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Monocyte chemoattractant protein 1 acts as a T-lymphocyte chemoattractant

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ABSTRACT **We have utilized a transendothelial lymphocyte chemotaxis assay to identify and purify a lymphocyte chemoattractant in supernatants of mitogen-stimulated peripheral blood mononuclear cells. Amino acid sequence analysis revealed identity with monocyte chemoattractant protein 1 (MCP-1), a chemoattractant previously thought to be specific for monocytes. Recombinant MCP-1 is chemoattractive for purified T lymphocytes and for CD3$^+$ lymphocytes in peripheral blood lymphocyte preparations. The T-cell response to MCP-1 is dose-dependent and chemotactic, rather than chemokinetic. Phenotyping of chemoattracted T lymphocytes shows they are an activated memory subset. The response to MCP-1 by T lymphocytes can be duplicated in the absence of an endothelial monolayer and the majority of T-lymphocyte chemotactic activity in mitogen-stimulated peripheral blood mononuclear cell supernatants can be neutralized by antibody to MCP-1. Thus, MCP-1 is the major lymphocyte chemoattractant secreted by mitogen-stimulated peripheral blood mononuclear cells and is capable of acting as a potent T-lymphocyte, as well as monocyte, chemoattractant. This may help explain why monocytes and T lymphocytes of the memory subset are always found together at sites of antigen-induced inflammation.**

In antigen-specific inflammatory responses, monocytes and T lymphocytes adhere to and migrate between endothelial cells of the postcapillary venules and accumulate in the challenged site. Lymphocytes in the inflammatory site are predominately of the memory phenotype as distinguished by monoclonal antibodies to CD45RO or CD29 (1, 2). However, only a small fraction of T lymphocytes present in these lesions are antigen-specific (3). Thus, it has long been speculated that antigen-stimulated lymphocytes or antigen-presenting cells must recruit lymphocytes into an inflammatory site in a non-antigen-specific manner, presumably by secreting chemotactic factors.

In the past several decades, many compounds have been described as having lymphocyte chemotactic activity (4–6). However, despite interesting initial observations, lymphocyte-specific chemoattractant factors have yet to be purified to homogeneity or characterized as to amino acid sequence. In contrast, a number of chemoattractive cytokines, termed chemokines, have recently been found to induce chemotaxis of specific leukocyte subsets in modified Boyden chamber chemotaxis assays. These heparin-binding 70- to 80-amino acid proteins were initially identified as monocyte or neutrophil specific, but recently, some have been found to act on lymphocytes as well. These include the α chemokines, interleukin 8 and IP-10 (7, 8), and the β chemokines, RANTES and macrophage inflammatory protein 1α and 1β (9–12).

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To identify lymphocyte chemoattractants, we have developed an assay measuring lymphocyte chemotaxis across endothelial monolayers grown on polycarbonate filters. This assay more accurately simulates emigration of lymphocytes from the bloodstream than do assays that measure chemotaxis across filters alone (S.J.R., M.W.C., and T.A.S., unpublished data). The assay enhances measurement of lymphocyte chemotaxis, because it has a signal-to-noise ratio >10. Using this transendothelial assay, we have purified to homogeneity and sequenced the major lymphocyte chemoattractant secreted by mitogen-stimulated peripheral blood mononuclear cells (PBMCs). Surprisingly, it is monocyte chemoattractant protein 1 (MCP-1), the prototypic member of the β chemokine family (13, 14), that has previously been thought to be chemoattractive only for monocytes.

