7	8.6 ± 1.3
	30	23.1 ± 1.9	29.9 ± 1.6‡
	60	13.3 ± 1.2	48.5 ± 3.1
Gey's			
	30	19.2 ± 1.2	15.8 ± 2.8

*Monocytes (1.8×10^6/ml) were incubated under conditions of directed (Gey's in the upper compartment; 10^{-7} M LTB_4 in the lower compartment) LTB or random (Gey's in upper and lower compartments) migration at 37 C for the indicated times.

†The data represent the mean ± SEM (n = 4) number of cells which had adhered to or migrated across the monolayer per millimeter of endothelium (see Material and Methods).

‡The significance of the difference between monocyte accumulations under conditions of directed versus random migration was $P < 0.01$.

In response to 10^{-7} M f-Met-Leu-Phe (Table 4), monocytes at a concentration of 4×10^6 cells/ml had adhered to the monolayer and had begun to migrate across by 10 minutes. Monocytes continued to accumulate in the connective tissue with time, and by 30 minutes their numbers had increased 3.8-fold. As seen with LTB_4, the average rate of accumulation peaked at 30 minutes and by 60 minutes was beginning to decline (1.6/min at 30 minutes versus 1.3/min at 60 minutes). Like LTB_4, a gradient of f-Met-Leu-Phe stimulated nearly twice as many monocytes to traverse the monolayer as buffer alone. By 60 minutes approximately 26% of the suspended cells had accumulated in the connective tissue.

Studies of monocyte migration across endothelia have been performed at various cell concentrations.

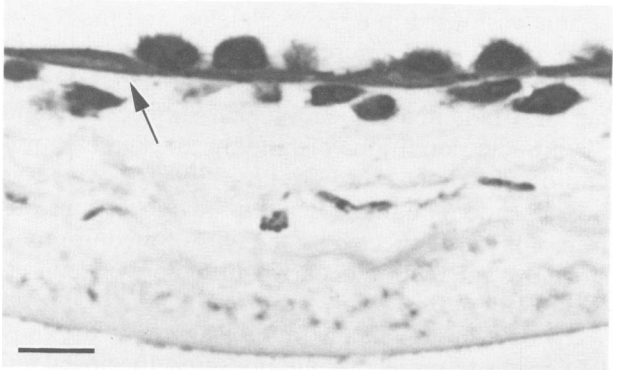

Figure 3—Light micrograph of the transendothelial migration of human monocytes for 60 minutes in response to 10^{-7} M LTB_4. Monocytes can be seen adhering to the thin monolayer of endothelial cells (*arrow*) and in the underlying connective tissue. Bar = 12.5 μ.

Table 4—Time Course of Monocyte Transendothelial Migration in Response to f-Met-Leu-Phe*

	Minutes	Adherence/mm†	Traversion/mm†
f-Met-Leu-Phe			
	10	25.5 ± 2.1	12.3 ± 0.4
	30	27.2 ± 1.6	46.8 ± 3.1‡
	60	24.8 ± 1.3	76.8 ± 2.0
Gey's			
	30	46.9 ± 3.1	26.8 ± 1.5

*Monocytes (4×10^6/ml) were incubated under conditions of directed (Gey's in the upper compartment; 10^{-7} M f-Met-Leu-Phe in the lower compartment) or random (Gey's in both compartments) migration at 37 C for the indicated times.

†The data represent the mean ± SEM (n = 4) number of cells which had adhered to or migrated across the monolayer per millimeter of endothelium (see Materials and Methods).

‡The significance of the difference between monocyte accumulations under conditions of directed versus random migration was $P < 0.001$.

The data presented here show that although the absolute number of accumulated cells increased when the starting concentration of monocytes was increased, the time sequence and the basic pattern of migration in response to both f-Met-Leu-Phe and LTB_4 remained similar (Tables 3 and 4).

Chemokinesis Versus Chemotaxis

To confirm that the effect of LTB_4 was truly chemotactic and not a reflection of its ability to increase random migration, monocyte transendothelial migration in response to LTB_4 was tested in "checkerboard" assays (Table 5). Various concentrations of LTB_4 were added either to both the upper and lower compartments or to the lower compartment alone. At all concentrations tested, monocyte adherence to and migration across the endothelium under chemokinetic conditions was not significantly greater than that observed in the presence of buffer. Monocyte transendothelial migration in response to a gradient of LTB_4 was dose-dependent, with $10^{-7} > 10^{-8} > 10^{-9}$ M. Maximal monocyte migration in response to a chemotactic gradient of 10^{-7} M LTB_4 was 3.3 times greater than that observed under conditions of chemokinesis (10^{-7} M LTB_4/10^{-7} M LTB_4) and 4.3 times greater than that observed under conditions of random migration. Similar findings were also observed in response to f-Met-Leu-Phe.

