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Characterization of transendothelial chemotaxis of T lymphocytes

Stephen J. Roth [a,d], Michelle Woldemar Carr [b,d], Shayla S. Rose [d], Timothy A. Springer [b,c,d,*]

[a] *Department of Cardiology, Children's Hospital, Boston, MA, USA*
[b] *Committee on Immunology, Harvard Medical School, Boston, MA, USA*
[c] *Department of Pathology, Harvard Medical School, Boston, MA, USA*
[d] *The Center for Blood Research, Boston, MA, USA*

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Abstract

We have adapted a chemotaxis assay using human umbilical vein endothelial cell (HUVEC) monolayers on microporous membranes for studying lymphocyte transendothelial chemotaxis in vitro. Supernatants of peripheral blood mononuclear cells stimulated with phytohemagglutinin (PHA) were identified as an excellent source of lymphocyte chemoattractant activity. The activity in PHA supernatant typically caused 2–6% of peripheral blood lymphocytes (PBL) to transmigrate compared to 0.1–0.3% to media control. Checkerboard analysis demonstrated that transmigration was directional and not attributable to random locomotion. Purified T lymphocytes also underwent transendothelial chemotaxis to PHA supernatant. Using monoclonal antibodies to several human adhesion receptors, we found that the interaction between LFA-1 and ICAM-1/ICAM-2 was more important for transendothelial lymphocyte chemotaxis than the interaction between VLA-4 and VCAM-1. A monoclonal antibody to the β_1 integrin subunit inhibited chemotaxis more than antibodies to the VLA α_2, α_3, α_4, or α_5 subunits. The transendothelial assay was used to guide purification of the lymphocyte chemoattractant activity, which we reported previously to be monocyte chemoattractant protein-1 (MCP-1) (Carr et al., Proc. Natl. Acad. Sci. USA (1994) 91, 3652). The adhesion molecules required for chemotaxis to MCP-1 were similar to those with PHA supernatant. The use of HUVEC in the assay enhances the signal-to-background ratio of chemotaxis and provides a model that is physiologically relevant to lymphocyte emigration from the bloodstream into sites of inflammation.

Keywords: Chemotaxis; T lymphocyte; Transendothelial

1. Introduction

The targeted transmigration of leukocytes from blood into extravascular tissue is an important component of both immune surveillance and the inflammatory response. For monocytes and neutrophils, transendothelial migration appears to occur in a sequential, three-step process which depends upon adhesive and chemoattractive signals localized to mi-

Abbreviations: ECM, extracellular matrix; HUVEC, human umbilical vein endothelial cell; ICAM-1, -2, intercellular adhesion molecule-1 and -2; LFA-1, lymphocyte function-associated antigen-1; MCP-1, monocyte chemoattractant protein-1; PECAM-1, platelet endothelial cell adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; VLA, very late antigen.

* Corresponding author. At: The Center for Blood Research, 200 Longwood Avenue, Boston, MA 02115, USA.

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crovascular endothelium of the inflamed site [2,3]. In the initial step, monocytes and neutrophils roll along the endothelium through transient attachments between selectin molecules and their carbohydrate ligands. While briefly tethered to endothelium, rolling cells are exposed to specific activating molecules called chemoattractants on or near the endothelial surface. The interaction of chemoattractants such as IL-8 and *N*-formyl-Met-Leu-Phe (fMLP) with their receptors on neutrophils and monocytes activates several processes, including enhanced adhesiveness of leukocyte integrin molecules [4–6]. The activation of integrin adhesiveness leads to the firm attachment of tethered leukocytes on endothelium by integrin-immunoglobulin family adhesion molecule interactions, the third step of the process. Once arrested on the endothelial surface, leukocytes are directed by a chemoattractant gradient to transmigrate between endothelial cells and into the perivascular extracellular matrix (ECM).

Lymphocytes also undergo targeted transmigration. The patterns of lymphocyte transmigration are more complex than those of monocytes and neutrophils, however. Lymphocytes are relatively heterogeneous as a cell population, and they participate in antigen-specific immune responses as well as recirculation pathways in lymphoid and non-lymphoid tissues. Naive lymphocytes (no prior specific antigen exposure) emigrate preferentially through the 'high' or cuboidal endothelial cells of venules in peripheral and mucosal-associated lymph node tissue [7,8]. In contrast, memory lymphocytes (prior specific antigen exposure and activation) predominately transmigrate through tissue endothelium, showing a preference for the tissue where specific antigen exposure initially occurred [9,10]. Interactions between adhesion molecules on lymphocytes including LFA-1 and VLA-4 with their respective counter-receptors ICAM-1/ICAM-2 and VCAM-1 on endothelium are important both in vivo and in vitro for lymphocyte binding and transmigration [11–14]. However, these adhesive interactions alone cannot explain the specificity of lymphocyte emigration patterns. It appears likely that specific activating molecules, analogous to the monocyte and neutrophil chemoattractants, exist for lymphocytes and act in concert with adhesion molecules to direct lymphocyte recirculation and antigen-specific responses.

The study of lymphocyte transmigration and attempts to identify lymphocyte chemoattractants using in vitro chemotaxis assay systems have been limited by several factors. Unless activated, a population of lymphocytes contains far fewer motile cells than a population of neutrophils or monocytes [15,16]. Lymphocytes are also more heterogeneous as a cell population than other leukocytes in both their phenotypes and functions [17]. Together, these differences lead to small percentages of lymphocytes migrating (< 10% of input cells) in assays despite migration periods of several hours [16]. Conventional lymphocyte chemotaxis assays are performed with a modified Boyden chamber apparatus [16,18,19]. This apparatus consists of a chamber separated by a microporous (5 μm or 8 μm pores) polycarbonate or nitrocellulose membrane into a lower compartment for the chemoattractant and an upper compartment for input lymphocytes. The membranes are often coated with collagen. Diffusion of the chemoattractant into the upper compartment occurs rapidly and creates a gradient towards which lymphocytes migrate. Among the non-physiologic features of this assay is the absence of vascular endothelium. Physiologic interactions of lymphocyte adhesion molecules with endothelium and components of the ECM secreted and organized by endothelium cannot occur in this assay. Additionally, certain chemoattractants may bind to proteoglycans both on the endothelial surface [20–22] and in the subendothelial ECM [20]. The binding of chemoattractants to proteoglycans may facilitate the presentation of chemoattractants to circulating and transmigrating lymphocytes. In conventional assay systems, presentation of chemoattractants either does not occur or is likely to be non-physiologic.

In an effort to create more physiologic in vitro transmigration assays, HUVEC monolayers cultured on microporous membranes or collagen matrices have been utilized by several groups. Neutrophil transendothelial chemotaxis assays with HUVEC-covered membranes [20,23–25] and transendothelial assays measuring spontaneous monocyte [26] and lymphocyte [27,28] migration into collagen matrices have been performed. We have adapted a transmigration assay which employs HUVEC-covered Transwell (Costar, Cambridge, MA) cell culture chamber inserts [20,23] for in vitro studies of lymphocyte

transendothelial chemotaxis. Using the transendothelial assay, we screened potential sources for human lymphocyte chemoattractant activities and identified a potent activity in supernatants of cultured, mitogen-stimulated peripheral blood mononuclear cells. This activity was purified and demonstrated to be MCP-1 [1]. We report here the functional characterization of T lymphocyte transendothelial chemotaxis using both culture supernatants and purified MCP-1.

2. Materials and methods

2.1. Reagents

The following recombinant human cytokines were used: interleukin-1α (IL-1α), interleukin-2 (IL-2), interleukin-4 (IL-4), interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) (all from Genzyme, Cambridge, MA) and MCP-1 (Peprotech, Rocky Hill, NJ). Fluorescein isothiocyanate-dextran (FITC-dextran) of average MW 4400, Wright stain, modified, and Giemsa stain, modified, were all from Sigma, St. Louis, MO. 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) was purchased from Molecular Probes, Eugene, OR.

2.2. Monoclonal antibodies

The following previously described murine monoclonal antibodies (mAb) against human antigens were used: TS1/22 (anti-CD11a, IgG1) [29], TS1/18 (anti-CD18, IgG1) [29], R6.5 (anti-ICAM-1, IgG2a) [30], CBR-IC2/2 (anti-ICAM-2, IgG2a) [31], and X63 (nonbinding antibody, IgG1) were produced in our laboratory. IF7 (anti-CD26, IgG1) [32], 2H4 (anti-CD45RA, IgG1) [33], 4B4 (anti-CD29, IgG1) [33], and UCHL-1 (anti-CD45RO, IgG2a) [34] were generous gifts of C. Morimoto, Dana-Farber Cancer Institute, Boston, MA. HAE-2a (anti-VCAM-1, IgG1) [35] was a generous gift of T. Tedder, Duke University, Durham, NC. BB11 (anti-E-selectin, IgG2b) [36] was a generous gift of R. Lobb, Biogen, Cambridge, MA. WAPS 12.2 (anti-P-selectin, IgG1) [37] was a generous gift of E. Butcher, Stanford University Medical Center, Stanford, CA. PECAM-1.2 (anti-CD31, IgG1) [38] was a generous gift of P.J. Newman, Blood Research Institute, Milwaukee, WI. B1 (anti-CD20, IgG2a) [39], MY4 (anti-CD14, IgG2b) [40], T3 (anti-CD3, IgG1) [17], T4 (anti-CD4, IgG1) [17], and T8 (anti-CD8, IgG1) [17] were obtained from Coulter Immunology, Hialeah, FL. HP2/1 (anti-CD49d, IgG1) [41] and 3G8 (anti-CD16, IgG1) [42] were obtained from AMAC, Westbrook, ME. OKM1 (anti-CD11b,IgG2b [43] was obtained from Ortho Diagnostic Systems, Raritan, NJ. ZPO1 (anti-horseradish peroxidase, nonbinding antibody, IgG1) was obtained from Zymed Laboratories, So. San Francisco, CA. W6/32 (anti-HLA-A,B,C, IgG2a) [44], 12F1 (anti-CD49b, IgG2a), J143 (anti-CD49c, IgG1), and mAb 16 (anti-CD49e, IgG2a) were obtained from the 5th International Workshop on Human Leukocyte Differentiation Antigens.