MATERIALS AND METHODS

Preparation of Peripheral Blood Lymphocytes (PBLs) and Purified T Cells. PBMCs were obtained as described (15). Two steps of plastic absorption to remove monocytes yielded PBLs. During the second absorption step, PBLs were fluorescently labeled with 2′,7′-bis-(2-carboxyethyl)-5(and -6)-carboxyfluorescein (BCECF; Molecular Probes) at 0.5 μg/ml for 30 min (16), then washed, and resuspended at 5×10^6 cells per ml in assay medium (a 1:1 mixture of RPMI medium 1640/M199 plus 0.25% human serum albumin). T cells were purified using the magnetic cell separation (MACS; Miltenyi Biotec, Sunnyvale, CA) protocol (17). Briefly, PBMCs were incubated for 30 min on ice with the antibodies, MY4 (Coulter; 5 μg/ml), OKM1 (5 μg/ml), B1 (Coulter; 5 μg/ml), and 3G8 (5 μg/ml), to deplete monocytes, B cells, and natural killer cells, respectively. After incubation, cells were washed and incubated with 20 μl of MACS rat anti-mouse IgG1 and IgG2a+2b microbeads (Miltenyi Biotec) per 10^7 cells for 20 min on ice. After the incubation, cells were loaded onto a preequilibrated magnetic separation column coupled to the MACS magnet. T cells were stained with BCECF, washed, and resuspended in assay medium as described for PBLs. Flow cytometry studies showed that PBLs typically contained ≈5% monocytes whereas purified T cells contained <1% monocytes, B cells, or natural killer cells.

Transendothelial Chemotaxis Assay. Isolation and culture of human umbilical vein endothelial cells (HUVECs) were performed as described (18). HUVECs were cultured on collagen-coated 6.5-mm-diameter Transwell culture inserts (Costar) with an 8-μm pore size (or 5 μm where noted) as described (S.J.R., M.W.C., and T.A.S., unpublished data). Chemotactic factors (diluted in assay medium) or control

Abbreviations: PBMC, peripheral blood mononuclear cell; MCP-1, monocyte chemoattractant protein 1; PBL, peripheral blood lymphocyte; BCECF, 2′,7′-bis-(2-carboxyethyl)-5(and -6)-carboxyfluorescein; HUVEC, human umbilical vein endothelial cell; PHA, phytohemagglutinin; PE, phycoerythrin.

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medium were added to 24-well tissue culture plates in a final volume of 600 μl. HUVEC-coated Transwells were inserted into each well and BCECF-labeled PBLs or T cells were added to the top chamber in a final volume of 100 μl. A 1:20 dilution of these cells added to a well containing medium alone, but without a Transwell insert, served as a measure of the input number of cells. The assay plates were incubated for 4 hr at 37°C in 5% CO_2/95% air. After a 4-hr incubation, the Transwells were removed, the cells in each well bottom were counted by fluorescent microscopy. Four 10 × 10 grids (0.1 mm per grid) were counted per well. All samples were tested in duplicate and each experiment has been repeated a minimum of three times with similar results. In each experiment, the ability of the endothelial cell monolayer to act as a barrier against the upward diffusion of low molecular weight substances was assessed (S.J.R., M.W.C., and T.A.S., unpublished data). A neutralizing polyclonal rabbit anti-human MCP-1 antibody (Genzyme) and normal rabbit IgG (R&D Systems) were used in some assays. Purified recombinant MCP-1, expressed in *Escherichia coli*, was purchased from Peprotech (Rocky Hill, NJ) and purified recombinant MCP-1 expressed transiently in COS cells was a kind gift from B. Rollins (Dana–Farber, Boston).

Generation of Chemoattractant Supernatant. PBMCs were prepared from peripheral blood leukopaks (Children's Hospital, Boston) as described above. PBMCs were washed and resuspended at 2–4 × 10^6 cells per ml in serum-free X-VIVO medium (BioWhittaker) supplemented with 2 mM L-glutamine, gentamicin (50 μg/ml), indomethacin (1 μg/ml), 3 mM lithium chloride, 50 μM hydroxyurea, and phytohemagglutinin P (PHA-P; Sigma; 2.5 μg/ml). After 3 days at 37°C, culture supernatant (termed PHA supernatant) was collected, centrifuged, filtered, and stored at −70°C.

Purification of Lymphocyte Chemoattractant. PHA supernatant (4–6 liters) was adsorbed to a 40-ml heparin-Sepharose CL-6B column (Pharmacia) and protein was eluted using a 300-ml salt gradient from 0.15 to 1.5 M NaCl in 0.01 M Tris·HCl (pH 7.5). Pooled fractions from heparin-Sepharose chromatography were concentrated 20 times and applied to a 125-ml Sephadex G-75 column (Pharmacia). Active fractions from Sephadex G-75 chromatography were concentrated 40 times and applied to a 4.6 × 150 mm C_4 reverse-phase column (5 μm; Vydac, Hesperia, CA), equilibrated with 0.1% trifluoroacetic acid in water. Protein was eluted using a three-step gradient [0–25% (vol/vol) acetonitrile with 1% change in acetonitrile concentration per min, 25–45% acetonitrile with 0.2% change per min, and 45–80% acetonitrile with 1% change per min], in water containing 0.1% trifluoroacetic acid. Flow rate was 1 ml/min and all protein peaks were collected individually. HPLC fractions were subjected to N-terminal sequence analysis or proteolytic digestion in solution, followed by HPLC separation and sequencing, as has been described (19).