Monocyte–Endothelial Cell Interaction

Monocytes readily adhered to the endothelial surface and were observed in various stages of flattening or spreading along the monolayer at all time intervals examined (10–60 minutes; Figure 3). Ultrastructural examination of the interaction between the two cell types revealed that some of the adherent monocytes projected single or multiple pseudopods of varying length into depressions of the endothelial cell surface (Figures 4 and 5). The degree of indentation varied from minor intrusions to deep depressions, which sometimes brought the apical and basal endothelial membranes into close apposition (Figure 5). These indentations were not restricted to any particular region of the endothelial cell; however, the deepest indentations seemed to occur along the thinner regions of the cell. Although a given monocyte tended to interact with a single endothelial cell, many examples were observed in which a monocyte straddled an intercellular junction and sent pseudopods into indentations in adjacent cells (Figure 5).

To further characterize this indenting behavior, we examined the frequency of monocyte indentations under various experimental conditions. At least one hundred adherent monocytes were examined per group. In response to a chemotactic gradient of 10^{-7} M f-Met-Leu-Phe or LTB_4, approximately 40% of the adherent monocytes indented the endothelial surface with single or multiple pseudopods (Table 6). Under conditions of random migration, slightly fewer adherent cells exhibited this behavior. Therefore, the interaction does not seem to be caused specifically by the presence of a chemoattractant. This behavior was not a function of the method of monocyte isolation. In addition to cells isolated by differential adhesion, monocytes isolated on gradients of Nycodenz were also found to indent the endothelial surface under conditions of chemotaxis, chemokinesis, and random migration (Table 5). Furthermore, mononuclear cells (monocytes, lymphocytes, and platelets) obtained from Ficoll–Hypaque gradients also showed this behavior. This isolation procedure eliminated the serum wash used to remove platelets and the Nycodenz gradient or differential adhesion which removed lymphocytes. Mononuclear cells isolated in this manner also indented the endothelial surface 51% and 39%, under conditions of random and directed migration, respectively. In addition, this behavior was not dependent on the presence of bovine serum albumin, nor was it affected by the type of medium used since this interaction occurred in the presence of either M199 or Gey's.

To determine whether monocytes were the only leukocytes to project pseudopods into the apical endothelial surface, the interaction of adherent neutrophils with the endothelial surface was also examined. Monocytes and neutrophils isolated from the same

Table 5—Monocyte Transendothelial Migration Under Conditions of Chemotaxis, Chemokinesis, and Random Migration

Compartment*				
Upper	Lower	Adherence†	Traversion†	Indent‡
Gey's	10^{-7}	9.9 ± 1.8	21.4 ± 2.5§	48
Gey's	10^{-8}	9.2 ± 1.6	13.7 ± 1.4§	ND
Gey's	10^{-9}	15.1 ± 1.8	7.6 ± 0.7	ND
10^{-7}	10^{-7}	11.9 ± 1.9	6.4 ± 0.9	50
10^{-8}	10^{-8}	8.9 ± 1.7	2.9 ± 0.2	ND
10^{-9}	10^{-9}	10.5 ± 1.6	3.7 ± 0.4	ND
Gey's	Gey's	10.5 ± 1.4	5.0 ± 1.2	49

*Monocytes ($1.8 = 10^6$/ml) isolated by Nycodenz were incubated under conditions of chemotaxis (Gey's in upper compartment, LTB_4 in the lower compartment), chemokinesis (LTB_4 in both compartments), or random migration (Gey's in both compartments) at 37 for 30 minutes.

†The data represent the mean ± SEM (n = 4) number of cells which had adhered to or migrated across the monolayer per millimeter of endothelium (see Materials and Methods).

‡The data represent the percent of at least 100 adherent monocytes which indented the endothelial surface with single or multiple pseudopods as observed by transmission electron microscopy on 60–80-nm sections.

§The significance of the difference between monocyte accumulations under conditions of chemotaxis versus random migration was $P < 0.005$.