2.3. Leukocyte purification

Human peripheral blood mononuclear cells (PBMC) were purified from the citrate-anticoagulated whole blood of healthy volunteers by dextran sedimentation and centrifugation through Ficoll-Hypaque (1.077; Sigma, St. Louis, MO) [45]. PBL were prepared from PBMC by adhering monocytes/macrophages at 3×10^6/ml in RPMI 1640 (BioWhittaker, Walkersville, MD)/5% fetal bovine serum (FBS) (Sigma, St. Louis, MO) on 150×20 mm tissue culture-treated plastic Petri dishes for 1 h at 37°C, 5% CO_2 with one dish change.

Purified T lymphocytes were prepared from PBMC by negative selection using magnetic cell sorting, as described [46,47]. Briefly, PBMC at 1×10^7/ml in PBS with 1% bovine serum albumin (Intergen, Purchase, NY) plus 5 mM EDTA (PBE buffer) were incubated with a combination of mAb at 5 μg/ml which bind monocytes (MY4 and OKM1), B lymphocytes (B1), and natural killer cells (3G8) for 30 min on ice. After 3 washes in PBE, cells were incubated with magnetic microbeads conjugated to rat anti-mouse IgG1 and IgG2a + 2b (MACS, Miltenyi Biotec, Sunnyvale, CA) for 20 min on ice and then passed over a magnetic column. Immunofluorescence flow cytometry showed the purified T lymphocyte preparations contained $> 95\%$ T lymphocytes ($CD3^+$) and $< 1\%$ monocytes ($CD14^+$), B lymphocytes ($CD20^+$), or natural killer ($CD16^+$) cells.

For assays in which migrated cells were not analyzed by flow cytometric techniques, cells were labelled with 1 μg/ml of BCECF for 30 min at 37°C, 5% CO_2 in RPMI 1640/5% FBS [31]. Unlabelled lymphocytes can be difficult to differentiate from endothelial cell debris in the bottom compartment of assay chambers by light microscopy. BCECF labelling allowed lymphocytes to be identified easily by fluorescence and did not significantly affect lymphocyte viability or migration.

Both PBL and T lymphocyte preparations routinely had > 95% cell viability as determined by trypan blue exclusion and were used in assays on the day they were prepared.

2.4. Culturing of cells on Transwell filters

HUVEC were harvested from the umbilical cords of healthy newborns and cultured as previously described [48,49]. Confluent primary cultures were trypsinized and split 1 : 3 on tissue culture-treated flasks coated with type I collagen (5 μg/cm^2; Organogenesis, Canton, MA). Passage 1–3 HUVEC were grown in medium 199 (M199) (BioWhittaker, Walkersville, MD) supplemented with 15% heat-inactivated, low endotoxin FBS (Sigma, St. Louis, MO), 100 μg/ml heparin (Sigma, St. Louis, MO), 100 μg/ml endothelial mitogen (Biomedical Technologies, Stoughton, MA), 25 mM Hepes, 5 mM L-glutamine, and 50 μg/ml gentamicin at 37°C, 5% CO_2. When confluent, passage 1–3 HUVEC were trypsinized and seeded at $1-2 \times 10^5$ cells/cm^2 on collagen-coated, 6.5 mm or 24.5 mm diameter polycarbonate tissue culture inserts (filters) of 5 or 8 μm pore size (Transwell, Costar, Cambridge, MA). Filters were coated on their upper surface with type I collagen at 40 μg/ml in HBSS during a 0.5–1 h incubation at 37°C and washed in HBSS before seeding. HUVEC on filters were cultured identically to those at passage 1–3 on flasks. Since HUVEC cannot be visualized adequately on Transwell inserts by phase-contrast microscopy, sample inserts were stained with Wright-Giemsa to assess monolayer confluence. Inserts were incubated for 3 min with modified Wright stain followed by incubation for 7 min with modified Giemsa stain diluted 1/6 in distilled water. Confluence was achieved at day 5–7 of culture. All filters used in chemotaxis experiments were in culture 7–14 days and had confluent HUVEC monolayers.

Cytokine stimulation of HUVEC was performed by adding either IL-1α (10 U/ml), IL-2 (30 U/ml), IL-4 (30 U/ml), IFN-γ (1000 U/ml), or TNF-α (200 U/ml) to culture medium 4 or 18 h before supernatant harvesting or use of filters in chemotaxis assays. The effectiveness of stimulation was monitored by immunofluorescence flow cytometric analysis of ICAM-1, VCAM-1, and E-selectin expression on stimulated versus unstimulated cells [50].

Six additional cell types or cell lines were cultured on uncoated or collagen-coated Transwell filters for comparison experiments with HUVEC-covered filters. These included human dermal fibroblasts (American Type Culture Collection, Rockville, MD), the spontaneously transformed HUVEC line ECV304 [51] (a generous gift of C. Mackay, LeukoSite, Cambridge, MA), the murine embryonic fibroblast line NIH 3T3 (American Type Culture Collection) and endothelioma lines e End.1 and s End.1 [52], and the porcine endothelial cell line 144 (a generous gift of J. Kurnick, Massachusetts General Hospital, Boston, MA).

2.5. Lymphocyte transendothelial chemotaxis assay

Each lymphocyte transendothelial chemotaxis assay was performed with lymphocytes from one donor and groups of HUVEC-covered Transwell filters which were prepared and cultured identically. Assay media consisted of a 1 : 1 mixture of M199 (without FBS, heparin, and endothelial mitogen) and RPMI 1640 plus 0.5% human serum albumin (HSA) (Plasbumin-25, Miles, Elkhart, IN). Cluster plates with 24 wells (6.5 mm filter) were used for most assays; 6 well plates (24.5 mm filter) were used when migrated cells were to be harvested and subjected to flow cytometry. The sample to be tested for chemoattractant activity was diluted with assay media, distributed in duplicate or triplicate into 24 well (600 μl/well) or 6 well (2.5 ml/well) cluster plates, and warmed to 37°C in 5% CO_2 for 30 min. After complete removal of culture media, HUVEC-covered filters were transferred into wells of the pre-warmed plate(s). To each filter insert, 5×10^5 lymphocytes in 100 μl of assay media (6.5 mm filter) or 5×10^6 lymphocytes in 1.5 ml of assay media (24.5 mm

filter) were added. Two additional wells without filters also received a 1/20 dilution of input lymphocytes in assay media to allow for calculation of the percent input cells migrated. The plates were incubated at 37°C, 5% CO_2 for the period of chemotaxis.

Following chemotaxis, each Transwell insert was removed and the bottom filter surface gently scraped against the well edge to retrieve any lymphocytes remaining in the small volume of medium on the undersurface of the filter. In contrast to assay systems using polycarbonate membranes coated with type IV collagen, where only 1–3% of migrated lymphocytes detach from the membrane [53], < 10% of cells migrating through HUVEC-covered filters remained bound after treating the filters as described above. The majority of migrated cells accumulated directly beneath the filter on the surface of the bottom well. All migrated cells were resuspended by pipetting and then allowed to settle to the bottom surface over 45–60 min. This created a more homogenous distribution of cells for quantification. Fluorescent cells in each well were counted in 4 separate locations using a 10×10 grid (0.1 mm^2) on an inverted phase-contrast fluorescent microscope (Diaphot-TMD, Nikon, Garden City, NJ). The average number of cells per grid in duplicate or triplicate wells was calculated and divided by the average input cell count to determine the percent input cells migrating into the bottom well for each sample.

2.6. *Sources screened for lymphocyte chemoattractant activity*

Using the transendothelial assay, supernatants from a variety of unstimulated and stimulated cell cultures and human tissues were screened for their ability to promote PBL migration. Supernatants from the following human cell lines were tested: A549 (lung carcinoma), BT20 (breast carcinoma), MG-63 (osteosarcoma), HT 1080 (fibrosarcoma), LOX (melanoma), SW480 (colon adenocarcinoma) (all generous gifts of M. Hemler, Harvard Medical School, Boston, MA); A735 and RPMI 7951 (melanoma), THP-1 (monocytic leukemia), MOLT-3 and MOLT-4 (acute lymphoblastic leukemia), EB-3 and Ramos (Burkitt lymphoma), HeLa (epitheloid carcinoma), K562 (erythroleukemia), HL-60 (promyelocytic leukemia), SKW-3 (T cell lymphoma) and JY (B lymphoblastoid) (all from the American Type Culture Collection). Supernatants of cultured human fibroblasts from 3 types of skin were tested: normal, a patient with dermatomyositis (Ce Wo), and a patient with keloid (Kel Fib) (all from the American Type Culture Collection, Rockville, MD). Cytokine stimulation of HUVEC, fibroblasts, and some of the cell lines was performed by culturing cells with either IL-1α (10 U/ml), IL-2 (100 U/ml), IL-4 (30 U/ml), IFN-γ (1000 U/ml), or TNF-α (200 U/ml).

