Monocyte chemotactic protein-1 is a proinflammatory chemokine in rat skin injection sites and chemoattracts basophilic granular cells

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Abstract
Chemokines may control mast cell infiltrates found in many inflammatory diseases. These cells act through at least two main functions: migration and degranulation. Here we show that human recombinant monocyte chemotactic protein (MCP)-1 (10 ng/50 µl) induces, after 4 h, an inflammatory vascular permeability and cellular extravasation reaction, determined by Evan’s blue dye (1% in saline) injected into the tail vein of the rat, when injected intradermally in the rat skin. The blue color accumulating at the sites of injection provides evidence of vascular permeability and cellular extravasation. The colored areas of the skin were then enucleated and immersed in a fixative solution. Slides were prepared with sections of tissue colored with toluidine blue and analyzed under an optical microscope. A significant number of basophilic cells migrated to the injected area where MCP-1 (10 ng/50 µl) was used compared to the control PBS treatment. Cell recruitment was slightly less than N-formyl-methionine-leucyl-phenylalanine (used at 10⁻⁶ M/50 µl). Electron microscopy studies confirmed the presence of basophilic granular cells where MCP-1 was intradermally injected. After preparation of a histidine decarboxylase (HDC) probe, a Northern blot analysis was determined for HDC mRNA in the enucleated tissue injected with MCP-1 (10 ng/50 µl). Steady-state levels of HDC mRNA levels were induced after 4 h. These results were confirmed by the higher amount of histamine release, compared to the control PBS, in the enucleated tissue from the MCP-1 injection sites. Our results suggest that MCP-1 could play a significant role in diseases characterized by basophilic cell accumulation and migration to sites of tissue damage. Moreover, we show for the first time that MCP-1 is a pro-inflammatory chemokine that induces basophilic cell migration in rat skin injection sites.

Introduction
Monocyte chemotactic protein (MCP)-1, which has been purified and cloned from different sources, is the prototype of the CC chemokine subfamily and has chemoattractant properties on monocytes, T lymphocytes, but not on neutrophils (1–3). Along with MCP-1, other MCP chemokines, such as MCP-2 and -3, were purified, cloned and characterized (4,5). CC chemokines have a much wider range of biological activity than CXC chemokines, and they activate basophilic and eosinophilic leukocytes (6–8). Basophils are the circulating counterpart of tissue mast cells, and both express high-affinity receptors for IgE and, therefore, avidly bind IgE antibodies (9,10). Interaction of antigens with these IgE molecules stimulate basophils and mast cells to secrete their granular contents, which are the chemical mediators histamine, peptido-leukotrienes and cytokines involved in immediate hypersensitivity diseases (8–12).

Currently available evidence suggests an important role for MCP-1, -2 and -3 in a number of pathologic conditions,
including delayed-type hypersensitivity reactions, parasitic infections and rheumatoid arthritis (13–17). Recently, MCP-1 along with other chemokines, such as macrophage inflammatory protein-1α (MIP-1α), activate and are potent secretagogues for basophils (18–20). However, the effect of MCP-1 on mast cell and basophil activation and locomotion has yet to be established (21). Since it has been reported that the activation and the histamine releasing activity of MCP-1 on basophils is comparable with that of N-formyl-methionine-leucyl-phenylalanine (FMLP) (20), here we report that MCP-1 is a potent pro-inflammatory chemokine compared to FMLP in rat skin injection sites, with chemoattractant property for basophilic granular cells. MCP-1 induces transcription of histidine decarboxylase (HDC) mRNA and the appearance of HDC (the sole enzyme responsible for histamine from histidine) mRNA+ cells is related to the accumulation of basophils and histamine secretion.