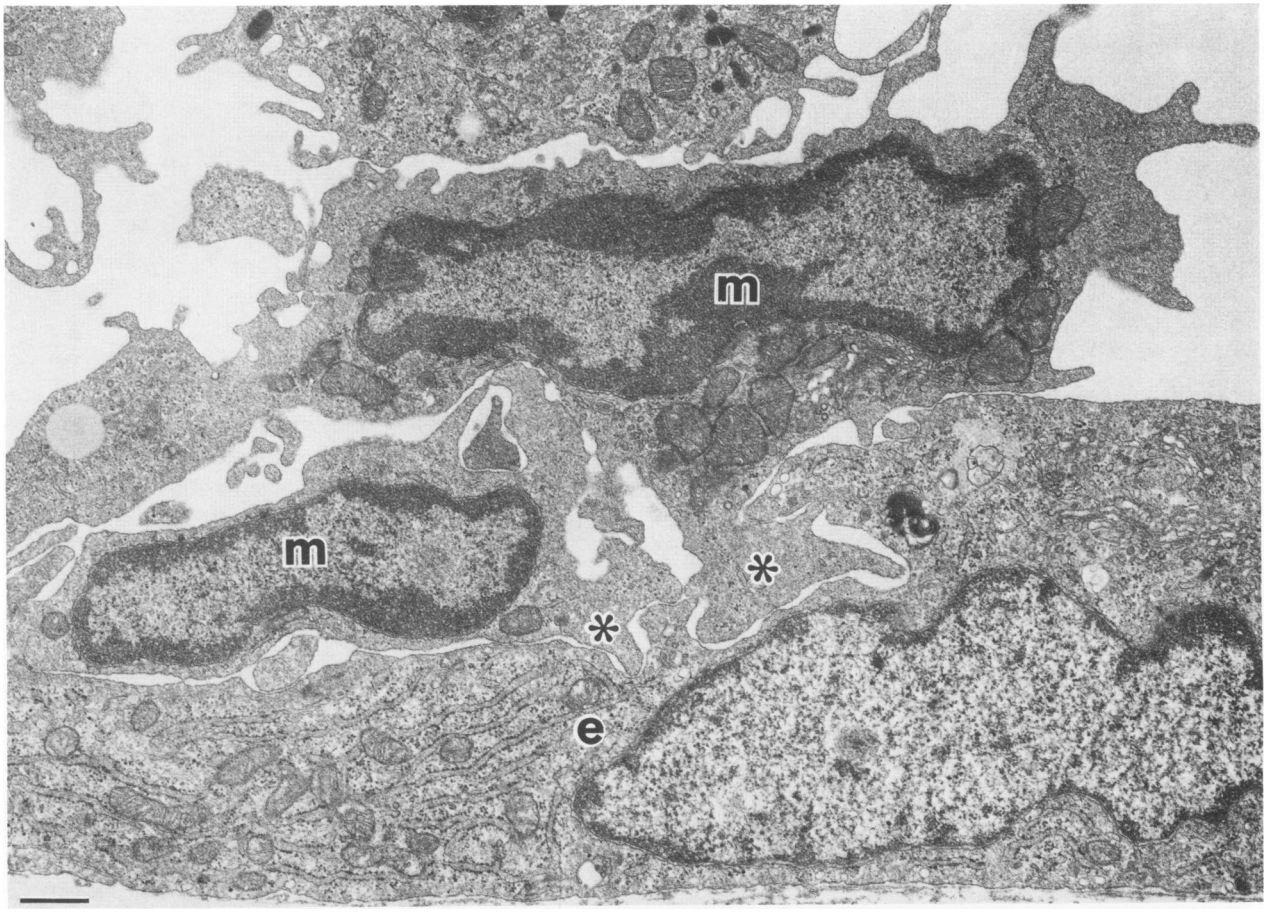

Figure 4—Transmission electron micrograph of two adherent monocytes (*m*) in the process of transendothelial migration in response to 10^{-7} M LTB_4. Both monocytes protrude pseudopods (*) into indentations in the apical surface of the endothelial cell (*e*). Bar = 0.71 μ.

donor were stimulated to traverse the endothelium for 30 minutes. The indenting behavior of these two leukocytes was examined at 10 minutes, during the initial stages of the interaction, and at 30 minutes, when adhesion for both cell types was at a maximum (Table 7). The percentage of monocytes and neutrophils with pseudopods that indented the endothelium increased nearly twofold between 10 and 30 minutes. However, at 30 minutes twice as many monocytes as neutrophils showed this behavior. In addition, at 30 minutes, monocytes were more likely to indent the endothelium with multiple pseudopods than were neutrophils.

While adherent monocytes frequently projected pseudopods into the apical endothelial surface (40–50% of the adherent cells), only approximately 1% of the adherent cells projected pseudopods into the junctional region between cells. Although it was not possible to tell whether the monocytes traveled through and/or between the endothelial cells, it was obvious that the process of monocyte migration across the endothelium did not disrupt the integrity of the monolayer. This conclusion was based on both light- and electron-microscopic observations. Light-microscopic examination of 1-μ sections from 19 experiments (total of 640 monolayers) revealed an intact endothelium during and after the process of monocyte emigration. Ultrastructural examination of thin sections cut at a minimum of five levels (separated by greater than 30 μ) from a total of 16 monolayers from 8 different experiments showed monocytes forming close contact with endothelial cells at sites of invasion (Figure 6). As seen by both light and electron microscopy, the endothelium appeared to reseal after monocyte traversion. After crossing the endothelium, monocytes initially projected pseudopods or wedged themselves between the endothelial cell and the basal lamina (Figures 5 and 6). The cells subsequently penetrated the basal lamina and entered the underlying connective tissue (Figures 3 and 5). The basal lamina produced by these endothelial cells appeared to form a relatively formidable barrier, because monocytes became constricted as they squeezed through it (Figure 5).