Flow Cytometry of Migrated Leukocytes. The transendothelial chemotaxis assay was performed as described above using HUVEC-coated 24.5-mm-diameter 8-μm (pore size) Transwell filters. Recombinant MCP-1 was added to the bottom chambers in a final volume of 2 ml and 1 ml of non-BCECF-labeled PBLs at 5–7 × 10^6 cells per ml was added to the top chamber. After incubation, the migrated cells were harvested, washed, and resuspended at 10^7 cells per ml in L-15 medium (GIBCO) with 2% (vol/vol) fetal calf serum. Cells from the starting and migrating populations were stained with the following mouse anti-human monoclonal antibodies directly conjugated to fluorochromes: anti-CD3 (Caltag, South San Francisco, CA); UCHL-1 to CD45RO (Harlan Bioproducts, Indianapolis); MY4 to CD14, T4 to CD4, T8 to CD8, B1 to CD20, 2H4 to CD45RA, Ta1 to CD26, 4B4 to CD29, and mouse IgG (Coulter); and anti-Leu-8 to L-selectin and anti-Leu-11 to CD16 (Becton Dickinson). All antibodies were coupled to fluorescein isothiocyanate with the exceptions of anti-CD3 coupled to R-phycoerythrin (PE)-Cy5 tandem conjugate and MY4 coupled to either fluorescein isothiocyanate or PE. Cells were gated based on their forward vs. 90° scatter profile. PE-conjugated antibodies specific to monocyte and endothelial cell antigens were added to confirm that all gated cells were lymphocytes. Antibody staining was done according to manufacturer's instructions.

RESULTS

By using a lymphocyte transendothelial chemotaxis assay, a lymphocyte chemotactic response was obtained to supernatants of PHA-stimulated PBMCs that was 10 times greater than the response to medium control or to PHA alone (S.J.R., M.W.C., and T.A.S., unpublished data). This lymphocyte chemotactic activity was purified using the transendothelial chemotaxis assay to identify active fractions. The activity was eluted from heparin-Sepharose in 0.5–0.6 M NaCl. Purification by size-exclusion chromatography on Sephadex G-75 showed it had a molecular mass of ≈16 kDa. In the final step of purification, a single peak of lymphocyte chemotactic activity that corresponded to a prominent protein doublet was eluted in 28–29% acetonitrile from a C_4 reverse-phase HPLC column (Fig. 1).

The molecular masses of the two active peaks were 9336 and 8657 Da, as determined by laser desorption mass spectrometry. The N termini of both active peaks were found to be blocked; therefore, these fractions were subjected to tryptic digestion followed by reverse-phase HPLC separation and microsequencing. Each peak yielded sequences of the peptides EICADPKQKXVQDXMX(H) and (W)VQDSM-

