Human Macrophage Inflammatory Protein α (MIP-1α) and MIP-1β Chemokines Attract Distinct Populations of Lymphocytes

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Summary
Lymphocyte trafficking is an essential process in immune and inflammatory functions which can be thought to contain at least two main components: adhesion and migration. Whereas adhesion molecules such as the selectins are known to mediate the homing of leukocytes from the blood to the endothelium, the chemoattractant substances responsible for the migration of specific subsets of lymphocytes to sites of infection or inflammation are largely unknown. Here we show that two molecules in the chemokine (for chemoattractant cytokine) superfamily, human macrophage inflammatory protein 1α (MIP-1α) and MIP-1β, do not share identical attractant activities for lymphocyte subpopulations. When analyzed in vitro in microchemotaxis experiments, HuMIP-1β tends to attract CD4+ T lymphocytes, with some preference for T cells of the naive (CD45RA) phenotype. HuMIP-1α, when tested in parallel with HuMIP-1β, is a more potent lymphocyte chemoattractant with a broader range of concentration-dependent chemoattractant specificities. HuMIP-1α at a concentration of 100 pg/ml attracts B cells and cytotoxic T cells, whereas at higher concentrations (10 ng/ml), the migration of these cells appears diminished, and the migration of CD4+ T cells is enhanced. Thus, in this assay system, HuMIP-1α having greater effects than HuMIP-1β, particularly on B cells.

The chemokines are a superfamily of small proteins (8,000–14,000 M,) secreted primarily by leukocytes and related by a conserved four-cysteine motif (1, 2). These proteins have been implicated in a wide range of acute and chronic inflammatory processes, as well as other immunoregulatory functions. The superfamily’s two branches are classified as the C-X-C and the C-C groups, as defined by the spacing of the first two cysteines in the conserved motif. Early characterizations of the two branches of the chemokine superfamily showed that, generally, C-X-C chemokines, such as the neutrophil attractant and activating factors (IL-8), melanoma growth-stimulatory activity (MGSA/gro), neutrophil-activating peptide 2 (NAP-2), and a neutrophil-activating protein derived from epithelial cells (ENA-78), are potent chemoattractants and activators of neutrophils but not monocytes (3–6), whereas the C-C chemokines, including such molecules as human monocyte chemotactic protein 1 (MCP-1) RANTES, and the macrophage inflammatory proteins 1α and 1β (HuMIP-1α and -1β) proteins exhibit chemoattractant potential for monocytes, but not neutrophils (7–9). C-C chemokines also have effects on other blood leukocytes. MCP-1 and RANTES are potent direct mediators of the release of histamine by human basophils (10–12), whereas RANTES, and to a lesser extent HuMIP-1α, are chemoattractants and activators of eosinophils (13, 14). The regulatory signals impaired by C-C chemokines can also be negative. MIP-1α has been reported to be a unique and specific inhibitor of the proliferation of hematopoietic stem cells (15).

A poorly understood area of lymphocyte biology is the specific targeting of lymphocyte effector cell subpopulations during an immune challenge or inflammatory response. We have shown that the C-C chemokine RANTES is a selective chemoattractant for T lymphocytes of the memory phenotype in vitro (9), and IL-8 has been reported to attract T cells in vitro and in vivo (16). A recent report has implicated human MIP-1β in the induction of the adhesive properties of T lymphocytes, and found this molecule to be localized to lymph node endothelium (17). In addition, we have discussed the possible roles of the MIP-1 proteins in differential attraction of lymphocytes (1), and their roles in a three-step model of leukocyte adhesion has been postulated (18). A detailed analysis of their chemoattractant properties, how-
ever, has never been presented. Here we detail the differential chemotactic potentials of the two closely related human chemokines HuMIP-1α and -1β for human lymphocytes, as determined using purified recombinant proteins in an in vitro microchemotaxis system.