Fresh tonsil from patients undergoing elective tonsillectomy (Children's Hospital, Boston, MA) and spleen from a normal patient and a patient with an infiltrating lymphoma (Brigham and Women's Hospital, Boston, MA) were teased apart and cultured in serum-free X-vivo 10 medium (M.A. Whittaker, Walkersville, MD) supplemented with 0.35 mg/ml $NaHCO_3$, 25 mM Hepes, 2.5 mM L-glutamine, 50 μg/ml gentamicin, and 100 U/ml penicillin for 1–4 days at 37°C, 5% CO_2. Urine from a healthy volunteer and patients with newly diagnosed, untreated Kawasaki disease (Children's Hospital, Boston, MA) were dialyzed against HBSS in dialysis tubing with a MW cutoff of 3500.

Stimulated mononuclear cell supernatants were prepared from PBMC cultured with phytohemagglutinin-P (PHA-P) (Sigma, St. Louis, MO) or in a mixed lymphocyte reaction (MLR). PBMC were isolated as described above from peripheral blood leukopaks obtained during platelet pheresis of healthy volunteers (Blood Donor Center, Children's Hospital, Boston, MA). After five washes in RPMI 1640, cells were resuspended at $2–4 \times 10^6$/ml in X-vivo 10 supplemented with 0.35 mg/l $NaHCO_3$, 25 mM Hepes, 2.5 mM L-glutamine, 50 μg/ml gentamicin, 1 mg/l indomethicin, 3 mM lithium chloride, 50 μM hydroxyurea, and 2.5 μg/ml PHA-P. Cells were cultured in tissue culture-treated flasks for 72 h at 37°C, 5% CO_2. MLR supernatant was prepared by culturing mitomycin C-treated and untreated PBMC from 2 healthy volunteers for 6 days, as described [54]. Cell-free supernatants were prepared by centrifugation at 1200 rpm $\times$ 5 min followed by filtration through a 0.22 μm filter. Supernatants were concentrated 25–35-fold using an Amicon Model 8400 ultrafiltration cell with a YM1 membrane (1000 MW cutoff) (Amicon, Beverly, MA) and stored at either 4°C or −80°C before use.

2.7. Flow cytometry

HUVEC were detached from 24.5 mm Transwell membranes by incubation with 5 mM EDTA in HBSS for 10 min at 37°C. Immunofluorescence flow cytometry was as described [55] with a first incubation of 1 : 100 ascites or 5 μg/ml purified mAb and staining with 1/20 dilution of FITC-conjugated second antibody (Zymed Laboratories, So. San Francisco, CA).

2.8. Assessment of barrier function of HUVEC monolayers

In addition to microscopic inspection following Wright-Giemsa staining, the integrity of confluent HUVEC monolayers was assessed for each group of filters by determining the ability of sample filters to restrict diffusion of a dextran of average MW 4400 conjugated to FITC (FITC-dextran). FITC-dextran was diluted 1/500 in assay media and 600 μl (24 well plate) or 2.5 ml (6 well plate) were added to duplicate wells. Either HUVEC-covered or untreated inserts of the same pore size were then placed into the wells. Immediately following insertion, 100 μl (6.5 mm filter) or 1.5 ml (24.5 mm filter) of assay media were added to inserts and incubation at 37°C, 5% CO_2 begun. At the completion of the incubation period, the amount of FITC-dextran which had diffused into the inserts was quantitated by measuring the fluorescence of a 50 μl aliquot from each insert using a Pandex fluorescence concentration analyzer (Baxter Healthcare, Mundelein, IL). The relative diffusion of FITC-dextran was determined by dividing the average fluorescence of these aliquots into the fluorescence of an equal volume of a mixture containing the same proportions of 1 : 500 FITC-dextran and assay media as was placed into assay wells. The fluorescence associated with untreated or HUVEC-covered membranes was determined by excising them and measuring their fluorescence by Pandex, and was $< 1\%$ of input fluorescence.

In experiments assessing the effect of PHA supernatant on HUVEC monolayer barrier function, FITC-dextran was diluted 1/500 in an optimal chemotactic dilution of PHA supernatant instead of assay media.

2.9. Lymphocyte chemotaxis inhibition experiments with monoclonal antibodies

L-15 medium with 0.5% HSA (L-15/HSA) was used as assay medium so that assays could be performed without pre-incubations in 5% CO_2 to adjust media pH. BCECF-labelled PBL were prepared at 1×10^7/ml in assay media. HUVEC-covered or untreated filters (6.5 mm) were overlaid with 50 μl of L-15/HSA with or without a concentration of mAb that was $2\times$ saturating as shown by flow cytometry (1 : 50 ascites or 10 μg/ml purified mAb). Lymphocytes (5×10^5 in 50 μl) were added to each insert. mAb remained in the wells for the duration of the assay. These assays were otherwise performed and analyzed as described above.

2.10. Statistical analysis

A square root transformation of cell count data was performed prior to analysis. Nested analysis of variance was used to compare mean counts of cells migrated in the presence or absence of mAb (Figs. 8–10). If analysis of variance detected a statistically significant difference in mean counts, the Tukey method of multiple comparisons was applied. A p value of < 0.05 was considered to be statistically significant.

3. Results

3.1. PHA-stimulated PBMC produce an activity which promotes transendothelial lymphocyte chemotaxis

To identify potential sources of lymphocyte chemoattractant activity, we screened supernatants from the 29 cell lines, cell types, tissues, and fluids described in Materials and methods using PBL in transendothelial assays. The most potent activities from these initial screenings were detected in supernatants from MLR and from PBMC stimulated for 72 h with PHA-P (termed PHA supernatant). PBL migration was promoted by dilutions of a concentrated ($25\times$) PHA supernatant, with a bell-shaped dose response curve and optimal migration at a

1/1000 dilution of concentrated supernatant (Fig. 1). PBL migration was reduced by 75–80% upon heating PHA supernatant at 100°C for 10 min (data not shown).

PBL migration to PHA supernatant was not dependent on the presence of endothelium, as shown by comparing migration through filters which were either untreated (Fig. 2A) or treated with collagen and covered by a HUVEC monolayer (Fig. 2B). Between 4–6 h of incubation, PBL migration through both untreated and HUVEC-covered filters appeared to plateau, with approximately 2.5–3.0 times as many PBL migrating through untreated filters. The ratio of PBL migrating to PHA supernatant versus control medium was consistently > 10 at 4–6 h with HUVEC-covered filters, whereas this ratio was consistently < 10 for untreated filters. Thus, the signal-to-background ratio for migration was enhanced using HUVEC-covered filters. Because PBL migration appeared to plateau after 4 h in repeat experiments, we chose this time as the standard assay incubation period.

We compared monolayers of other cell types to HUVEC in the chemotaxis assay (data not shown). Human dermal fibroblasts grown on uncoated filters and spontaneously transformed human umbilical vein endothelial ECV304 cells grown on uncoated or collagen-coated filters were similar to HUVEC in percent of PBL migration to PHA supernatant and low background migration. The porcine endothelial

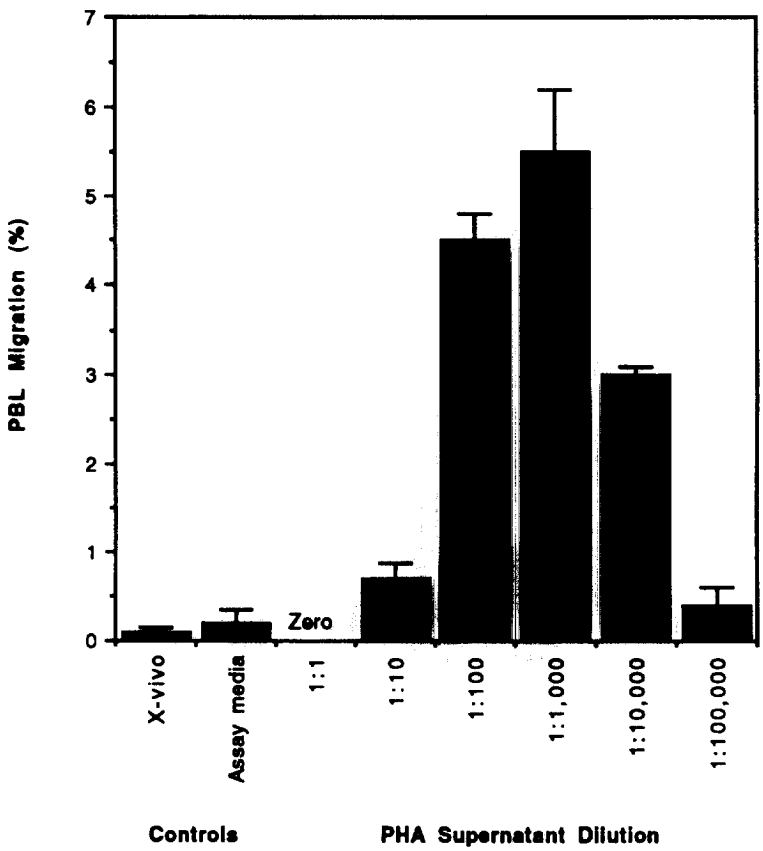


Fig. 1. Effect of titration of PHA supernatant on PBL transendothelial migration. A concentrated (25 ×) PHA supernatant was diluted with assay medium to the indicated titers and tested in duplicate in a 4 h PBL transendothelial migration assay. Controls include assay medium and a 1:1 mixture of X-vivo medium and assay medium. These data represent mean PBL migration ± range from one of three independent experiments with similar results.