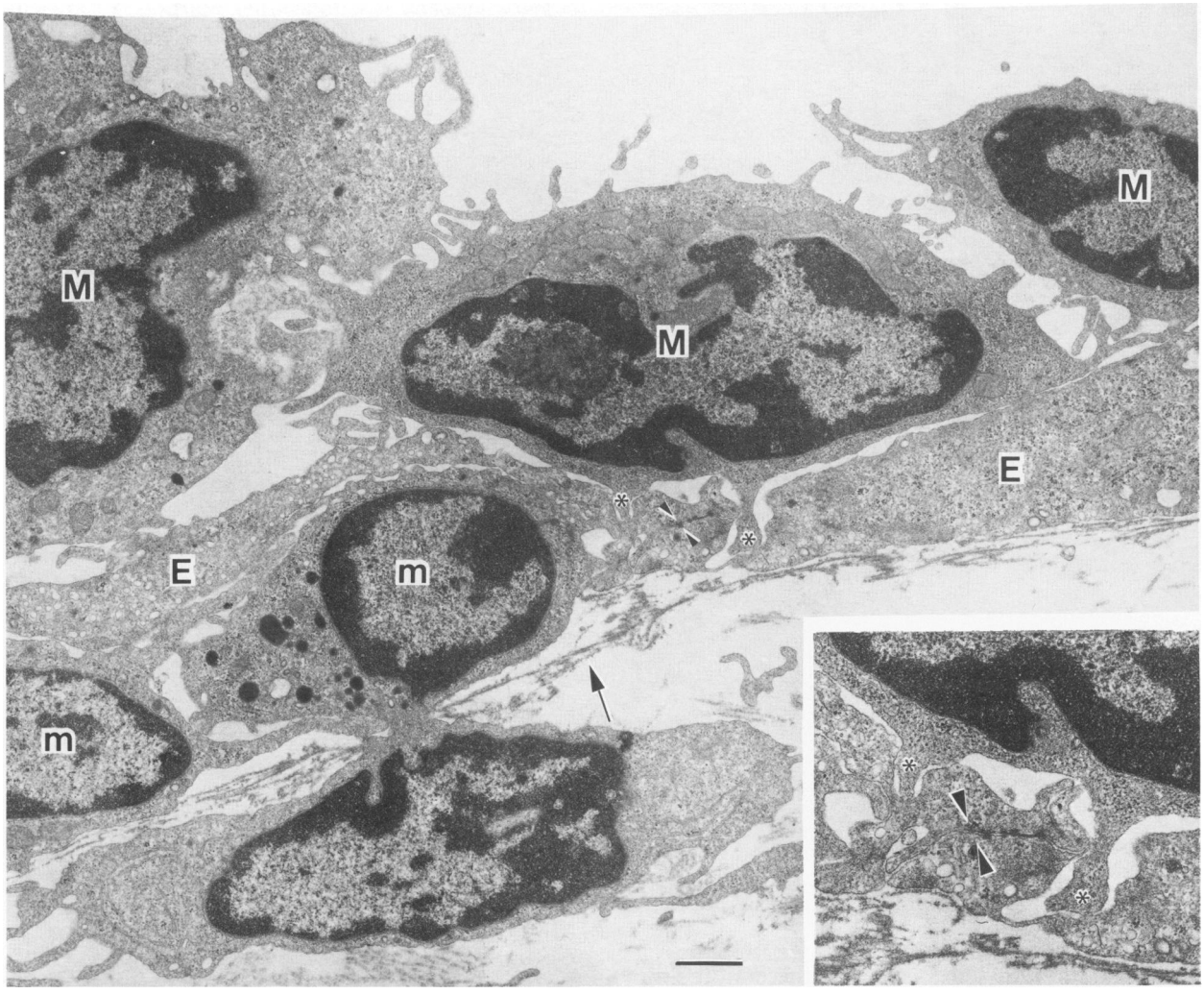

Figure 5—Transmission electron micrograph of the transendothelial migration of monocytes for 30 minutes in transendothelial migration of monocytes for 30 minutes in response to 10^{-7} LTB_4. Three monocytes (*M*) are adherent to the surface of two adjacent endothelial cells (*E*) whose junction is indicated by the *arrowheads*. The monocyte in the center is projecting a pseudopod (*) into an indentation in each endothelial cell. Two monocytes (*m*) have traversed the endothelial monolayer. One of these monocytes is in the process of squeezing itself through an aperture in the basal lamina (*arrow*). Bar = 1 μ. **Inset**—Higher magnification of α monocyte projecting pseudopods (*) into the apical surface of adjacent endothelial cells. The endothelial intercellular junction is indicated by the *arrowheads*. Bar = 0.91 μ.