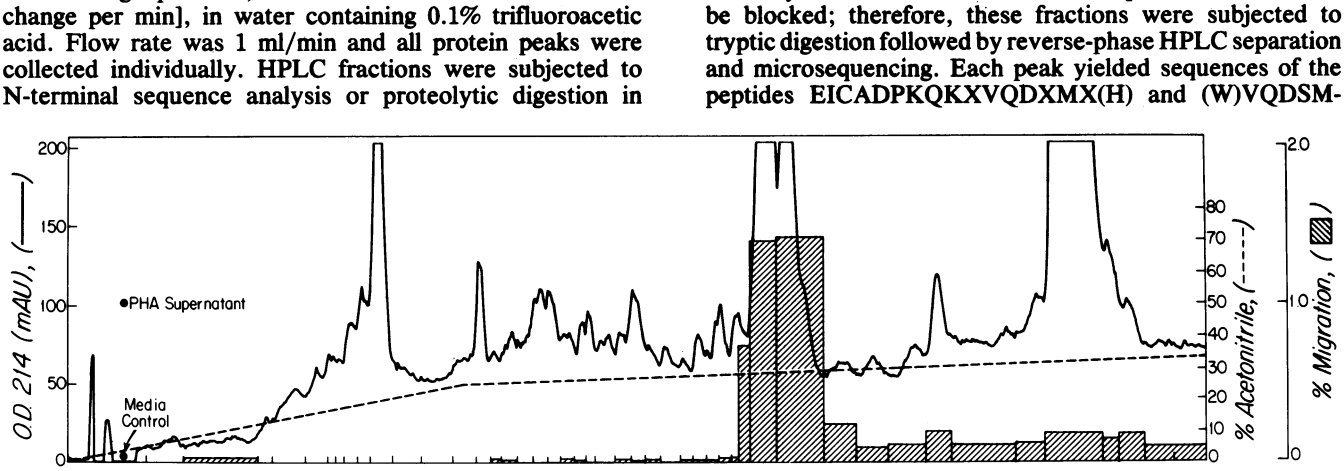

FIG. 1. Reverse-phase HPLC. Lymphocyte chemotactic activity purified by heparin-Sepharose and size-exclusion chromatography was applied to a C_4 reverse-phase column and eluted with acetonitrile in 0.1% trifluoroacetic acid. One-tenth of each fraction was lyophilized and one-half of each lyophilized fraction was tested in duplicate in the transendothelial chemotaxis assay (hatched bars). The experiment shown is representative of three purifications. mAU, arbitrary units (×10^{-3}).

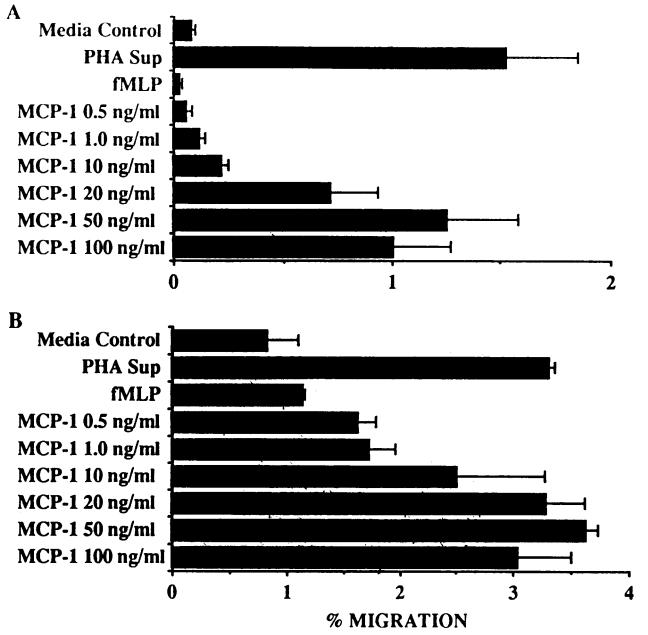

FIG. 2. T lymphocytes respond to recombinant MCP-1 in the presence or absence of an endothelial monolayer. Transendothelial migration of purified T lymphocytes in response to increasing doses of recombinant prokaryotic MCP-1 was assessed in the presence (*A*) or absence (*B*) of an endothelial monolayer using 5-μm-pore Transwells. MCP-1 was assayed at the concentrations indicated; fMet-Leu-Phe (fMLP) was 10 nM; PHA supernatant (Sup) was assayed at a 1:30 dilution. The data are representative of three experiments. Bars show the range of duplicates.

DHLDKQTQTPKT. These sequences are identical to the sequence of human MCP-1 (20, 21). The mass of the second eluting peak, 8657 Da, is in good agreement with the mass predicted for MCP-1 with the N-terminal glutamyl residue converted to a pyroglutamyl residue, 8664 Da. The mass of the first peak suggests a covalent addition, perhaps three O-linked carbohydrate residues. Two MCP-1 species with identical amino acid composition have been reported (22) with the earlier eluting peak in reverse-phase HPLC having a higher molecular mass in SDS/PAGE.