**Methods**

**Intradermal injections in the rat**

Sprague-Dawley rats obtained from Tacomic (Germantown, NY) were kept in virus-free sections of a modern animal facility (Tufts University), and were allowed access to food and water *ad libitum*. Male rats 250–300 g in weight were anesthetized with ketamine–HCl (1 ml/kg body wt) 0.2 ml injection (USP, Fort Dodge, IA) plus xylazine–HCl (100 mg/ml) 0.05 ml injection (Fermenta Animal Health, Kansas City, MO). The injection was made in the lower right quadrant of the abdomen. After 5 min the abdomens of the rats under anesthesia were shaved in four places and were injected with a 50 µl intradermal injection of MCP-1 (catalog no. 279-MC; pack sizes: 10 and 50 g), PBS (negative control) and FMLP (Sigma, St Louis, MO) (positive control) in different concentrations. In another set of experiments, after the injections of MCP-1, PBS and the positive control (FMLP), a 0.6 ml solution of Evan’s blue dye (1% in saline) was injected into the tail vein of the rats attaining a pale blue color in the areas where the blue dye (1% in saline) was injected, into the tail vein of the rats. After 4 h the blue dye, an inflammatory state was established due to the injected areas of the negative controls there was no discoloration in the skin tissues of the rats. The skin tissues were immersed in a fixative solution [5% formaldehyde (Polyborate, Ft. Worth, TX)] and cut by a microtome followed by decapitation. The areas colored with Evan’s blue dye (Sigma E2129) were measured, and we assumed that the greater the color intensity and the size of the area formed at the intradermal injection site, the greater was the vascular permeability and cellular extravasation. After 4 h from the intradermal injections, the animals were sacrificed by inhalation of CO₂ followed by decapitation. The areas colored with Evan’s blue dye (Sigma E2129) were measured, and we assumed that the greater the color intensity and the size of the area formed at the intradermal injection site, the greater was the vascular permeability and cellular extravasation, and therefore inflammation and chemoattraction (15). The colored areas of the skin were then enucleated and immersed in a fixative solution [5% formaldehyde (Polyscience, Warrington, PA) plus 0.2 M PBS for at least 10–15 min before use]. Slides were prepared with sections of the tissue and colored with toluidine blue (0.1% in 1% sodium borate for 2 min) and analyzed under an optical microscope (x10, x20 or x40; Nikon Diaphore THD microscope). The basophilic cells were counted in an optical field using a grating size of 5×5 mm.

Recombinant human MCP-1 was purchased from R&D Systems (Minneapolis, MN). Preparation of MCP-1 contained <1 endotoxin U/mg of protein as determined by the Limulus amebocyte lyase reaction.

**Electron microscopy studies**

Other tissue samples of injected compounds were removed for electron microscopy analysis (14,19). The samples were enucleated and fixed for 1 h at room temperature with 3% glutaraldehyde in 0.1 M cacodylate–HCl buffer (pH 7.2). The tissues were then infiltrated with two 10 min changes of 100% propylene oxide followed by an overnight exposure to a 1:1 mixture of propylene oxide and DMP-30. The next day the skin tissues were embedded in Epon with DMP-30. Embedded tissues were placed in a 56°C oven to polymerize for 48 h. Thick and thin sections were cut on Sorval MT-1 and MT-2B ultramicrotomes equipped with glass and diamond knives respectively. Sections (1000 Å) were picked up on 300 mesh copper grids, and stained with both uranyl and lead salts. The sections were then examined and photographed using a JEOL JEM-100s transmission electron microscope operated at an accelerating voltage of 80 kV.

**Preparation of the HDC probe**

We used a probe made from a reverse transcribed rat brain polyA⁺ RNA. Total cellular RNA was extracted from New England Deaconess Hospital rat brain. PolyA⁺ mRNA was purified by one-step chromatography on an oligo-dT column. A sample of 2 µg polyA⁺ mRNA was reverse transcribed at 42°C for 40 min in a 20 µl mixture containing 4 µl of 5×RT buffer (250 mM Tris–HCl, pH 8.3, at 42°C, 50 mM MgCl₂, 250 mM KCl, 15 mM dithiothreitol, 10 U placial RNase inhibitor, 0.5 mM each dNTP, 50 pmol oligo-dT primer and 20 U avian myeloblastosis virus RT). After RT the HDC cDNA was amplified by PCR using two specific primers synthesized on a gene assembler plus (Pharmacia LKB, Bromma, Sweden): 5’ primer, 5’-ATGATGAGGCGCAGTGAATTCC and 3’ primer, 5’-CCGAATTCGATCAGTCTGAGTAG. Then a 40 µl single-stranded cDNA mixture was supplemented with 50 pmol of each 5’ (sense) and 3’ (antisense) primers in a volume of 50 ml denatured for 2 min in a boiling bath and added to a 50 ml mix prewarmed at 72°C containing 0.25 mM each dNTP, 10 µl 10×Taq polymerase buffer (500 mM KCl, 100 mM Tris–HCl, pH 8.3, at 25°C, 15 mM MgCl₂ and 0.1% gelatin) and 1.5 U of Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT). The PCR program consisted of one cycle of 1 min at 94°C and 15 min at 72°C followed by 35 cycles of 30 s at 94°C, 30 s at 55°C and 4 min at 72°C, and was completed by an additional annealing at 55°C for 30 s and a final elongation at 72°C for 15 min. PCR was performed in a Techne PHC-2 programmable heating block. Amplified products were purified by glass-milk procedure (Geneclean Kit; BIO 101, Vista, CA), blunt-ended with the Klenow fragment and cut by EcoRI. The resulting blunt-end EcoRI fragments were cloned in the p-MAL vector (Biolabs) cut by both EcoRI and PstI restriction enzymes. The resulting recombinant plasmid was amplified in the TB1 *Escherichia coli* strain. Plasmid DNA was sequenced by the double-stranded protocol of the Sequenase kit (USB, Cleveland, OH). Plasmid...
containing amplified HDC cDNA was prepared according to the alkaline lysis method and purified on a CL4B column.