Table 6—Monocyte Indentation of Apical Endothelial Membrane*

	Single†	Multiple†	Total†
f-Met-Leu-Phe	27%	14%	41%
LTB_4	22%	20%	42%
Gey's	13%	23%	36%

*Monocytes incubated under conditions of directed (Gey's in the upper compartment; 10^{-7} M f-Met-Leu-Phe or LTB_4 in the lower compartment) or random (Gey's in both compartments) migration for 30 minutes at 37 C.

†The data represent the percent of at least 100 adherent monocytes which indented the endothelial cell surface with single or multiple pseudopods as observed by transmission electron microscopy on 60–80-nm sections.

Table 7—Effect of Time on Neutrophil and Monocyte Migration and Indentation of the Endothelial Surface*

	Minutes	Single†	Multiple†	Total†	Traversion‡
Neutrophils					
	10	12%	4%	16%	62
	30	21%	4%	25%	240
Monocytes					
	10	22%	7%	29%	1
	30	24%	27%	51%	17

*Neutrophils or monocytes (1.8×10^6/ml) isolated from the same donor were incubated under conditions of directed migration (M199 in the upper compartment; 10^{-7} M LTB_4 in the lower compartment) at 37 C for the indicated times.

†The data represent the percent of at least 100 adherent leukocytes which indented the endothelial cell surface with single or multiple pseudopods as observed by transmission electron microscopy on 60–80-nm sections.

‡The data represent the number of cells which had migrated across the monolayer per millimeter endothelium.

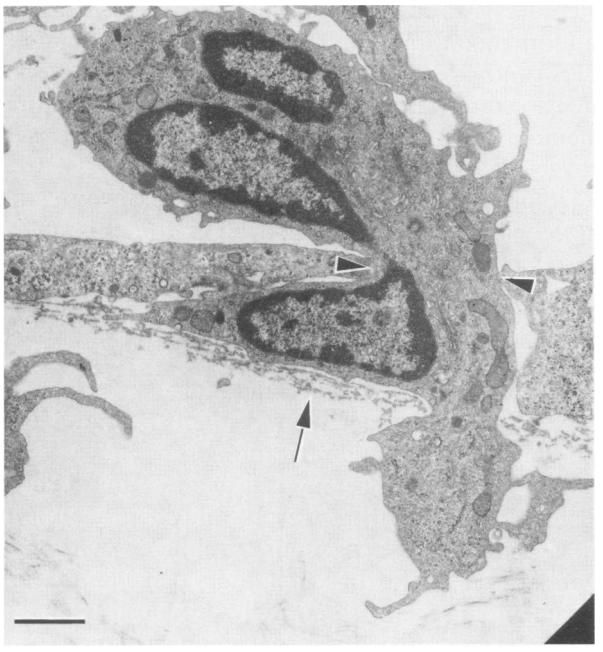

Figure 6—Transmission electron micrograph of a monocyte crossing the endothelial monolayer in response to 10^{-7} M LTB_4. Close contacts are made between the migrating monocyte and the endothelial cells (*arrowheads*). The monocyte has sent one pseudopod between the basal surface of the endothelium and its basal lamina (*arrow*). A second pseudopod has penetrated the basal lamina and has entered the connective tissue. Bar = 1.1 μ.

Discussion

The process of monocyte migration across vascular endothelium in response to LTB_4 was examined in a human *in vitro* system. The model, based on the work of Foltz et al[19] and Furie et al,[20] simulates a human vessel wall and is composed of primary umbilical vein endothelial cells and amnion connective tissue. Unlike some endothelial cell lines, primary umbilical vein endothelial cultures remain responsive to various inflammatory stimuli. These cells retain the capacity to produce prostacyclin in response to histamine,[1] thrombin,[12,27] and leukotriene C_4 and D_4.[12,28] They also become more adhesive for neutrophils and monocytes in response to interleukin-1.[29] The amnion connective tissue provides a natural autologous substrate for endothelial cell growth. The cells adhere well to the connective tissue, produce a basement membrane, and develop a transendothelial electrical resistance. In addition, the design of the system enables one to determine the confluency and permeability of the endothelial monolayer prior to each experiment, control the fluid environment above and below the monolayer, and simultaneously examine leukocyte adherence and transendothelial migration.