The N-terminal sequence of the active peaks from HPLC of another lymphocyte chemoattractant preparation was DAINAPV, which corresponds to the previously published sequence of MCP-1 beginning at residue 3 and, therefore, demonstrates distinct N-terminal processing of MCP-1. Sequencing of the other major peak from HPLC, eluting in 32% acetonitrile, identified it as interleukin 8. This peak was consistently inactive in the transendothelial lymphocyte chemotaxis assay.

To confirm identification of MCP-1 as a lymphocyte chemoattractant, purified recombinant human MCP-1 was tested in our transendothelial chemotaxis assay, using purified T cells as the migrating cells (Fig. 2*A*). fMet-Leu-Phe was included as a control for residual monocyte migration and PHA supernatant was included as a positive control. T cells were found to migrate to MCP-1 in a dose-dependent manner. Optimal T-cell chemotaxis was seen at 50 ng/ml but a good T-cell response was also seen at 20 ng/ml, the concentration for optimal monocyte chemotaxis. Eukaryotic and prokaryotic recombinant MCP-1 gave similar dose–response curves for T-cell chemotaxis (data not shown).

T cells were allowed to migrate to MCP-1 through 5-μm-pore Transwells without an endothelial monolayer to determine whether the endothelium was necessary to observe T-cell chemotaxis to MCP-1 (Fig. 2*B*). T cells were able to migrate to MCP-1 in the presence or absence of endothelial cells. Although the background migration was higher without endothelium, the dose-dependent migration to MCP-1 was similar with or without an endothelial monolayer. To further understand the role of endothelium in the lymphocyte response to MCP-1, endothelial cells were incubated with MCP-1 (20 ng/ml) for 4 hr and their level of cell adhesion molecule expression was examined by flow cytometry. MCP-1 caused no significant upregulation in the expression of cell adhesion molecules ICAM-1, ICAM-2, VCAM-1, E-selectin, or P-selectin (data not shown). Additionally, supernatants collected from 4-hr cultured endothelial cells or monocytes after pretreatment with MCP-1 (20 ng/ml) for 1 hr and washing showed no activity above background in the lymphocyte transendothelial chemotaxis assay (data not shown).

Both T lymphocytes and residual monocytes present in PBL preparations migrated to MCP-1 as shown by staining with CD3 and CD14 monoclonal antibodies, in agreement with migration to fMet-Leu-Phe by PBLs but not purified T cells (data not shown). A higher percent of PBLs always migrated to MCP-1 than to fMet-Leu-Phe.

Purified T cells were subjected to checkerboard analysis to examine whether MCP-1-induced migration is chemotactic or chemokinetic (Table 1). When a greater concentration of MCP-1 was in the bottom chamber, migration was 10–30 times more than background, but there was little migration when the concentration of MCP-1 in the top and bottom chambers was equal. Thus, the T-cell response to MCP-1 is due to directed migration rather than to increased random migration.

Polyclonal rabbit anti-human MCP-1 antibody was consistently found to neutralize the majority, but not all, of the T-cell chemotactic activity present in PHA supernatant (Fig. 3). In contrast, MCP-1 antibody was able to completely neutralize the T-cell response to recombinant MCP-1. Thus, MCP-1 is the major, but probably not the sole, lymphocyte chemoattractant in PHA supernatant.

The phenotype of cells in PBL preparations migrating to recombinant MCP-1 was determined by flow cytometry of the starting population of cells as compared to cells that migrated into the lower chamber during the transendothelial chemotaxis assay. Lymphocytes were separated from monocytes based on forward and 90° light scatter, as verified by the lack of CD14 expression in the gated population, and then

Table 1. Checkerboard analysis of T-cell migration to MCP-1

	Migration, %			
	50 ng/ml	20 ng/ml	10 ng/ml	0 ng/ml
50 ng/ml	0.12 (± 0.04)	0.56 (± 0.03)	1.17 (± 0.00)	3.69 (± 0.96)
20 ng/ml	0.10 (± 0.01)	0.16 (± 0.06)	0.68 (± 0.06)	1.06 (± 0.08)
10 ng/ml	0.01 (± 0.00)	0.11 (± 0.07)	0.09 (± 0.04)	0.53 (± 0.01)
0 ng/ml	0.01 (± 0.00)	0.13 (± 0.00)	0.17 (± 0.04)	0.11 (± 0.08)

Purified T lymphocytes were assayed for transendothelial chemotaxis, with the indicated concentrations of recombinant MCP-1 (0–50 ng/ml) in the top and bottom compartment of the wells, shown above and to the left of the matrix, respectively. Values in parentheses show range of duplicates. The experiment is representative of three similar experiments.

