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Biochemical and Biologic Characterization of Murine Monocyte Chemoattractant Protein-1

Identification of Two Functional Domains[1]

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Monocyte chemoattractant protein-1 (MCP-1) is a member of the chemokine-β (or C-C) family of cytokines. Murine MCP-1, first identified as the *JE* gene, differs from human MCP-1 in molecular size and extent of glycosylation. We have used Chinese hamster ovary cells to express recombinant murine MCP-1 and find that its predominant form is a microheterogeneous protein of M_r ≈25,000. Most of MCP-1's microheterogeneity is due to variable amounts of sialic acid that are terminally attached to a constant number of *O*-linked oligosaccharide chains per molecule. This carbohydrate, along with a small amount of N-linked carbohydrate, accounts for 50% of the apparent molecular size of murine MCP-1 and is not required for in vitro monocyte chemoattractant activity. Mutational analysis shows that most of the carbohydrate is added to a 49-amino acid C-terminal domain that is not present in human MCP-1 and is not required for in vitro biologic activity, suggesting that murine MCP-1 consists of an N-terminal domain containing monocyte chemoattractant activity and a heavily glycosylated C-terminal domain of as yet unknown function. MCP-1 produced in COS cells contains a small amount of sulfate, but Chinese hamster ovary-produced MCP-1 does not. The absence of sulfate does not alter MCP-1's in vitro chemoattractant properties. In vitro, highly purified murine MCP-1 attracts monocytes, but not neutrophils, with a specific activity similar to human MCP-1 (EC_{50} ≈ 0.5 nM). Equilibrium binding experiments with human monocytes reveal the presence of ≈3000 binding sites per cell with a K_d of 0.77 nM. In vivo, injection of up to 1 μg murine MCP-1 in a variety of murine strains induces the appearance of a sparse mixed inflammatory infiltrate. The disparity between MCP-1's in vitro and in vivo effects suggests that other factors may be required to elicit a full-blown monocyte chemotactic response to MCP-1 in vivo. *Journal of Immunology,* 1994, 152: 3541.

Chemokines are structurally and genetically related proinflammatory cytokines, many of which are leukocyte-specific chemoattractants (1, 2). Chemokine synthesis can be detected at a variety of inflammatory sites, and chemokine activity is presumed to be at least partly responsible for the accumulation of leukocytes at those sites (3–12). Chemokines can broadly be divided into two subclasses, namely chemokine-α (or C-X-C) and chemokine-β (or C-C), based on amino acid sequence and genetic mapping criteria. For the most part, members of the chemokine-α subclass, e.g., IL-8, tend to attract and activate neutrophils, whereas members of the chemokine-β subclass tend to act on monocytes. Nevertheless, these boundaries are not absolute, and there is evidence that both subfamilies exert effects on lymphocytes (13, 14).

MCP-1,[3] a member of the chemokine-β subfamily, attracts and activates monocytes at subnanomolar concentrations (15, 16). MCP-1 has no detectable effects on neutrophils, but can induce histamine release from basophils (17, 18). MCP-1 was first cloned in its murine form as *JE*, a gene whose expression is induced in mouse fibroblasts

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[3] Abbreviations used in this paper: MCP-1, monocyte chemoattractant protein-1; CHO, Chinese hamster ovary.

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by platelet-derived growth factor (19, 20). However, most of the characterization of MCP-1 has been performed on the human homologue (21, 22), which was first purified from human cell lines on the basis of its monocyte chemoattractant properties (15, 16).

Although our knowledge about the properties of MCP-1 and other chemokines has expanded greatly in the past five years, most of this information has been gained through in vitro studies. A number of questions remain about chemokine physiology in whole animals, and in order to address these questions, suitable animal models must be developed. Murine models are likely to be most productive, but their use requires a greater understanding of the properties of murine chemokines. In the case of MCP-1, molecular analysis has revealed that murine and human MCP-1 have some striking differences despite being unique homologues and sharing extensive structural similarities. For example, the murine MCP-1 core protein is larger and more highly glycosylated than human MCP-1 (20, 23). Nevertheless, there has been no detailed analysis of the structure and biologic properties of murine MCP-1. In order to characterize murine MCP-1, we have analyzed recombinant murine MCP-1 produced in a eukaryotic expression system and purified to homogeneity.