**Materials and Methods**

**Preparation of Recombinant Chemokines.** A cDNA for HuMIP-1β was isolated by screening a human cultured T cell cDNA library with an oligonucleotide of 50 residues corresponding to the first 50 coding sequence nucleotides of the hH400 molecule (19). For HuMIP-1α, a cDNA was isolated using PCR, employing an antisense primer made against nucleotides 283 to 236 in the published LD78 sequence (20) and was used in the reverse transcription of 7.5 mg of total RNA from an antigen-induced cultured functional T cell line. The sense primer was made to match nucleotides −19 to 3 in the LD78 sequence and used in concert with the antisense primer in a PCR of the cDNA. Mammalian expression system–derived material was used for preliminary determinations of HuMIP-1α and -1β attractant potentials. In these experiments, the cDNA inserts were subcloned into the pRK5 expression vector, transfected into the human embryonic kidney cell line 293, and transiently expressed as described for RANTES (9). Confirmation and quantitation of the chemotactic activities was obtained using purified recombinant HuMIP-1α and -1β chemokines. These were produced in *Escherichia coli* by linking cDNAs encoding the mature, secreted forms of the molecules (devoid of the mammalian signal sequence) to the bacterial STII promoter in an expression plasmid.

HuMIP-1α was purified from *E. coli* fermentation medium after first removing the cells by low-speed centrifugation. The pH of the supernatant was then adjusted to ~3 with phosphoric acid and the resulting precipitate was removed by a second low-speed spin. After readjustment to pH 5, the HuMIP-1α contained in the clear supernatant was captured on a bare silica column and eluted with an ethanol/sodium chloride buffer at the same pH. The silica pool was dialyzed into a dilute Tris-hydroxy amino methane buffer at neutral pH and loaded onto a DEAE-Sepharose column, from which HuMIP-1α was eluted at a step up in ionic strength. The product was further purified by C4 reversed-phase HPLC using a linear gradient of acetonitrile. Solvent was removed by capture and elution from DEAE-Sepharose as described above, and the purified product was formulated for administration by dialysis into an isotonic citrate/sodium chloride buffer. HuMIP-1α was purified using this procedure contained <1 EU/mg as determined in the limulus amoebocyte lysate test. HuMIP-1β was purified from recombinant *E. coli* by dispersing cell paste in phosphate buffer at neutral pH, followed by homogenization at ~20,000 psi. The homogenate was adjusted to pH 3 and centrifuged briefly to pellet cell debris. The supernatant was decanted and adjusted to pH 6 with sodium hydroxide, initiating the precipitation of HuMIP-1β. After centrifugation, the supernatant was discarded and the pellet dissolved with ~1 M NaCl/20 mM acetic acid, pH 3. This solution was then applied to a column of phenyl Toyopearl equilibrated in the same buffer. After analysis by reversed-phase HPLC, selected fractions of the column flow-through were pooled and dialyzed across a 5-kD cellulose membrane versus 20 mM acetic acid, pH ~3.2. Purity as judged by HPLC was >99%. Pyrogen was not detectable in LAL assay at a sensitivity limit of 5 EU/mg.

**Lymphocyte Migration Assays.** Lymphocyte migration in response to either HuMIP-1α and -1β conditioned media or purified recombinant chemokines was assessed. Leukocytes isolated from whole blood were depleted of monocytes by a two-step adherence to plastic, resulting in a cell distribution (as evaluated by immuno-cytometric detection) routinely <1% monocytes (9, 21). Test substances and controls are tested in the same assay, each in duplicate wells of the microchemotaxis chamber. After incubation for 1 h, fixing, and staining as described (9, 21), lymphocyte migration is evaluated using either an image analyzer, or by counting the cells by eye. In the first instance, a migration index (MI) is calculated as the area of lower surface of filter occupied by cells after stimulation with chemotactic peptide divided by the area of filter occupied by unstimulated, randomly migrating cells (9, 21). In the second, the MI is the actual number of cells migrating in response to the test substance divided by the number of cells that have randomly migrated in the control chambers.

**Results and Discussion**

Initial experiments were done with recombinant HuMIP-1α and -1β proteins obtained from a mammalian expression system as described for RANTES (9). Though the mature secreted forms of HuMIP-1α and -1β are nearly 70% identical in predicted sequence, they display biochemically distinct behaviors, possibly due to glycosylation differences (1). After transfection of the human embryonic kidney cell line 293 with either HuMIP-1α or -1β cDNA expression constructs, proteins of ~8,000 and 12,000 daltons, respectively, are released into the cell culture medium (1). We initially tested the ability of such 293 cell supernatants containing recombinant HuMIP-1α and -1β to attract lymphocytes isolated from peripheral blood using a microchemotaxis assay system (9, 21). In *n* = 6 initial experiments, conditioned media from 293 cells producing recombinant HuMIP-1α and -1β both contained a potent lymphocyte attracting activity (> the positive control, 5% zymosan-activated plasma, at optimal dilution of chemokine-containing cell culture supernatants), whereas media from control transfected cells had no effect (1, and data not shown).