Northern blot analysis
Northern blot analysis was performed on the enucleated tissue from the intradermal injection sites tested with MCP-1, the negative control (PBS) and the positive control (FMLP).

Total RNA was isolated by guanidine hydrochloride as previously described (20,21). Total RNA (10 mg/lane) was fractionated by electrophoresis on a 2% agarose gel, transferred to nylon membranes (Hybond N; Amersham, Les Ulis, France) and hybridized with \( {^{32}}P \) (2×10^8 c.p.m./mg). It was then washed 4 times at room temperature for 15 min in 2×SSC and 0.1% SDS, heated to 48°C for 30 min, and then washed twice in 0.1×SSC and 0.1% SDS. Membranes were finally exposed to Kodak XAR5 for 3 days at –70°C. Signals were compared with ribosomal RNA to evaluate an equal quantity of RNA for each lane.

Histamine measurement
Histamine in tissue supernatants was estimated by a radioenzymatic method, essentially a modification of the method described by Kaplan et al. and others (22,23).

Tissues were minced in cold PBS and then resuspended in water (1 g/0.2 ml). This suspension was sonicated in a Branson 1200 ultrasound devise for 10 min, vortexed for 5 min and pelleted by 10 min centrifugation at top speed in an Eppendorf microcentrifuge. Total histamine in tissue homogenates taken from the injection sites was determined after centrifugation at 400 g for 5 min. To 20 µl of supernatant taken from disrupted tissue, 2 µl 20% perchloric acid, 6 µl 1 N NaOH and 100 µl 0.05 M sodium phosphate, pH 7.4, was added. The precipitate was pelleted by 5 min centrifugation at top speed in an Eppendorf microfuge and aliquots were taken from the supernatant for further histamine assay. In 50 µl of the reaction mixture for histamine assay 5–25 µl sample, 5 µl rat kidney histamine methyl transferase and \( {^{3}}H \)adenosyl-L-methionine (73.8 Ci/mM; Amersham) were added.

Isotope and enzyme were diluted with 0.05 M sodium phosphate, pH 7.4, for optimal conditions. The reaction mixture was incubated for 1 h at 37°C, following which 20 µl 1.5 M perchloric acid, 20 µl 10 M NaOH and 500 µl freshly prepared toluene:isoamylalcohol (4:1 v/v) were added, and the mixture was shaken for 10 min. After 10 min centrifugation at 200 g, 0.3 ml was taken from the upper phase and 4 ml of Aquasol (New England Nuclear, Boston, MA) scintillation fluid was added. Radioactivity was measured in a \( \beta \) counter and the concentration of histamine was computed by using a standard curve. Samples were always performed in duplicate from duplicate cultures. Histamine methyltransferase was a gift from the laboratory of Dr David Cochrane (Medford, MA) and DNP-BSA was provided by Dr Fu-Tong Liu (Scripps Clinic, La Jolla, CA).

Statistical analyses
Data from different experiments were combined and reported as the mean ± SD. Student's \( t \) test for independent means was used to provide a statistical analysis (\( P > 0.05 \) was considered as not significant).

Results

Injection sites of human recombinant MCP-1
In order to establish the inflammatory potential and basophilic granular cell chemoattractant activities of MCP-1, purified recombinant human MCP-1 was tested in rat skin injection sites. FMLP was used as a positive control for leukocyte migration (24,25), while PBS (vehicle) was used as a negative control. In Fig. 1 we show that MCP-1 (5, 10 or 20 ng/50 µl) provokes an inflammatory reaction based on the intensity of the colored, inflamed area (Evans's blue dye 1% in saline), after 4 h intradermal injections of MCP-1. The migration time studies were based upon previous publications where different authors show that the peak of accumulation of white cells is reached in 4 h (24,28,30) after MCP-1 treatment in vivo, as also confirmed in our previous experiments. The maximum inflammatory effect (most intense color) was achieved with MCP-1 at 10 and 20 ng/50 µl (sites nos 3 and 4 respectively); while MCP-1 at 5 ng/50 µl (site no. 2) demonstrated only a modest response in vascular permeability and cellular extravasation. Similar inflammatory effects to MCP-1 at 10 and 20 ng/50 µl were also observed after 4 h injection of FMLP (10⁻⁶ M/50 µl) (site no. 5).