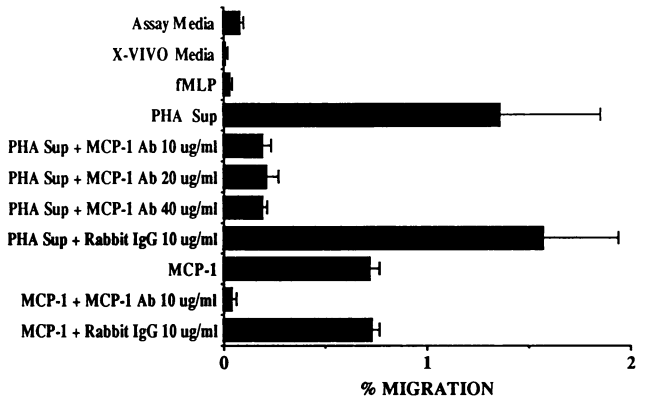

FIG. 3. Antibody to MCP-1 neutralizes lymphocyte chemotactic activity in PHA supernatant. Transendothelial migration (8-μm-pore Transwells) of purified T lymphocytes to recombinant MCP-1 or PHA supernatant (Sup) was assayed in the presence or absence of neutralizing rabbit anti-human MCP-1 polyclonal antibody (Ab) or normal rabbit IgG control. MCP-1 was at 20 ng/ml; PHA supernatant was a 1:10 dilution; fMet-Leu-Phe (fMLP) was at 10 nM. Data are representative of three experiments. Bars show the range of duplicates.

were examined as to cell surface phenotype (Fig. 4). Virtually no naive T cells, as defined by expression of CD45RA, respond to MCP-1. In contrast, memory cells appear to be selected as shown by expression of CD45RO, increased expression of CD29, and the increased percentage of L-selectin-negative cells. The vast majority of migrating lymphocytes appear to be activated, as demonstrated by their expression of the activation marker CD26. Both $CD4^+$ and $CD8^+$ T cells migrate to MCP-1, with a slight preference for $CD4^+$ cells seen in some but not all experiments. B cells and natural killer cells, as defined by the cell-specific antigens CD20 and CD16, respectively, were detected in the starting population but not in the migrated cell population.

Kinetic studies of cells migrating in response to MCP-1 indicate that significant levels of T lymphocytes do not respond until 4 hr. This is in contrast to monocytes that respond significantly to MCP-1 in as little as 1 hr (data not shown).

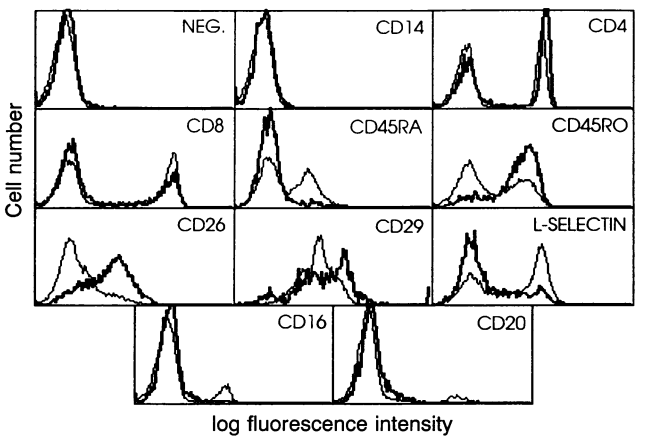

FIG. 4. Phenotype of lymphocytes migrating to MCP-1 through endothelial monolayers. Starting input cells (thin line) and cells migrating to MCP-1 (50 ng/ml; thick line) were collected at 4 hr, incubated with antibodies, and subjected to three-color flow cytometry (three-decade logarithmic scale). PE-coupled antibodies to monocyte and endothelial cell antigens were used to determine that the scatter-gated population included only lymphocytes. The CD14 results are included to confirm that the gated populations of cells are free of monocytes. Histograms are from one of two similar experiments using three-color immunofluorescence. Similar results were obtained in six experiments using single-color indirect immunofluorescence.