Because the method of monocyte isolation has the potential to influence the behavior of the cells, monocytes have been isolated either by differential adhesion or Nycodenz gradients in these experiments. Both methods generated monocyte populations of approximately 95% purity. Monocytes isolated by differential adhesion exhibited greater adherence to and migration across the endothelium than monocytes isolated by Nycodenz gradients (Table 3 versus Table 5). This is particularly noticeable under conditions of random migration. However, the basic behavior of monocytes prepared by either method is the same. Regardless of the isolation procedure, monocytes readily adhered to the endothelium, indented its surface, and were stimulated to traverse the endothelial monolayer by LTB_4 and f-Met-Leu-Phe.

Several groups have recently reported on the adherence of monocytes[29-31] and monocytelike cells[29,32] to endothelial cells cultured on plastic substrates. There is general agreement in the literature that even in the presence of buffer alone, there is a strong attraction between monocytes (regardless of isolation procedure) and endothelial cells. Monocyte adherence to endothelium is greater than that observed with either neutrophils[30,31] or lymphocytes.[30] While monocyte adherence to endothelial monolayers is greater than that of neutrophils, monocyte accumulation in the connective tissue is slower (Table 7).[16] The reasons for this difference in the rates of accumulation are not clear but may relate to the manner in which monocytes and neutrophils interact with the endothelial cell surface. In light of these observations, it was of interest to examine the interaction of monocytes with endothelium by electron microscopy to determine whether there was something unique about the physical interaction between these two cell types. These studies have shown that extensive contacts were made between monocytes and endothelial cells resulting in endothelial surface indentations. The number of monocytes exhibiting this behavior increased with time of incubation and was not affected by the method of isolation, carrier medium, or presence or location of the chemoattractant. Monocytes were twice as likely to show this behavior as neutrophils and were more likely to indent the surface with multiple, rather than single, pseudopods. This behavior appears to be a unique interaction between leukocytes, because leukocytes do not indent the apical surface of kidney epithelial cells (Madin-Darby canine kidney epithelial cells, MDCK; unpublished results). This indenting behavior has been observed for both monocytes and neutrophils *in vivo* and does not seem to be an artifact of the *in vitro* system. Indentation of the surface of aortic endothelial cells by monocytes has been demonstrated in atherosclerotic lesions of the pig.[6] Similar behavior has been reported for

neutrophils migrating across small venules of the rat mesentery[7] and across postcapillary venules of the cat cerebral meninges.[4]

Monocytes were frequently observed to project pseudopods into indentations of the endothelial surface and, less often, into the junctional region between endothelial cells, suggesting two possible routes of transendothelial migration. The controversy between transcellular versus intercellular leukocyte migration has not yet been resolved. *In vivo* studies have provided evidence for both pathways.[4-11] The mechanisms of transendothelial migration and the reasons for choosing one pathway versus another remain to be determined. Regardless of the route taken, the process of monocyte migration does not seem to be disruptive to the endothelium. Close contacts were observed between endothelial cells and migrating monocytes, and resealing of the endothelium occurred after monocyte emigration. Similar results have been obtained *in vivo*[15] and in studies of neutrophil migration across endothelium *in vitro* performed by our laboratory (unpublished results). Monocytes, like neutrophils,[7,34] initially tend to wedge themselves or send pseudopods between the endothelium and the basal lamina and then squeeze through perforations in the basal lamina. Whether monocytes or neutrophils traverse the basal lamina by mechanical force, enzymatic digestion, or some other process remains to be determined.

LTB_4 has been shown to be a potent chemoattractant for neutrophils both *in vivo*[5,13-15] and *in vitro*.[13,16,35,36] LTB_4 has also been found to stimulate monocyte migration in an agarose microdroplet assay[37] and in the 48-well chemotactic assay presented in this study. However, whether this inflammatory mediator can directly stimulate monocyte migration across endothelial cells had not previously been examined. The present *in vitro* study demonstrates that physiologic concentrations of LTB_4 directly stimulate monocyte migration across vascular endothelium within a time frame similar to that observed for the synthetic chemoattractant f-Met-Leu-Phe. In both cases, monocyte accumulation began as early as 10 minutes and increased over the 60 minutes of incubation. The *in vitro* rate of neutrophil accumulation in the connective tissue is faster than that observed for monocytes.[16] This is consistent with the rates of accumulation for monocyte and neutrophils *in vivo*,[1] where significant monocyte infiltration into acute bacterial lesions was observed by 1 hour, even though 95% of the entering leukocytes were neutrophils.

Approximately two to three times as many monocytes traversed the endothelium under conditions of chemotaxis as under conditions of chemokinesis or random migration. This response was similar for both LTB_4 and f-Met-Leu-Phe, indicating that the chemoattractants function as chemotactic rather than chemokinetic agents when stimulating monocytes to traverse an endothelial monolayer.