Materials and Methods

MCP-1 expression and purification

Murine MCP-1 was expressed in Chinese hamster ovary (CHO) cells stably transfected with the expression plasmid pXM-JE10 as described (24). The specific cell line used for expression is denoted 10A-10. Cells were maintained in the alpha modification of minimal essential medium (MEM-α) supplemented with 10% bovine calf serum and 10 μM methotrexate. MCP-1 was purified from serum-free medium conditioned for 48 h by confluent monolayers of 10A-10 cells. Clarified medium was concentrated on YM-10 membranes (Amicon, Danvers, MA) then clarified again by centrifugation. Material soluble in 45% $(NH_4)_2SO_4$ was dialyzed extensively against 50 mM NaH_2PO_4 (pH 6.5) then filtered through a 0.22-μ cellulose acetate filter. The filtrate was applied to a Mono S column (Pharmacia, Piscataway, NJ) in the same buffer, and the column was developed using a 0 to 300 mM NaCl gradient in 50 mM NaH_2PO_4 (pH 6.5). Material eluting at 120 to 150 mM NaCl was concentrated using a Centricon-10 device (Amicon, Danvers, MA), then applied to an ODS-Hypersil column (Hewlett-Packard, Andover, MA). After washing the bound material with 0.1% trifluoroacetic acid, the column was developed using a 0 to 100% acetonitrile gradient. MCP-1 eluted as a symmetrical peak at 40% acetonitrile. This material was evaporated to dryness, resuspended in 50 mM sodium borate (pH 8.0), 150 mM NaCl, then chromatographed on Superdex (Pharmacia, Piscataway, NJ) in the same buffer. Pure MCP-1 was quantified using an amino acid analyzer (Applied Biosystems Inc., Foster City, CA). In some experiments, MCP-1 was produced in COS cells transiently transfected with the expression plasmid pXM-JE10 or in 3T3 cells treated with PDGF as described (20).

Chemotaxis

Monocyte chemoattractant activity was determined on human PBMCs using a 48-well microchamber device as described (25). MCP-1 attracted only monocytes as determined by nuclear morphology, cell size, and the presence of nonspecific esterase in 100% of migrating cells. In order to isolate human neutrophils, peripheral blood was diluted 1:4 with HBSS, then underlayed with Ficoll-Hypaque (Pharmacia, Piscataway, NJ). After centrifugation at 3000 rpm for 15 min, pelleted cells were resuspended in one volume of HBSS. One-tenth volume 4% Dextran T500 (Pharmacia, Piscataway, NJ) was added, and RBCs were allowed to sediment by gravity for 45 min. The supernatant containing neutrophils was removed, cells were washed once with HBSS, the pelleted cells were resuspended in ACK (1.55 M NH_4Cl, 72 mM K_2CO_3, 1 mM EDTA), incubated at 4°C for 10 min, and then diluted in HBSS. Cells were washed twice in Gey's balanced salt solution (GBSS) with 2% BSA, and resuspended at 3×10^6 cells/ml in GBSS/0.2% BSA. Chemotaxis was performed in microchambers as described for monocytes, except that a polyvinylpyrrolidone-free membrane was used, and the chamber was incubated for 1 h.

Iodination of MCP-1

Murine MCP-1 (5 μg) was radioiodinated using the Bolton-Hunter reagent (4400 Ci/mmol) (NEN-DuPont, Boston, MA) in 10 μl of 100 mM sodium borate (pH 8.5). Iodinated MCP-1 was then separated from unincorporated Bolton-Hunter reagent by gel filtration chromatography. Specific activities were in the range of 2500 to 3000 Ci/mmol with an average of 95% of all MCP-1 molecules substituted with one ^{125}I. Iodinated MCP-1 showed a single band of $M_r \approx 25{,}000$ on SDS-PAGE, and its biological specific activity was identical to noniodinated MCP-1 in in vitro monocyte chemotaxis assays.

Equilibrium binding assays

Human monocytes were isolated from leukopaks obtained from the Blood Component Laboratory at the Dana-Farber Cancer Institute. Leukopak contents were diluted 1:4 with HBSS, underlayed with Ficoll-Hypaque (Pharmacia, Piscataway, NJ), and centrifuged at 3000 rpm for 15 min. PBMCs were recovered from the interface, and monocytes were isolated from these cells by counterflow centrifugal elutriation essentially as described (26, 27). Preparations contained >95% monocytes as determined by staining for nonspecific esterase. Varying amounts of ^{125}I-labeled MCP-1 were added to 1 to 10×10^6 monocytes in 0.3 ml of binding buffer (50 mM HEPES (pH 7.2)/1 mM $CaCl_2$/5 mM $MgCl_2$/0.5% BSA), with or without a 125-fold molar excess of unlabeled MCP-1. Cells were incubated at 27°C for 90 min, then the entire binding mixture was filtered through GF/C filters (Whatman, Hillsboro, OR) that had been pretreated with 0.3% polyethyleneimine, and retained material was washed twice with binding buffer plus 0.5 M NaCl. Dried filters were counted in a gamma counter (Packard, Meriden, CT). Data were analyzed using Ligand software (28).