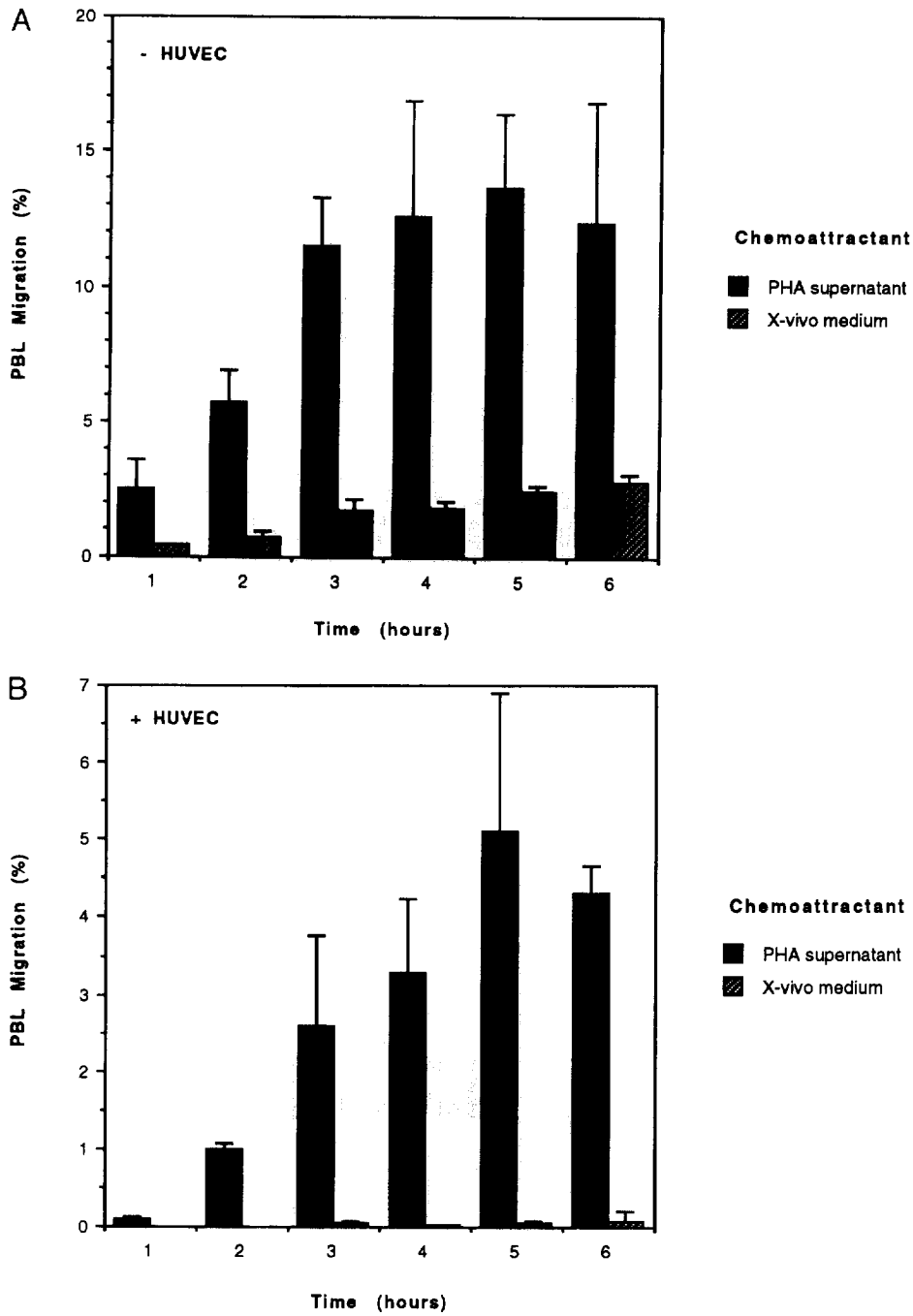


Fig. 2. Kinetics of PBL migration to PHA supernatant through untreated versus HUVEC-covered Transwell filters. PBL were added to duplicate inserts of 8 μm pore size/time point which were either untreated (*A*) or covered with HUVEC monolayers (*B*). Migration to a dilution of PHA supernatant promoting optimal migration versus a 1 : 1 mixture of X-vivo and assay media was determined every hour for 6 h. These data represent mean PBL migration $\pm$ range from one of three independent experiments with similar results.

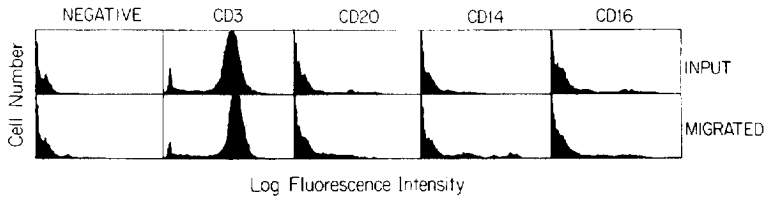


Fig. 3. Immunofluorescence flow cytometry of input and transmigrated PBL. Following 4 h of transmigration to PHA supernatant (migrated PBL) or incubation in assay media (input), cells were harvested. Input and migrated PBL were stained with either a non-binding control (ZPO1), a mAb to CD3 (T3), a mAb to CD14 (MY4), a mAb to CD16 (3G8), or a mAb to CD20 (B1). These data represent one of five experiments with similar results.

cell line 144, mouse fibroblast NIH 3T3 cells, and two mouse endotheliomas, e End.1 and s End.1, did not support PBL migration to PHA supernatant.

To confirm that T lymphocytes were the predominant cell type migrating to PHA supernatant, we performed flow cytometric analyses of the input and migrated cells from transendothelial assays (Fig. 3). Input PBL contained approximately 70–75% T lymphocytes ($CD3^+$), 5–10% B lymphocytes ($CD20^+$), 10–15% natural killer cells ($CD16^+$), and $< 5\%$ monocytes ($CD14^+$). Of the 2–6% of input cells which typically migrated during 4 h, approximately 80% were T lymphocytes. There was no enhancement of the percentage of natural killer cells or B

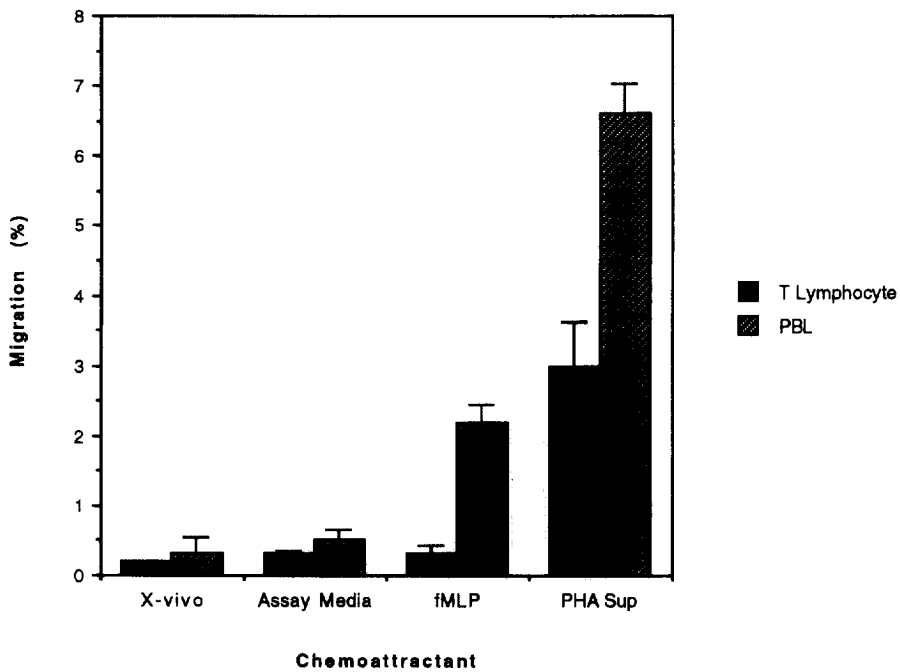


Fig. 4. PBL versus purified T lymphocyte transendothelial migration. Blood from a single donor and Transwell filters with HUVEC which were passaged and cultured identically were used. The percentage of input PBL and T lymphocytes migrated over 4 h to an optimal chemotactic dilution of PHA supernatant, fMLP at 10^{-8} M, and controls including assay medium and a 1 : 1 mixture of X-vivo medium and assay medium are shown, as indicated. These data represent mean cell migration ± range from one of three independent comparison experiments with similar results.

lymphocytes in the migrated population. No significant change in the ratio of $CD4^+$ to $CD8^+$ lymphocytes occurred with transmigration (data not shown). A small but detectable enhancement of the low number of monocytes in the input population was observed in the migrated cells (Fig. 3, CD14 panels). When PBMC, which contained on average 20–30% monocytes, were added as input cells instead of PBL, a prominent monocytic migratory response occurred (data not shown). When purified T lymphocytes were compared directly with PBL in transendothelial assays, approximately half as many input T lymphocytes ($3.0 \pm 0.3\%$) as PBL ($6.6 \pm 0.4\%$) migrated to PHA supernatant (Fig. 4). T lymphocytes did not migrate above control levels to the monocyte/neutrophil chemoattractant fMLP [56], which promoted migration of approximately one third the number of PBL migrating to PHA supernatant. By flow cytometric analysis, $< 1\%$ of the input and migrated cells in assays using purified T lymphocytes were $CD14^+$ (data not shown). These results demonstrate that the majority of input PBL which migrate are T lymphocytes, and that highly purified T lymphocytes can also migrate in response to PHA supernatant.