Despite the fact that fewer monocytes traversed the endothelium under conditions of random migration, there was still a significant amount of monocyte adherence to and migration across the endothelium. *In vivo,* monocytes, like neutrophils, are thought to marginate along the blood vessel wall.[38] The monocytes ultimately leave the circulation to form macrophages throughout the connective tissues of the body. The random adherence and migration of monocytes seen in these experiments may reflect these physiologic events. Why monocytes traverse the endothelium in the absence of an exogenous stimulus is unknown. One cause of this intrinsic migratory behavior may be the chemoattractants reportedly produced by endothelial cells.[39,40] In contrast, the presence of the exogenous chemoattractants (LTB_4 and f-Met-Leu-Phe) in our system may simulate an inflammatory state in which one sees increased numbers of migrating monocytes.

The results presented in this paper provide some insight into the process of monocyte transendothelial migration. The flexibility of the model system also permits the study of the effects of other inflammatory mediators on this process. In addition, once monocytes have crossed the endothelial monolayer, they accumulate in the connective tissue, where they may differentiate into macrophages. This system also enables one to examine the monocyte/macrophage transformation and the factors which regulate it.

References

1. Issekutz T, Issekutz A, Movat H: The *in vivo* quantitation and kinetics of monocyte migration into acute inflammatory tissue. Am J Pathol 1981, 103:47–55
2. Paz RA, Spector WG: The mononuclear cell response to injury. J Pathol Bacteriol 1962, 84:85–103
3. Ryan GB, Majno G: Leukocyte infiltration, Inflammation. Upjohn Co., Kalamazoo, Mich, 1977, p. 24
4. Faustmann PM, Dermeitzel R: Extravasation of polymorphonuclear leukocytes from the cerebral microvasculature. Cell Tissue Res 1985, 242:399–407
5. Thureson-Klein A, Hedqvist P, Lindbom L: Ultrastructure of polymorphonuclear leukocytes in postcapillary venules after exposure to leukotriene B_4 *in vivo.* Acta Physiol Scand 1984, 122:221–224
6. Gerrity R: The role of the monocyte in atherogenesis: I. Transition of blood-borne monocytes into foam cells in fatty lesions. Am J Pathol 1981, 103:181–190
7. Marchesi VT, Florey HW: Electron microscopic observations on the emigration of leucocytes. J Exp Physiol 1960, 45:343–348
8. Cho Y, De Bruyn PPH: Transcellular migration of