In vivo assays

Animals were anesthetized with methoxyflurane and, for intradermal injections, flank hair was removed using clippers. Twenty-five microliters of MCP-1 diluted in endotoxin-free PBS was injected intradermally either into flank skin or footpads using a 26-ga needle. Control sites received 25 μl PBS only. At times ranging from 6 to 24 h after injection, animals were killed by CO_2 asphyxiation, and skin samples were placed in 10% buffered formalin. Sections were stained with hematoxylin and eosin, and the extent of inflammatory cell infiltration in several randomly chosen microscopic fields was graded in a blinded fashion by one of us (C.L.C.) on a scale of 0 to 2+. The endotoxin content of MCP-1 preparations was always less than 0.05 ng endotoxin/μg MCP-1.

Endoglycosidase digestions

Confluent monolayers of 10A-10 cells were washed with methionine-free medium, then incubated in methionine-free medium for 1 h. Medium was then changed to methionine-free medium supplemented with 0.5 mCi/ml [^{35}S]methionine (NEN DuPont, Boston, MA) for 4 h. Medium was collected, made 1 mM PMSF, and clarified by centrifugation. To digest N-linked carbohydrate, an aliquot of labeled supernatant was boiled in 0.5% SDS/42 mM β-mercaptoethanol. It was made of 20 mM $NaPO_4$ (pH 8.6) and 1.25% Nonidet P-40, then 4.8 μl *N*-glycanase (Genzyme, Boston, MA) was added, and the mixture was allowed to incubate at 37°C overnight. To digest sialic acid, an aliquot of labeled supernatant was made 20 mM in Tris acetate (pH 6.5), 1 mM calcium acetate, and 10 mM D-galactono-γ-lactone; then 0.1 unit neuraminidase (Genzyme, Boston, MA) was added, and the mixture was incubated at 37°C for 1 h. Digestion of *O*-linked sugars was carried out by adding 8 μl *O*-glycanase (Genzyme, Boston, MA) to 100 μl of neuraminidase-treated samples and incubating at 37°C for 6 h. For each digestion, control mock digestions

were performed by adding water in place of enzyme. Samples were analyzed by immune precipitation.

Sulfate radiolabeling

Confluent monolayers of 10A-10 cells, transfected COS cells, or PDGF-treated 3T3 cells were incubated with serum-free and sulfate-free Joklik's medium for 1 h, then fresh medium was added with 0.5 μCi/ml sodium [^{35}S]sulfate for 4 h. Medium was made of 1 mM PMSF, clarified by centrifugation, and analyzed by immune precipitation.

Construction of truncated MCP-1

Two sets of PCR reactions were performed using pcJE-1 (full length murine MCP-1 cDNA cloned in pGEM-1 (Promega, Madison, WI)) as template (20). One set comprised the 17-base T7 primer (flanking the 3′ side of the murine MCP-1 cDNA) and the following primer: CCAAAT GAGATAAGAACC (bases 361–378 of murine MCP-1, changing base 372 from C to A). The other set comprised the 17-base SP6 primer (flanking the 5′ side of the murine MCP-1 cDNA) and the following primer: GGTTCTTATCTCATTTGG (bases 378–361 of murine MCP-1, changing base 372 from G to T on the opposite strand). The PCR products were gel purified, combined, and subjected to PCR again using SP6 and T7 primers. This resulted in a murine MCP-1 cDNA in which a TAA stop codon appears after the codon for arginine-73 in mature murine MCP-1. This engineered cDNA was cloned into the *Eco*RI site of plasmid pXM which was used for transient COS cell transfections.