A checkerboard analysis [57], modified by the use of HUVEC-covered instead of untreated filters, was performed to evaluate whether PBL migration to PHA supernatant was caused primarily by chemotaxis (directional locomotion) or by random locomotion. Significant migration occurred only when a gradient existed between the bottom and top compartments, with the more concentrated supernatant present in the bottom (Table 1). A similar experiment using purified T cells demonstrated that they also underwent chemotaxis, not random locomotion, in response to PHA supernatant (data not shown).

3.2. The effect of PHA supernatant on the HUVEC monolayer

Compared to an untreated filter, a filter covered with a confluent HUVEC monolayer would be ex-

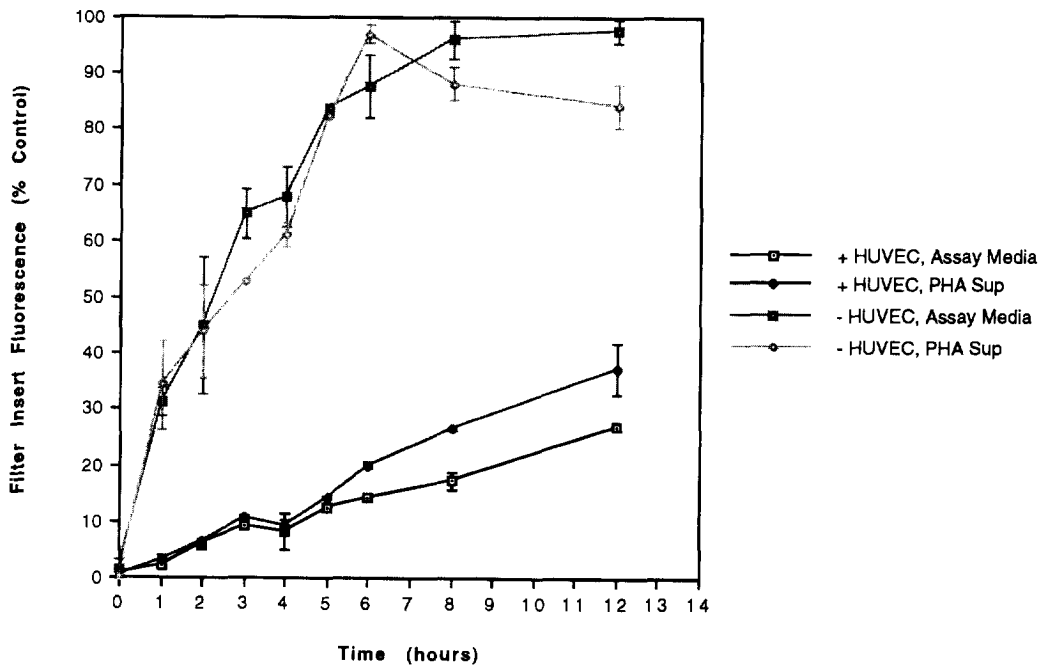


Fig. 5. Time course of FITC-dextran diffusion through untreated versus HUVEC-covered 8 μm Transwell filters. FITC-dextran (average MW 4400) in either assay media or an optimal chemotactic dilution of PHA supernatant was added in duplicate per time point to the bottom compartments of a Transwell apparatus. After filter incubation for the indicated times, aliquots of media were removed from the upper compartments and their fluorescence measured. These data represent the mean percentage fluorescence $\pm$ range relative to control (100% fluorescence) from one of three independent experiments with similar results.

Table 1
Effect of varying concentration gradients of PHA supernatant on PBL transendothelial migration

Bottom	PBL migration (%)			
	Top			
	Media	1/1000	1/100	1/10
Media	0.2±0.17	0.2±0.07	0.3±0.17	0.5±0.11
1/1000	0.8±0.12	0.6±0.01	0.4±0.08	0.4±0.01
1/100	3.4±0.10	3.5±0.10	0.7±0.51	0.6±0.46
1/10	2.7±0.70	2.3±0.40	0.8±0.21	0.2±0.12

The indicated dilutions of a concentrated (30×) PHA supernatant in assay medium or a media control (1:1 mixture of X-vivo medium and assay medium) were added to the top and bottom compartments of a Transwell apparatus. A 4 h transendothelial migration assay with 5×10^5 PBL/well was performed. Values represent the mean percentage ± range of PBL migrated in duplicate wells of one representative experiment of a total of three experiments.

pected to decrease the diffusion of soluble molecules, thereby maintaining a steeper concentration gradient of chemoattractant between compartments. To test this we examined the diffusion of a FITC-conjugated dextran of average MW 4400 from the bottom to the top compartment of wells. The fluorescence of media in the top compartment was < 10% of that measured in the bottom compartment after a 4 h diffusion period using HUVEC-covered filters (Fig. 5). In contrast, > 60% of the fluorescence measured in the bottom compartment was detected in the top compartment after a 4 h diffusion period with untreated filters of the same pore size. PHA supernatant at a dilution used to promote optimal chemotaxis did not alter the diffusion of FITC-dextran through HUVEC-covered filters compared to assay media until > 5 h (Fig. 5). FITC-dextran diffusion through untreated filters reached equilibrium within 6–8 h, whereas with HUVEC-covered filters, a gradient was maintained for greater than 12 h.

Because PHA supernatant is a complex mixture which contains multiple cytokines [58], we tested both individual cytokines and cytokine-stimulated HUVEC supernatants for the ability to promote PBL migration. IL-1α, IL-2, IL-4, IFN-γ, or TNF-α over a range of concentrations did not cause PBL transendothelial migration (Fig. 6). PHA-P also did not promote migration. Culture supernatants from unstimulated HUVEC or from HUVEC exposed to PHA-P (2.5 μg/ml) or these cytokines (see Materials and methods) for up to 18 h did not promote PBL

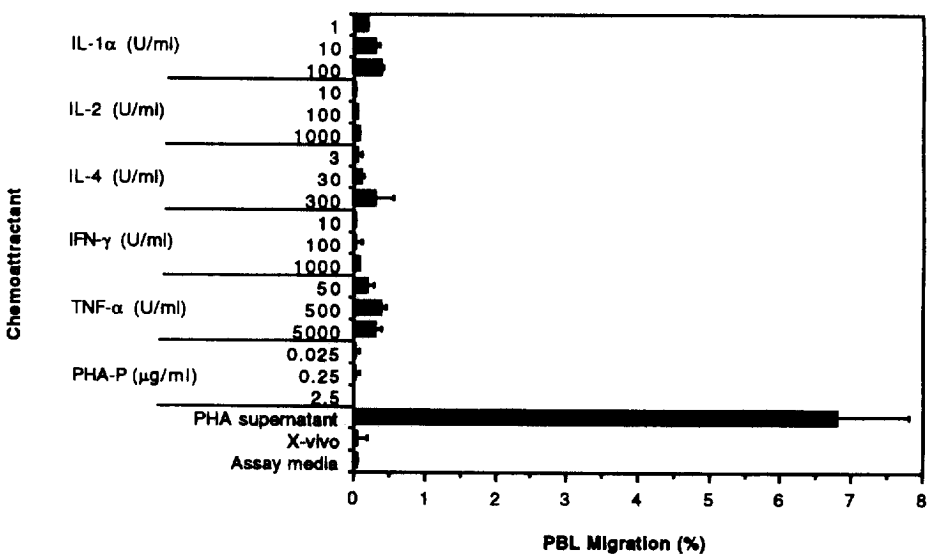


Fig. 6. PBL transendothelial migration to IL-1α, IL-2, IL-4, IFN-γ, TNF-α, or PHA-P. The indicated concentrations of each cytokine or PHA-P were prepared in assay media and tested in duplicate for their ability to promote PBL transendothelial migration in a 4 h assay. These data represent the mean percentage migration ± range in one of three independent experiments with similar results.