lymphocytes through the walls of the smooth-surfaced squamous endothelial venules in the lymph node. Evidence for the direct entry of lymphocytes into the blood circulation of the lymph node. J Ultrastruct Res 1981, 74:259–266
9. De Bruyn PPH, Michelson S, Thomas TB: Migration of blood cells of the marrow through the sinusoidal wall. J Morphol 1971, 144:417–437
10. Schwartz C, Sprague E, Kelley J, Valente A, Suenram CA: Aortic intimal monocyte recruitment in the normo and hypercholesterolemic baboon (Papio cynocephalus). Virchows Arch [Pathol Anat] 1985, 405:175–191
11. Marchesi V: The site of leukocyte emigration during inflammation. J Exp Physiol 1961, 46:115–118
12. Cramer EB, Pologe L, Pawlowski N, Cohn ZA, Scott WA: Leukotriene C_4 promotes prostacyclin synthesis by human endothelial cells. Proc Natl Acad Sci USA 1983, 80:4109–4113
13. Lewis RA, Goetzl E, Drazen J, Soter N, Austen KF, Corey EJ: Functional characterization of synthetic leukotriene B_4 and its stereoisomers. J Exp Med 1981, 154:1243–1248
14. Lindbom L, Hedqvist P, Dahlen SE, Lindgren JA, Arfors KE: Leukotriene B_4 induces extravasation and migration of polymorphonuclear leukocytes *in vivo*. Acta Physiol Scand 1982, 116:105–108
15. Lewis RE, Granger H: Neutrophil-dependent mediation of microvascular permeability. Fed Proc 1986, 45:109–113
16. Migliorisi G, Pawlowski N, Scott WA, Fulop A, Cramer E: Effect of leukotriene (LT)B_4 on the transendothelial migration of human monocytes and neutrophils (PMN's) (Abstr). Fed Proc 1984, 43:845
17. Marasco WA, Phan SH, Krutsch H, Showell HJ, Feltner DE, Nairn R, Beckner EL, Ward PA: Purification and identification of formyl-methionyl-leucyl-phenylalanine as the major peptide neutrophil chemotactic factor produced by *Escherichia coli*. J Biol Chem 1984, 259:5430–5439
18. Falk W, Goodwin RH, Leonard E: A 48-well microchemotaxis assembly for rapid and accurate measurement of leukocyte migration. J Immunol Methods 1980, 33:239–247
19. Foltz C, Russo R, Siegal G, Terranova V, Liotta L: Interactions of tumor cells with whole basement membrane in the presence or absence of endothelium, Interaction of Platelets and Tumor Cells. New York, Alan R. Liss, 1982, pp. 353–371
20. Furie M, Cramer E, Naprstek B, Silverstein S: Cultured endothelial cell monolayers that restrict the transendothelial passage of macromolecules and electrical current. J Cell Biol 1984, 98:1033–1041
21. Jaffe EA, Nachman RL, Becker CG, Minick CR: Culture of human endothelial cells derived from umbilical veins: Identification by morphologic and immunologic criteria. J Clin Invest 1973, 52:2745–2756
22. Poole JCF, Sanders AG, Florey HW: The regeneration of aortic endothelium. J Pathol Bacteriol 1958, 75:133–143
23. Pawlowski N, Kaplan G, Hamill A, Chon ZA, Scott WA: Arachidonic acid metabolism by human monocytes: Studies with platelet-depleted cultures. J Exp Med 1983, 158:393–412
24. Bevilacqua M, Amrani D, Mosesson M, Bianco C: Receptors for cold-insoluble globulin (plasma fibronectin) on human monocytes. J Exp Med 1981, 153:42–60
25. Li CY, Lam KW, Yam LT: Esterases in human leukocytes. J Histochem Cytochem 1973, 21:1–12
26. Baenziger NL, Forgerty F, Mertz LF, Chernuta LF: Regulation of histamine-mediated prostacyclin synthesis in cultured human vascular endothelial cells. Cell 1981, 24:915–923
27. Weksler BB, Ley C, Jaffe EA: Stimulation of endothelial cell prostacyclin production by thrombin, trypsin and ionophore A23187. J Clin Invest 1978, 62:923–930
28. Pologe L, Cramer EB, Pawlowski N, Abraham E, Cohn Z, Scott W: Stimulation of human endothelial cell prostacyclin synthesis by select leukotrienes. J Exp Med 1984, 160:1043–1053
29. Bevilacqua M, Pober J, Wheeler ME, Cotran R, Gimbrone M: Interleukin-1 activation of vascular endothelium: Effects on procoagulant activity and leukocyte adhesion. Am J Pathol 1985, 121:393–403
30. Pawlowski N, Abraham E, Pontier S, Scott WA, Cohn Z: Human monocyte-endothelial cell interaction in vitro. Proc Natl Acad Sci USA 1985, 82:8208–8212
31. Wallis W, Beatty P, Ochs H, Harlan J: Human monocyte adherence to cultured vascular endothelium. Monoclonal antibody-defined mechanisms. J Immunol 1985, 135:2323–2330
32. DiCorletto P, de la Motte C: Characterization of the adhesion of the human monocyte cell line U937 to cultured endothelial cells. J Clin Invest 1985, 75:1153–1161
33. Marchesi VT: Some electron microscopic observations on interactions between leukocytes, platelets, and endothelial cells in acute inflammation. Ann NY Acad Sci 1964, 116:774–788
34. Cramer EB, Migliorisi G, Pologe L, Abrahams E, Pawlowski N, Cohn Z, Scott WA: Effect of leukotrienes on endothelium and the transendothelial migration of neutrophils. J Allergy Clin Immunol 1984, 74:386–390
35. Goetzl EJ, Pickett WC: Novel structural determinants of the human neutrophil chemotactic activity of leukotriene B_4. J Exp Med 1981, 153:482–487
36. Palmblad J, Malmsted C, Uden A-M, Radmark O, Samuelsson B: Leukotriene B_4 is a potent and stereospecific stimulator of neutrophil chemotaxis and adherence. Blood 1981, 58:658–662
37. Smith MJH, Ford-Hutchinson AW, Bray MA: Leukotriene B_4: A potential mediator of inflammation. J Pharm Pharmacol 1980, 32:517–518
38. van Furth R, Diesselhoff-den Dulk MMC, Sluiter W, van Dissel JT: New perspectives on the kinetics of mononuclear phagocytes, Mononuclear Phagocytes. Boston, Martinus Nijhoff Publishers, 1985, pp. 201–208
39. Mercandetti A, Lane T, Colmerauer MEM: Cultured human endothelial cells elaborate neutrophil chemoattractants. J Lab Clin Med 1984, 104:370–380
40. Quinn M, Parthasarathy S, Steinberg D: Endothelial cell-derived chemotactic activity for mouse peritoneal macrophages and the effect of modified forms of low density lipoprotein. Proc Natl Acad Sci USA 1985, 82:5949–5953

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