Immune precipitation

Radiolabeled material was diluted with an equal volume of cold RIPA buffer and precleared by incubating at 4°C with normal rabbit serum (Sigma Chemical Co., St. Louis, MO) for 1 h followed by protein A-agarose beads (Bio-Rad, Richmond, CA) for an additional hour. After centrifugation, rabbit anti-mouse MCP-1 serum (22) was added to supernatants at 1:100 dilution and incubated at 4°C for 1 hr. Protein A-Sepharose was added at 4°C for 1 h. The beads were washed twice with RIPA buffer and once with 50 mM Tris-HCl (pH 7.0)/1 M NaCl. Beads were resuspended in SDS-PAGE sample buffer, boiled, and released material was analyzed on a 15% SDS-polyacrylamide gel. Gels were treated with Autofluor (National Diagnostics, Atlanta, GA) before drying. Gels containing ^{35}S sulfate-labeled material were analyzed on a Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Results

Purification of murine MCP-1

Murine MCP-1 was expressed in the stably transfected CHO cell line, 10A-10. Serum-free conditioned medium was concentrated, and MCP-1 was purified to apparent homogeneity by (1) removal of material insoluble in 45% $(NH_4)_2SO_4$, (2) cation exchange chromatography on Mono S, (3) reversed-phase HPLC, and (4) gel filtration chromatography on Superdex. Figure 1 shows the progressive purification of the M_r 25,000 species of murine MCP-1. In the presence of 150 mM NaCl, MCP-1 appeared as a single, symmetrical peak on gel filtration chromatography with a retention time consistent with an apparent molecular mass of 45,000 to 65,000 Da. This suggests that at physiologic salt concentrations, MCP-1 may exist as a dimer. Alternatively, MCP-1's extensive substitution with carbohydrate (see below) may cause anomalous chromatographic behavior on gel filtration columns. TenA-10 cells also produce a minor MCP-1 species of M_r 14,000 (<1% of total MCP-1 reactive material by immunoblotting), which had monocyte chemoattractant activity but was not pursued further in this study.

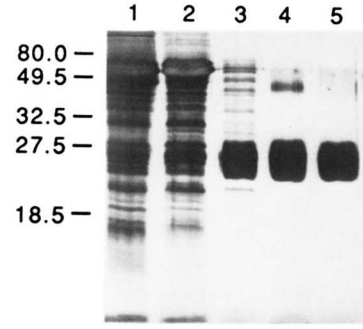

FIGURE 1. Conditioned medium from 10A-10 cells was subjected to purification steps described in the text. Aliquots from each step were boiled in SDS sample buffer containing β-mercaptoethanol and analyzed by electrophoresis through a 15% polyacrylamide gel followed by silver staining. *Lane 1*, concentrated medium (15 μg total protein applied to lane); *lane 2*, material soluble in 45% $(NH_4)_2SO_4$ (10 μg total protein); *lane 3*, material eluting from Mono S at 120 to 150 mM NaCl (1.5 μg total protein); *lane 4*, material eluting from ODS-Hypersil at 40% acetonitrile (0.8 μg total protein); *lane 5*, prominent peak on Superdex gel filtration chromatography (0.8 μg total protein).

In vitro monocyte chemoattractant activity of murine MCP-1

We tested the ability of material at each step of the purification scheme to attract human monocytes in vitro as described in *Materials and Methods* (Table I). Loss of active material at the Mono S step was due to stringent criteria for recovering the major Mono S peak. Immunoblotting of all fractions from Mono S showed no evidence for the M_r 25,000 species being processed into the M_r 14,000 species, which remained <1% of total immunoreactive MCP-1. Murine MCP-1 at all stages of purification attracted only monocytes from freshly isolated human PBMCs. Figure 2 shows a typical chemotaxis experiment in which purified murine MCP-1 attracted monocytes, but not neutrophils. The specific activity of pure recombinant murine MCP-1 is equal to that of natural human MCP-1 in attracting human monocytes in vitro (15).

Equilibrium binding of murine MCP-1 to human monocytes

Radioiodinated MCP-1 retaining full in vitro biologic activity was prepared as described in *Materials and Methods* and used in equilibrium binding assays with human monocytes purified by elutriation. Figure 3 shows a typical experiment that provides evidence for a single class of receptor with a K_d of 0.77 nM. On average, monocytes had 3300 ± 1680 (SE) binding sites per cell.