DISCUSSION

It has long been suspected that a chemoattractant secreted at sites of antigenic stimulation is capable of recruiting lymphocytes from the bloodstream into inflammatory lesions. Using a physiologically relevant transendothelial lymphocyte chemotaxis assay, we have purified a lymphocyte chemoattractant in the supernatant of PHA-stimulated PBMCs. Unexpectedly, amino acid sequence analysis of this lymphocyte chemoattractant showed it to be identical to the previously characterized monocyte chemoattractant, MCP-1 (22–24). To confirm this result, we tested recombinant MCP-1 in the transendothelial chemotaxis assay. We showed that MCP-1 attracts both purified T cells and T cells within PBL preparations. T cells respond to MCP-1 in a dose-dependent manner and by chemotaxis rather than chemokinesis. Furthermore, we demonstrated that MCP-1 is responsible for the majority, but probably not all, of the T-lymphocyte chemotactic activity within PHA supernatant, as shown by neutralization with antibody to MCP-1. The lymphocyte response to MCP-1 does not depend on the endothelium present in our assay system, since T lymphocytes migrate through filters to MCP-1 in the presence or absence of an endothelial cell monolayer. However, the endothelial cell monolayer greatly enhances the signal-to-noise ratio of the lymphocyte response to MCP-1 by decreasing nonspecific migration.

Only a subpopulation of lymphocytes respond to MCP-1. The chemoattracted lymphocytes are all of the memory phenotype, as shown by the finding that they are $CD45RA^-$, $CD45RO^+$, $CD29^+$, and mostly L-selectin-negative (25). Furthermore, they appear to be activated, as shown by increased expression of CD26, the ectoenzyme dipeptidyl peptidase IV expressed on activated, but not resting, T cells (26, 27). Both $CD4^+$ and $CD8^+$ T cells respond to MCP-1. Neither $CD20^+$ B cells nor $CD16^+$ natural killer cells migrate to MCP-1 at significant levels.

We show that MCP-1 acts as a T-cell, as well as a monocyte, chemoattractant. A number of groups have examined MCP-1 chemotactic specificity, yet, to our knowledge, none has reported a lymphocyte response (23, 24). Several possibilities may explain the difference in these results. Because lymphocytes are less motile than neutrophils or monocytes, their chemotaxis has always been more difficult to measure (28). Indeed, we found that the kinetics of the lymphocyte and monocyte responses to MCP-1 differ. Monocytes migrate easily in as little as 1 hr; however, T-cell migration is not significant until 4 hr. Many of the groups looking at migration to MCP-1 examined their chemotaxis assays at 90 min and, thus, would have seen little lymphocyte migration (22, 23, 29). Because the endothelial barrier in our assay greatly slows the dissipation of the chemotactic gradient (S.J.R., M.W.C., and T.A.S., unpublished data), chemotaxis can continue longer than in standard Boyden chamber assays where chemoattractants diffuse to equilibrium within 4 hr (30). Thus, the transendothelial variation of the traditional chemotaxis assay allows us to more easily detect the later-occurring T-cell migration. Additionally, the presence of endothelium in our assay decreases nonspecific background migration, allowing small numbers of migrating T cells to be detected. However, as we have demonstrated, endothelium is not required to observe T-cell chemotaxis to MCP-1.

MCP-1 has been found in many T-cell-mediated inflammatory conditions (31–33) including delayed-type hypersensitivity reactions and rheumatoid arthritis. In each of these pathologies, both monocytes and lymphocytes migrate to the inflammatory site. The monocytic infiltration usually pre-

cedes the major lymphocytic infiltration (34) and this is consistent with the kinetics we have observed. Additionally, the T cells migrating to these inflammatory sites are usually of the activated memory phenotype (1, 2, 35), the same phenotype as the T cells responding to MCP-1. The observations that MCP-1 can act as a chemoattractant for both lymphocytes and monocytes and is the major lymphocyte chemoattractant secreted by mitogen-activated mononuclear cells may have great relevance for the classic *in vivo* observation that lymphocytes and monocytes are found together at sites of antigen-induced inflammation. Further work is required to test the importance of MCP-1 in lymphocyte and monocyte emigration in antigen-specific reactions and autoimmune disease *in vivo*.

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