In Fig. 2 we show the mean of the major diameters ± SD of the area colored with Evans's blue formed at the intradermal injection site in four representative experiments. The mean ± SD of the inflamed areas treated with MCP-1 (10 and 20 ng/50 µl) were significantly higher (\( P < 0.05 \)) compared to
controls (PBS 50 µl); while the maximum effect was obtained with FMLP. When MCP-1 was used at 5 ng/50 µl no effect was found.

**Microscopic studies on MCP-1 biopsy of rat skin injection sites**

In order to quantify the migratory responses of basophilic granular cells in the rat skin injection sites, we studied intradermal accumulation of basophils after 4 h treatment with MCP-1 at 10 ng/50 µl, FMLP (10^{-6} M/50 µl) or PBS (control) 50 µl. The inflamed area was enucleated and fixed, which washed out all the Evan’s blue dye, thereby permitting the unaffected interpretation of toluidine blue staining. The infiltrated basophilic cells were then colored with toluidine blue (0.1%) and observed under an optical microscope ×20 (Fig. 3). A significant basophilic accumulation was obtained with MCP-1 10 ng/50 µl (Fig. 3c) compared to control. However, the effect was lower than FMLP (Fig. 3b). No effect was found when PBS was used (Fig. 3a). The infiltrated basophilic cells were counted in an optical field using a 5×5 mm grating under an optical microscope ×20 (Fig. 4). MCP-1 (5, 10 and 20 ng/50 µl) induced basophil migration in a dose-dependent manner. However, FMLP, used as a positive control, proved to be more potent than MCP-1, while PBS (vehicle) had no effect on the migratory response of basophilic granular cells.

**Electron microscopy studies of MCP-1 intradermal injections**

The presence of basophilic cells in the MCP-1 injected sites, revealed by optical microscopy was morphologically further analyzed by electron microscopy. In Fig. 5 we show an electron micrograph of a representative experiment of seven (∼37,500): two basophilic cells chemotraacted after 4 h from the intradermal injection of MCP-1 at 10 ng/50 µl in the rat. The cells stuck together (no secretion observed) and degranulation occurring.

**Generation of HDC mRNA levels in rat skin injection site after treatment with MCP-1**

A probe was prepared in order to detect mRNA encoding the HDC gene. After cloning into the p-Mal plasmid, this was used to detect HDC mRNA by Northern blot hybridization (27). Since MCP-1 has been presented as a chemoattractant for basophils and mast cells, and induces HDC *in vitro* on rat peritoneal mast cells (8), in this report we analyzed the production of HDC mRNA in rat skin injection sites. Steady-state levels of HDC mRNA in PBS-treated controls were low (Fig. 6, lane 1). In the presence of MCP-1 (10 or 20 ng/50 µl) (Fig. 6, lanes 2 and 3), a strong increase of HDC mRNA was found. When the animals were exposed to FMLP (10^{-6} M/50 µl) (positive control) maximal levels were reached (Fig. 6, lane 4) compared to the controls. The enhancement of HDC mRNA is explained by *de novo* synthesis of this enzyme induced by MCP-1 and FMLP. This effect was abolished in the presence of actinomycin D (data not shown), which inhibits mRNA transcription.

**Total histamine release in the rat skin injection sites after treatment with MCP-1**

Since MCP-1 has been presented as a potent chemotactic factor for basophils and mast cells (6,21), in this report we also studied the effect of MCP-1 (10 or 20 ng/50 µl) on the total histamine release in the skin tissue injection sites (Fig. 7). The animals (four experiments) were exposed to PBS (control) 50 µl, FMLP (10^{-6} M/50 µl) and MCP-1 (10 or 20 ng/50 µl) for 4 h. The inflamed area was enucleated, finely minced and sonicated in order to lyse the basophilic cells with the total release of histamine in the supernatants. The maximum histamine release was obtained with FMLP treatment (30 ± 5), while when the animals were exposed to MCP-1 20 and 10 ng/50 µl the release was 25±6 and 20±3 respectively. A very low effect was obtained with PBS treatment, 6.1 ± 4 (control).