To confirm that these effects were due solely to the presence of the recombinant chemokines, we next obtained purified recombinant HuMIP-1α and -1β using a bacterial expression system. The cumulative results of four experiments, each determination performed in duplicate using the purified recombinant chemokines, are shown in Fig. 1. In these experiments, HuMIP-1α and -1β were tested in parallel in the same assay, in order to make direct comparisons of their activities possible, and a MI was determined by directly counting the number of responding cells. Both HuMIP-1α and HuMIP-1β show potent chemotactic activity over a wide con-
centration profile, as exemplified from the bell-shaped curves representing the MI at each concentration of chemokine. However, interesting differences in the patterns of response to the two chemokines can be noted. The curve for HuMIP-1β (●, Fig. 1) shows a maximal MI of ~2.7 at 100 pg/ml of chemokine, with the curve decreasing uniformly at either higher or lower concentration of the chemokine. Such bell-shaped profiles are typical of in vitro chemotaxis assays. In the case of HuMIP-1α, however, the pattern is different (□, Fig. 1). Not only is HuMIP-1α more potent than HuMIP-1β, with a maximal MI of ~4.2 at 100 pg/ml, but the MI curve is consistently biphasic, with a second distinct peak at a concentration of 10 ng/ml (MI ~3.7) of the chemokine. In all cases, the control buffer tested in parallel showed no chemoattractant activity (△, Fig. 1).

Immunohistochemical phenotyping of the migrating lymphocytes revealed differences in the attractant properties of these two related cytokines. Initially the assays were run at the concentration of chemokine that produced the highest MI (100 pg/ml for each chemokine), and phenotyping performed on the migrating cells using a panel of primary mAbs that recognize B cells, MHC class II surface antigens, all T cells, or the T cell subset-specific markers CD4, CD8, CD45RO, or CD45RA (9, 21). CD45RA and CD45RO define two different isoforms of the leukocyte common antigen CD45. These isoforms have been thought of as markers that may differentiate naive from memory T cells (22, 23).

Upon stimulation of virgin or naive CD45RA T cells, a differentiation event occurs that results in the loss of the CD45RO isoform and the concomitant expression of the CD45RA isoform. It is within the CD45RO population of cells that T cell memory is thought to reside (24–28).

There is a marked migration of T but not B cells, in response to 100 pg/ml of HuMIP-1β (Fig. 2). Further, there is no significant migration of CD8+ cells to HuMIP-1β. Migration is largely preferential to cells that are CD4+.

HuMIP-1β also shows a modest, but statistically significant tendency to attract T cells of the CD45RA+ phenotype (Fig. 2). This can be contrasted with what has been seen with RANTES, which causes the preferential migration of CD45RO+ T cells while not affecting the CD4+/CD45RA+ cells (9). Lastly, the distribution of responding phenotypes is similar at other concentrations of the recombinant HuMIP-1β chemokine (data not shown).

Since maximal lymphocyte attraction to recombinant HuMIP-1α was biphasic, lymphocytes responding to each of these concentrations (100 pg/ml and 10 ng/ml) were separately phenotyped and distinct differences in concentration-specific attractive activities were noted. First, it was observed that HuMIP-1α was a more potent T lymphocyte attractant than HuMIP-1β. At 100 pg/ml, HuMIP-1α attracted 345 ± 47 T lymphocytes (Fig. 3) as compared with 227 ± 12 for HuMIP-1β (Fig. 2). Second, whereas neither HuMIP-1β, in these experiments, nor RANTES, in previous experiments (9 and Schall, T., and K. Bacon, unpublished data), had any attractant effects on B cells or CD8+ cells in the assay, HuMIP-1α at 100 pg/ml is a potent attractant of both of these lymphocyte populations, in addition to CD4+ cells (Fig. 3). At this concentration, the MI for B cells in response to HuMIP-1α was ~8.3 (125 ± 17 vs. 15 ± 10). In addition, nearly three and a half times the CD8+ T cells (175

![Figure 1](image1.png) Figure 1. PBL migration in response to purified recombinant HuMIP-1α and -1β chemokines. Concentration-response curves for lymphocyte migration to HuMIP-1β (●) and HuMIP-1α (□) or control (formulation buffer diluted identically into cell culture medium) (△). Points represent the migration index (MI) which is defined as the number of cells counted on the lower surface of the filter after incubation with the chemokines in the microchemotaxis chamber divided by the number of cells that have migrated to the lower surface of the filters in control wells of the chamber. (Dotted line) MI value for randomly migrating cells. Results are expressed as mean migration indices with error bars representing SEM for n = 4 experiments.