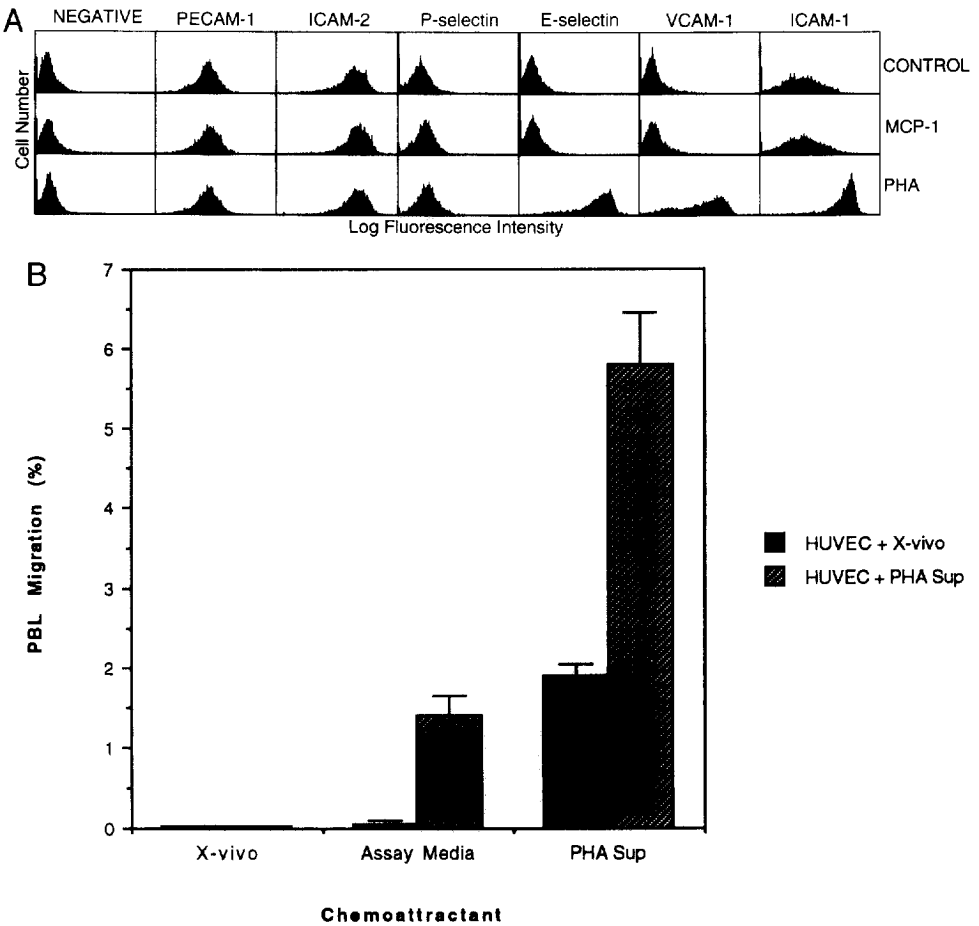
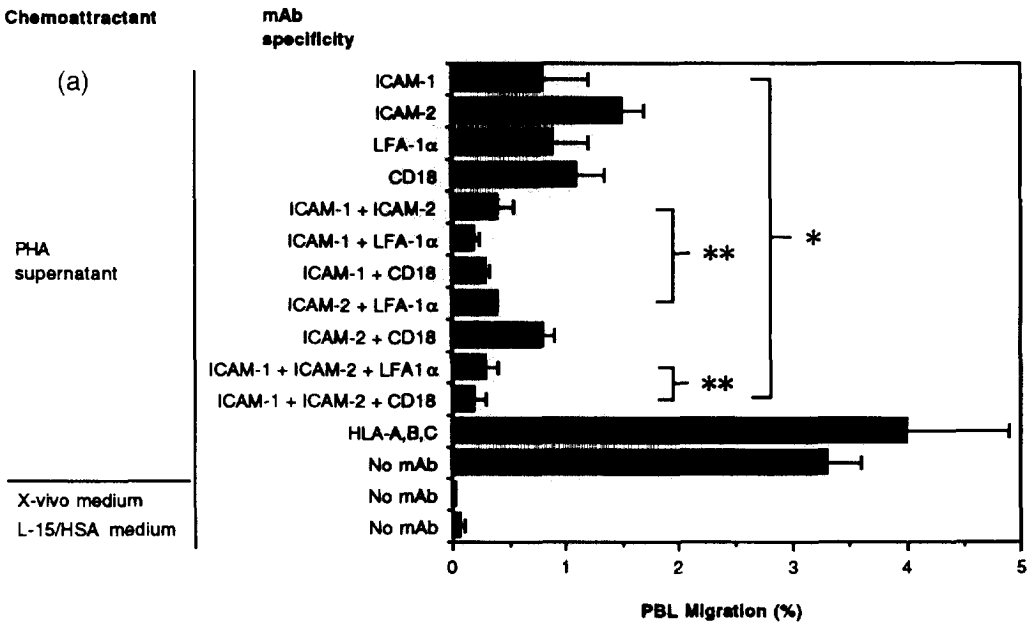
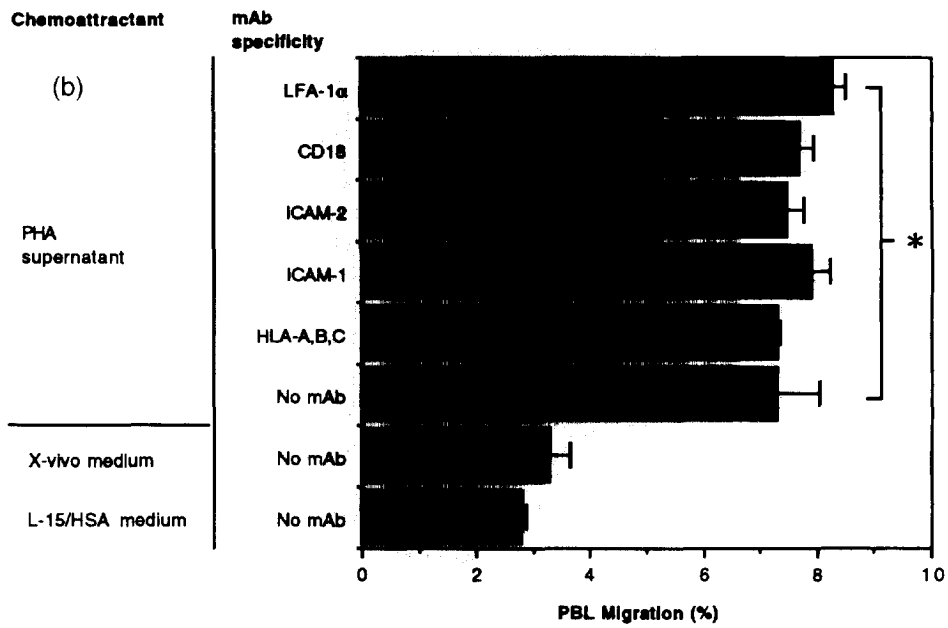


Fig. 7. Effect of pretreatment of HUVEC on expression of adhesion molecules and transendothelial chemotaxis. *A*: immunofluorescence flow cytometry. Following a 4 h incubation in culture media (control), MCP-1 50 ng/ml (MCP-1), or an optimal dilution of PHA supernatant (PHA), HUVEC were harvested with 5 mM EDTA in HBSS from Transwell membranes. Cells were stained with either a non-binding control (X63), a mAb to PECAM-1 (PECAM-1.2), a mAb to ICAM-2 (CBR-IC2/2), a mAb to P-selectin (WAPS 12.2), a mAb to E-selectin (BB11), a mAb to VCAM-1 (HAE-2a), or a mAb to ICAM-1 (R6.5). *B*: transendothelial chemotaxis to PHA supernatant. HUVEC-covered filters were cultured for 4–6 h after adding either an optimal chemotactic dilution of PHA supernatant or an equal dilution of X-vivo medium. Filters were washed and used in a 4 h PBL chemotaxis assay in duplicate with either the identical chemotactic dilution of PHA supernatant or control media, as indicated. The data represent the mean percentage PBL migrated ± range from one of three independent experiments with similar results.

Fig. 8. Effect of mAb to ICAM-1, ICAM-2, LFA-1α, and CD18 on PBL chemotaxis to PHA supernatant through HUVEC-covered (*a*) or untreated (*b*) filters. Saturating concentrations of mAb to ICAM-1 (R6.5), ICAM-2 (CBR-IC2/2), LFA-1α (TS1/22), or CD18 (TS1/18) were added either singly or in the indicated combinations to duplicate filter inserts. PBL were added and a 4 h chemotaxis assay using an optimal chemotactic dilution of PHA supernatant was performed. In both *a* and *b*, a control mAb to HLA-A,B,C (W6/32) was included. No mAb was present in the PHA supernatant control and both media controls (1 : 1 mixture of L-15/0.5% HSA and X-vivo medium or L-15/0.5% HSA alone). These data represent the mean percentage migration ± range from one of four independent experiments with similar results. *a*: *, $p < 0.05$ when compared to controls (PHA supernatant with mAb to HLA-A,B,C or no mAb); **, $p < 0.05$ when compared to any of the individual anti-adhesion mAb used (ICAM-1, ICAM-2, LFA-1α, or CD18). *b*: *, $p < 0.05$ when compared to media controls (X-vivo and L-15/HSA), but not significantly different within group.

transendothelial migration (data not shown). However, after 4 h of incubation with PHA supernatant, the expression of inducible adhesion molecules on HUVEC known to be important in lymphocyte binding and transendothelial migration, including ICAM-1 [45,59], VCAM-1 [11,60], and E-selectin [61] was significantly increased upon flow cytometric analysis (Fig. 7A). In contrast, levels of the constitutively expressed adhesion molecules PECAM-1 [62,63] and ICAM-2 [31], and inducible molecule P-selectin [64] were not increased. Incubation of HUVEC for 4 h with MCP-1 (50 ng/ml), the major lymphocyte