**Discussion**

Recently it has been found that a chemoattractant secreted at the site of antigenic stimulation is capable of recruiting many different cells, such as monocytes, lymphocytes, basophils and eosinophils, from the blood stream into inflammatory lesions (19–21,24–26). In this report, we have found that MCP-1 induces basophilic cell migration when injected in the rat skin. Basophils respond chemotactically to MCP-1 in a dose-dependent manner. Basophilic granular cells in slide sections of tissues colored with toluidine blue strongly respond and migrate in MCP-1 intradermal injection sites of the rat skin. A higher effect was obtained when FMLP (10^{-6} M/50 µl) was injected after 4 h and the intradermal migration of basophils in response to MCP-1 was dose dependent as revealed by optical microscopical study (∗×20). The presence of basophilic cells in rat skin sites injected with MCP-1 10 ng/50 µl was also shown in an electron micrograph (magnification ∗×37,500). Since the accumulation of mast cells and basophils in the tissue also means a high presence of histamine, we...
Fig. 3. Basophilic cell migration induced by human recombinant MCP-1 at 10 ng/50 µl (c) and FMLP (b) into sites of injection. The sections were stained with toluidine blue (0.1%) and analyzed under an optical microscope ×20. (a) A control PBS treatment 50 µl. This representative experiment was found to be reproducible and has been performed at least three times. The arrows indicate infiltrated basophilic granular cells.
found that HDC, the sole enzyme responsible for histamine from histidine, increases its mRNA production, as reported in a Northern blot analysis. The data obtained in our in vivo model indicates, for the first time, that MCP-1 provokes an increase in HDC mRNA synthesis in rat skin injection sites. In addition, since basophilic cells respond to the chemoattractant MCP-1, in the biopsy of the rat skin injection sites an increase in total histamine generation was found after 4 h from MCP-1 treatment. Therefore, it is possible that MCP-1 also activates basophilic production of histamine as evidenced by the HDC gene expression. MCP-1 could play a meaningful role where basophilic cells are recruited in disease sites such as the asthmatic lung or atopic dermatitis.

Our results are in accordance with Taub et al. who described...
mast cell migration in response to the chemokines MCP-1, regulated upon activation, normal T expressed and secreted (RANTES), and MIP-1α on extracellular matrices and its enhancement by IgE-dependent activation, providing a mechanism by which cells may be drawn to sites of inflammation (21). They also found that mast cells did not degranulate in response to the chemokines, a phenomena that was not tested in the rat skin tissue of our experiments (18,21).

Previous studies from several laboratories have revealed that MCP-1, along with MCP-2 and -3, provokes significant migration of CD4⁺ and CD8⁺ T cells into the site of injection, even after 4 h, demonstrating that MCP-1, -2 and -3 are inflammatory mediators that stimulate the directional migration of T cells, as well as monocytes, and play an important role in immune cell recruitment into sites of antigenic challenge (24–29). However, other authors state that MCP-3 is involved in the regulation of the early eosinophil response to specific allergen, whereas RANTES may have more relevance to the later accumulation of T cells and macrophages (19).

Our studies are also in accordance with Meurer et al., who found that in canine dermis human MCP-1 caused a mild perivascular inflammatory reaction. However, this effect was more pronounced when a single intradermal injection of RANTES was administered, resulting in an eosinophilic- and macrophage-rich inflammatory site within 4 h (30), demonstrating that chemokines can have cross-species activity and the association of each individual chemokine ligand with its cognate receptor is not exclusive: multiple chemokines can bind productively to a single receptor and vice versa. These data suggest that MCP-1 could play a significant role in diseases characterized by basophilic cell accumulation and mobilization to sites of tissue damage. Our studies show, for the first time, direct in vivo evidence that the migration of basophilic cells due to the CC chemokine MCP-1 in vitro has some correlation in vivo. This effect is not selective since MCP-1 attracts other cell types such as lymphocytes (24,28). The studies also suggest that MCP-1 presumably directs mast cells to local tissue sites and towards lymphoid tissue, playing different roles in attracting basophilic cells and monocytes along with other leukocytes to inflamed tissue. Since MCP-1 is generated by macrophages, the interaction between these cells and connective tissue mast cells is an important pathogenetic interaction in a wide variety of immunological disorders (24,27–28,30–32). However, more studies on the effect of MCP-1 in vivo and in vitro are required to clarify the specificity of the proinflammatory effect of this CC chemokine. In addition, studies involving the antagonism of MCP-1 through competitive receptor binding are underway in our laboratory to better understand the new evidence revealed by the data reported here.

Acknowledgements

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Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>FMLP</td>
<td>N-formyl-methionine-leucyl-phenylalanine</td>
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<td>HDC</td>
<td>histidine decarboxylase</td>
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<td>MCP-1</td>
<td>monocyte chemotactic protein-1</td>
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<td>MIP-1α</td>
<td>macrophage inflammatory protein-1α</td>
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<td>RANTES</td>
<td>regulated upon activation, normal T expressed and secreted</td>
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MCP-1 chemoattracts basophilic cells

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