![Figure 2](image2.png) Figure 2. Immunophenotyping of cells responding to HuMIP-1β. Phenotype of lymphocytes migrating in response to 100 pg/ml concentration of purified recombinant HuMIP-1β (solid bars) or identically diluted formulation buffer in cell culture media (control, hatched bars) as determined by immunocytochemical analysis. PBL assayed as in Fig. 1 were phenotyped using primary antibodies recognizing B cells, MHC class II surface antigens, all T cells, or the T cell subset-specific markers CD4, CD8, CD45RO, or CD45RA. Histograms represent mean ± SEM number of positively staining cells which have migrated to the undersurface of the filters as counted per five high power fields (n = 4 experiments).

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The recruitment of lymphocytes to areas of injury or infection is a hallmark of host defense. In addition, immune-mediated inflammatory disorders such as rheumatoid arthritis and asthma, among others, are characterized by the migration and persistence of specific subpopulations of lymphocyte effector cells. The data presented here, and those in a previous report (9), show that a small group of related cytokines differ from what we have shown for RANTES, which causes memory and naive phenotypes, and differentiates between the types of cells attracted in a concentration-dependent fashion. The specific attractant activities of the HuMIP-1 proteins also differ from what we have shown for RANTES, which causes the selective migration of monocytes and T lymphocytes of the memory phenotype, while not affecting T cells of other phenotypes (9). Thus, this one small family of cytokines displays attractant effects on most subpopulations of lymphocyte effector cells. A recent report by Tanaka et al. (17) suggests that MIP-1β specifically enhances the adhesive properties of T lymphocytes. Although the cells acted upon in that study appear to be primarily CD8+ T cells, the report provides no evidence that MIP-1β acts exclusively on these cells. In fact, since RANTES did not enhance adhesion in that report, but clearly is chemotactic for T cells (9, 29), the Tanaka et al. report may suggest that chemokines differentially induce adhesion and migration in lymphocyte subpopulations. Moreover, the possibility cannot be excluded that the lymphocyte purification and separation methods used in the adhesion study may have predisposed the cells to activation along an adhesion pathway in response to the chemokine. The finding that HuMIP1-β is localized on LN endothelium is compelling, but further experimentation is required to determine if this form of the chemokine is acting in inflammatory processes at all, and whether these processes are primarily adhesion, or migration, or some combination of both.

The mechanisms by which the chemokines attract different subpopulations of lymphocytes is not clear. The ability of HuMIP-1α to attract distinct cell types in a concentration-specific fashion has some precedent in the chemokine superfamily. The related RANTES cytokine attracts monocytes in vitro at ~100-fold higher relative concentration than that optimal for memory Th cell attraction (9), and neither cell population responds at the optimal concentration for the other. Similarly, IL-8 attracts neutrophils at concentrations 10-100-fold higher than those optimal for T cells (16). We have recently cloned a receptor that binds all of the C-C chemokines with varying affinities, but appears to bind and signal in response to HuMIP-1α most strongly (30). Experiments are currently underway to determine the distribution of this receptor among lymphocyte subpopulations, and to ascertain if it participates in the attractant specificities observed in these experiments.

The data in this study show differences in the lymphocyte chemoattractant potentials for the closely related cytokines HuMIP-1α and -1β. Whereas HuMIP-1β tends to attract CD4+ T lymphocytes, HuMIP-1α appears to be a more potent chemoattractant with a broader specificity, attracting B cells and cytotoxic T cells as well as CD4+ T cells. HuMIP-1α does not appear to distinguish between lymphocytes of the memory and naive phenotypes, and differentiates between the types of cell attracted in a concentration-dependent fashion. The specific attractant activities of the HuMIP-1 proteins also differ from what we have shown for RANTES, which causes the selective migration of monocytes and T lymphocytes of the memory phenotype, while not affecting T cells of other phenotypes (9). Thus, this one small family of cytokines displays attractant effects on most subpopulations of lymphocyte effector cells.
of lymphocyte effector cells. Moreover, their differential attractant effects on distinct populations of lymphocytes may be dependent on not only differences in structure and biochemistry but also on differences in the relative concentration of a single cytokine. Understanding these effects may provide clues as to the workings of lymphocyte trafficking in vivo, and may allow for therapeutic intervention in inflammatory and autoimmune disorders.

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