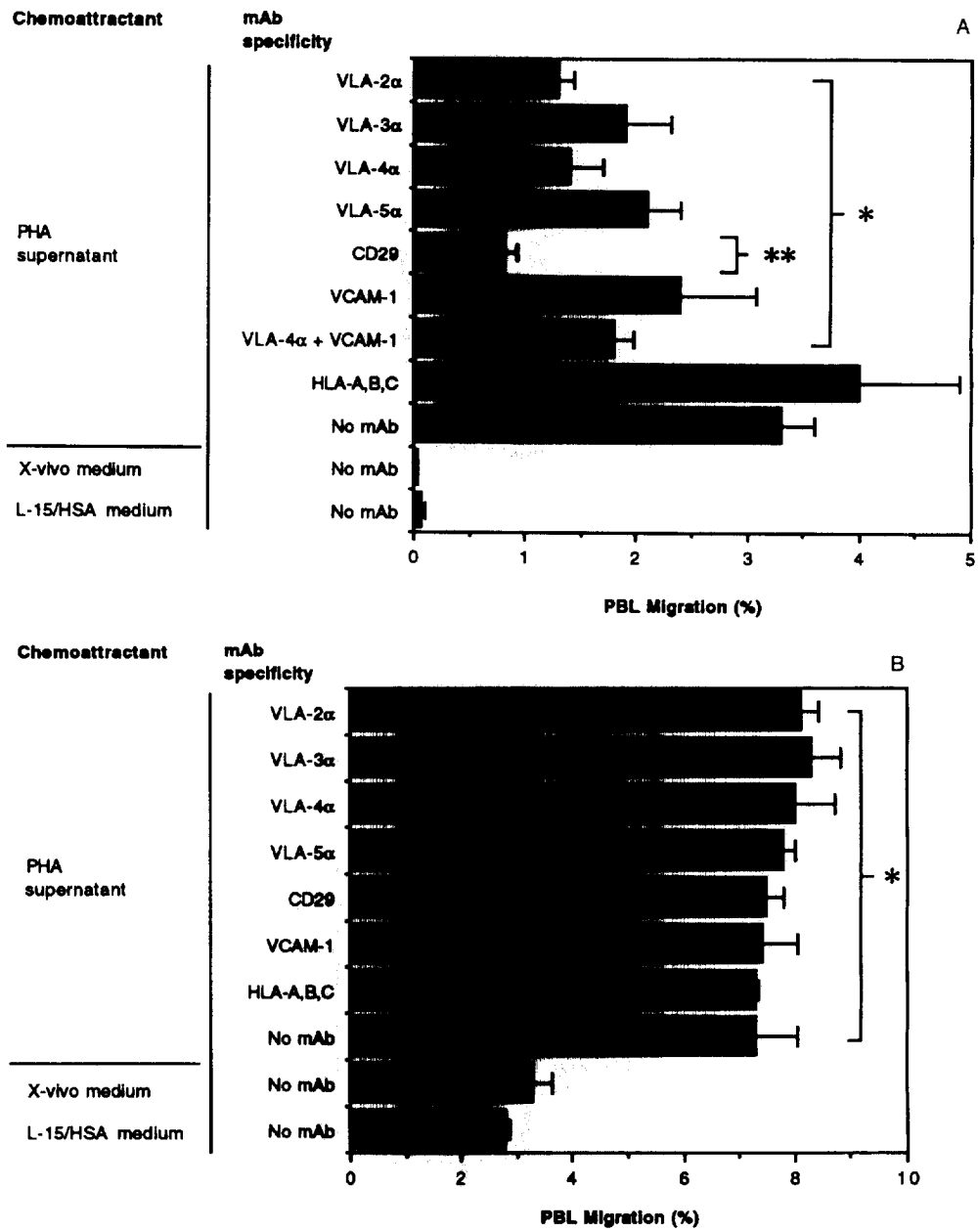


Fig. 9. Effect of mAb to VLA-2, VLA-3, VLA-4, VLA-5, and VCAM-1 on PBL chemotaxis through HUVEC-covered (*A*) or untreated (*B*) filters. Saturating concentrations of mAb to VLA-2α (12F1), VLA-3α (J143), VLA-4α (HP2/1), VLA-5α (mAb 16), or VCAM-1 (HAE-2a) were added either singly or in the indicated combinations to duplicate filter inserts and a 4 h PBL chemotaxis assay performed with controls as described in Fig. 8. These data represent the mean percentage migration ± range from one of four independent experiments with similar results. *A*: *, $p < 0.05$ when compared to controls (PHA supernatant with mAb to HLA-A,B,C or no mAb); **, $p < 0.05$ when compared to all mAb to adhesion molecules except VLA-2α. *B*: *, $p < 0.05$ when compared to media controls (X-vivo and L-15/HSA), but not significantly different within group.

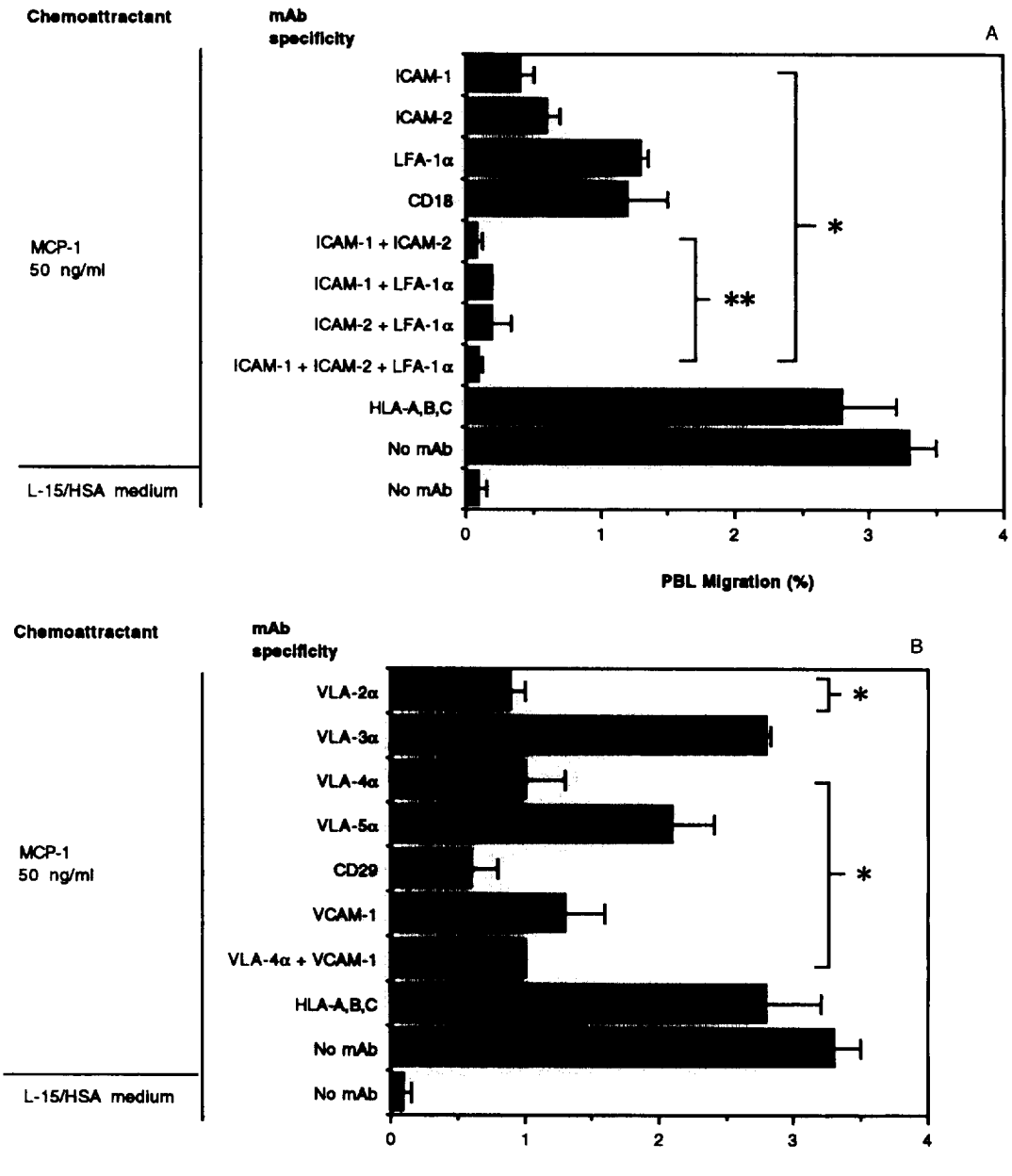


Fig. 10. Effect of mAb to leukocyte and endothelial cell molecules on PBL transendothelial chemotaxis to MCP-1. Saturating concentrations of the mAb listed in Fig. 8 (*A*) or Fig. 9 (*B*) were used with controls as described in Fig. 8 in a 4 h transendothelial chemotaxis assay to MCP-1 50 ng/ml. These data represent the mean percentage migration ± range from one of three independent experiments with similar results. *A*: *, $p < 0.05$ when compared to controls (MCP-1 with mAb to HLA-A,B,C or no mAb); **, $p < 0.05$ when compared to any of the individual anti-adhesion mAb used (ICAM-1, ICAM-2, LFA-1α, or CD18) and not significantly different than media control (L-15/HSA). *B*: *, $p < 0.05$ when compared to controls (MCP-1 with mAb to HLA-A,B,C or no mAb).

chemoattractant in PHA supernatant [1], did not increase expression of any of these four inducible adhesion molecules (Fig. 7A). When HUVEC-covered filters were cultured with PHA supernatant or TNF-α (200 U/ml) for 4–6 h, washed, and then used in assays, migration was increased approximately 1.5–3-fold compared to control filters (Fig. 7B and data not shown). Migration to control media was also increased, however, and the signal-to-background ratio was decreased.

3.3. Lymphocyte chemotaxis through HUVEC is inhibited by anti-adhesion-molecule mAb

We utilized mAb to several human leukocyte and endothelial adhesion molecules known to be important in lymphocyte-HUVEC interactions to investigate their role in lymphocyte transendothelial chemotaxis. PBL chemotaxis to PHA supernatant was significantly inhibited upon addition of mAb to either ICAM-1, ICAM-2, LFA-1α, or the leukocyte integrin β_2 subunit compared to either no mAb or a control mAb (anti-HLA-A,B,C), which binds both lymphocytes and HUVEC ($p < 0.05$) (Fig. 8a). Several combinations of 2 or 3 of these mAb produced significantly greater inhibition, with PBL chemotaxis reduced to $< 25\%$ of that seen with no mAb or a control mAb. When tested in assays with filters lacking endothelial monolayers, none of these antibodies caused significant inhibition of PBL chemotaxis to PHA supernatant (Fig. 8b). These results demonstrate that adhesion interactions between LFA-1 and ICAM-1/ICAM-2 are important for PBL transendothelial chemotaxis in the assay.

HUVEC cultured for 7–14 days on Transwell inserts synthesize and deposit fibronectin, laminin, and multiple types of collagen [23] (S. Roth, unpublished observations). Because lymphocytes must traverse the subendothelial ECM to transmigrate, we also evaluated the effect of mAb to several of the leukocyte β_1 integrins, which function as counter-receptors for these ECM components [13]. Significant inhibition of migration to PHA supernatant was produced by mAb to VLA-2α (CD49b), VLA-3α (CD49c), VLA-4α (CD49d), VLA-5α (CD49e), and the common β_1 integrin subunit (CD29) ($p < 0.05$) (Fig. 9A). The most pronounced inhibition was with mAb to β_1. Since VLA-4α also binds VCAM-1 on HUVEC [60], we assessed the effect of an anti-VCAM-1 mAb which blocks VLA-4-VCAM-1-mediated lymphocyte binding as well [35]. mAb to VCAM-1 significantly decreased chemotaxis, but was less inhibitory than the anti-VLA and anti-β_1 mAb ($p < 0.05$) (Fig. 9A). With mAb to VCAM-1 and VLA-4α combined, no significant additional inhibitory effect was observed. When tested in assays with filters lacking endothelial monolayers, these mAb did not significantly inhibit PBL chemotaxis (Fig. 9B). These data demonstrate that interactions of β_1 integrins with their ECM ligands also play an important role in lymphocyte transendothelial chemotaxis. The binding of VLA-4 to VCAM-1, however, contributes less to chemotaxis to PHA supernatant than the binding of LFA-1 to ICAM-1/ICAM-2.

In parallel experiments with these mAb using purified MCP-1 at 50 ng/ml as the chemoattractant, similar inhibition patterns were observed. Used individually, mAb to ICAM-1, ICAM-2, LFA-1α, and CD18 significantly inhibited PBL migration compared to either no mAb or the anti-HLA-A,B,C control mAb ($p < 0.05$) (Fig. 10A). When combined as indicated, migration was inhibited to media background levels by these mAb (Fig. 10A). mAb to VLA-2α, VLA-4α, VLA-5α and the β_1 subunit produced significant inhibition of migration to MCP-1, similar to the results obtained with PHA supernatant ($p < 0.05$) (Fig. 10B). The inhibition produced by mAb to VLA-3α was not statistically significant, however (Fig. 10B). Blocking VLA-4:VCAM-1 binding caused significant inhibition of migration to MCP-1 ($p < 0.05$) (Fig. 10B), but was again less inhibitory than blocking LFA-1:ICAM-1/ICAM-2 binding (Fig. 10A).

4. Discussion

We have developed an in vitro lymphocyte chemotaxis assay that models extravasation from the bloodstream and used it to characterize several important features of T lymphocyte transendothelial chemotaxis. HUVEC at passage 1–3 were seeded onto type I collagen-coated Transwell inserts and cultured until confluent. The HUVEC monolayer produced a physical barrier both to cell movement

and to diffusion of low molecular weight molecules. Background PBL transmigration was low and the signal-to-background ratio for chemotaxis was improved compared to assays using membranes without endothelium. Results with monolayers of other cell types and species suggested strong species restrictions in transendothelial chemotaxis. Human fibroblast monolayers also supported migration, which may be relevant to lymphocyte migration through tissues. HUVEC monolayers produced a barrier to diffusion that maintained a concentration gradient of low molecular weight dextran for more than 12 h. Diffusion to equilibrium occurred within 6 h in conventional chemotaxis chambers. The physical barrier generated by confluent HUVEC is expected to mimic more accurately the vascular barrier to cell emigration and chemoattractant diffusion present in vivo in blood vessels; the concentration gradient would be enhanced even more in vivo with removal of chemoattractant that diffuses across endothelium by blood flow.

The transendothelial assay was used in screening 29 potential sources for human lymphocyte chemoattractant activity. The greatest PBL migration was promoted by supernatants from PBMC stimulated for 3 days with PHA-P and from MLR. PHA supernatant stimulated PBL migration through both untreated filters and HUVEC-covered filters, demonstrating that endothelium is not required to detect the activity. Migration in both systems increased substantially between 2 and 4 h, and appeared to plateau between 4 and 6 h. In a checkerboard analysis using HUVEC-covered filters, the activity was shown to stimulate chemotaxis, not random locomotion of lymphocytes.

To confirm that T lymphocytes transmigrated to PHA supernatant, we used flow cytometry to analyze input and migrated PBL populations and performed assays with purified $CD3^+$ T lymphocytes as the input cell population. Flow cytometry of migrated PBL revealed them to be approximately 80% T lymphocytes. The proportion of monocytes ($CD14^+$) in the migrated population was consistently increased, suggesting the existence of a monocyte chemoattractant activity (-ies) in addition to a lymphocyte chemoattractant activity. Purified $CD3^+$ T lymphocytes also underwent chemotaxis to PHA supernant, although the percentage of cells migrating was reduced compared to PBL. This reduction in the number of purified T lymphocytes migrating was due, in part, to the contribution of monocytes to total PBL migration. While monocyte transmigration occurring either before or with T lymphocyte transmigration may enhance the number of lymphocytes migrating, monocytes are not required for lymphocyte chemotaxis in the assay.

We used the lymphocyte transendothelial chemotaxis assay to guide purification of the activity in PHA supernatant and demonstrated that MCP-1, a member of the C-C chemokine subfamily previously known as a monocyte chemoattractant [65], was the major T lymphocyte chemoattractant [1]. MCP-1 also is a chemoattractant for cloned $CD4^+$ and $CD8^+$ T lymphocytes [66] and peripheral blood T cells in modified Boyden assays [67]. Over 80% of the transmigratory response of purified T lymphocytes to PHA supernatant could be inhibited by a neutralizing antibody to MCP-1 [1]. Flow cytometric analysis of input PBL and lymphocytes which transmigrated to MCP-1 revealed that the responding subpopulation of T lymphocytes was chiefly composed of activated ($CD26^+$), memory-type ($CD45R0^+$, $CD29^+$, L-selectin-negative) cells [1]. Both $CD4^+$ (helper) and $CD8^+$ (cytotoxic) T lymphocytes migrated to MCP-1, but B and natural killer lymphocytes did not migrate at significant levels [1]. The ease of harvesting transmigrated cells for further characterization, such as these phenotypic analyses, is an advantage of this assay over modified Boyden chamber assays.

More recent studies with the lymphocyte transendothelial assay also show that the C-C chemokines MCP-2, MCP-3, RANTES, MIP-1α, and MIP-1β, but not the C-X-C chemokines IL-8 and IP-10, are active ([70]). Although cytokines such as IL-1, IL-2, and IL-4 have been reported to be active in some chemotaxis assays [16], we found no activity in the transendothelial assay.

PHA supernatant and MCP-1 are chemoattractive through a direct effect on lymphocytes, rather than through an indirect effect on endothelium. They were active in chemotaxis assays lacking an endothelial monolayer. Pertussis toxin pretreatment of lymphocytes inhibited $> 80\%$ transendothelial chemotaxis (data not shown). Pretreatment of endothelial cell monolayers with PHA supernatant or TNF as shown here, but not with MCP-1 [1], enhanced both chemo-

taxis and spontaneous migration and resulted in a decrease in the signal-to-background ratio. This enhancement with PHA supernatant may be due in part to the increased expression of ICAM-1 and VCAM-1. Further work is required to determine whether the endothelial monolayer plays an active role in presenting chemoattractants to T lymphocytes [20–22].

We examined the adhesive interactions required for T lymphocyte transendothelial chemotaxis. Results were similar for PHA supernatant and recombinant MCP-1. Chemotaxis was highly dependent on interaction of LFA-1 with its ligands on endothelium, ICAM-1 and ICAM-2. mAb to the LFA-1 αL and β_2 integrin subunits, and ICAM-1 and ICAM-2 molecules gave 50 to 90% inhibition of chemotaxis. mAb to the VLA α_2, α_4, and α_5 subunits, and to the common β_1 subunit, gave lesser but significant inhibition. mAb to β_1 gave more inhibition than mAb to α_2, α_4, or α_5, consistent with the sharing of the β_1 integrin subunit between the $\alpha_2\beta_1$, $\alpha_4\beta_1$, and $\alpha_5\beta_1$ molecules, and a role for each of these complexes. mAb to VCAM-1, a ligand for VLA-4 on endothelium, consistently produced less inhibition than mAb to ICAM-1 and ICAM-2. These results demonstrate the importance of β_1 integrins in interactions with the extracellular matrix, and to a lesser extent with VCAM-1, in transendothelial migration. Our results are consistent with studies showing that mAb to LFA-1α and ICAM-1 inhibit spontaneous migration of T lymphocytes through endothelial cell monolayers [11,12,14], and extend these results with the studies of ICAM-2 and β_1 integrins. By contrast to our results with transendothelial chemotaxis, none of the mAb we tested inhibited chemotaxis through uncoated polycarbonate filters. This supports the specificity of the effect in the transendothelial assay, and the congruence of this assay to results in vivo where mAb to LFA-1 inhibit lymphocyte emigration from the bloodstream [68,69].

The transendothelial chemotaxis assay provides a more physiologic model for studying lymphocyte emigration from the bloodstream than conventional in vitro chemotaxis assays lacking endothelium. It may be improved further, for example, by substituting microvascular endothelium for HUVEC, since lymphocyte emigration from the vasculature occurs primarily through post-capillary venules rather than through large veins such as the umbilical vein [3]. However, this chemotaxis assay with HUVEC provides remarkable parallels with in vivo emigration, including T cell responses to specific antigen in MLR or to mitogen that result in production of chemoattractive activity, dependence on LFA-1:ICAM-1 interactions, dependence on β_1 integrins, and selectivity for memory T lymphocyte subsets [1].

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