In vivo effects of murine MCP-1

Pure murine MCP-1 was injected intradermally into shaved flank skin or into footpads of C3H/HeJ (10 mice),

Table I. *Purification of murine MCP-1 from 10A-10 cells*[a]

Purification Step	Volume (mL)	Protein (mg)	MCA (Units)[b]	Recovery (%)	Specific Activity (Units/mg)
Concentrated medium	26.6	210.1	2,311,000	100	11,000
45% $(NH_4)_2SO_4$	85	47.6	1,680,000	73	35,300
Mono S	0.035	0.317	36,000	1.6	113,600
RP-HPLC	0.050	0.065	20,900	0.9	322,000
Superdex	0.030	0.024	28,000	1.2	1,167,000

[a] Medium conditioned by 10A-10 cells was concentrated and subjected to the purification steps described in the text. Data reflect the purification of the M_r 25,000 species of murine MCP-1.
[b] An aliquot from each purification step was tested for monocyte chemoattractant activity as described in *Materials and Methods*.

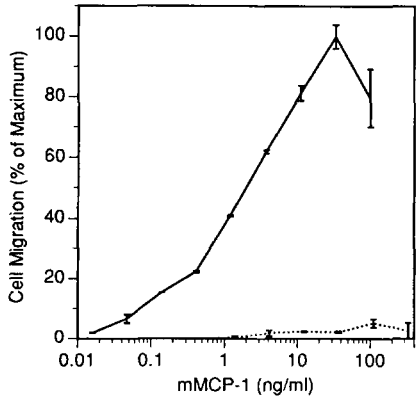

FIGURE 2. PBMCs and neutrophils were prepared from human donors as described in *Materials and Methods*. Chemotaxis assays were performed using a 48-well microchamber apparatus, and for every concentration of MCP-1 tested, migrated cells in five high-power fields were counted in duplicate. Percentage of maximal migration was determined using the maximal response of monocytes to MCP-1 (119 cells/hpf at 33.3 ng/ml MCP-1; a number greater than the response to 10^{-8} M FMLP) and the maximal response of neutrophils to FMLP (246 cells/hpf at 10^{-8} M FMLP). Solid line, monocytes; dotted line, neutrophils.

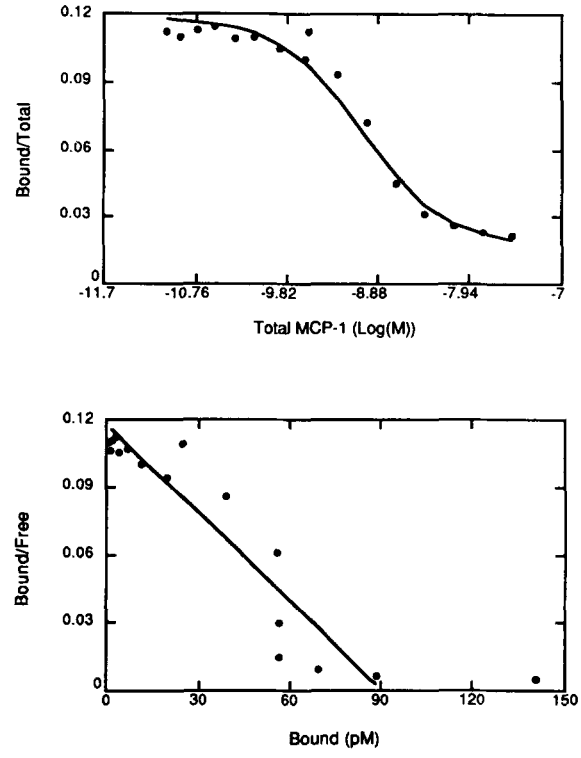

FIGURE 3. Varying amounts of radioiodinated MCP-1 (2–250 pM) were incubated with 7.8×10^6 human monocytes per incubation in the presence or absence of a 125-fold molar excess of unlabeled MCP-1. After collecting bound radioactivity on filters as described in *Materials and Methods*, radioactivity was determined by gamma counting. Data were analyzed using Ligand software (28). *Upper panel*, dissociation curve; *lower panel*, Scatchard analysis. Each point represents the average of two replicate incubations. These data are representative of two independent experiments.

BALB/c (10 mice), FVB (20 mice), and outbred Swiss Webster mice (20 mice). Injected quantities ranged from 10 ng to 1 μg. Mice were killed at times varying from 4 to 24 h after injection, and the injection site was analyzed histologically. In no case did injection of MCP-1 produce footpad swelling. MCP-1 induced the appearance of a very mild mixed inflammatory cell infiltrate at the injection site. MCP-1 never induced the appearance of a vigorous, solely monocytic infiltrate.

Sites of carbohydrate addition in murine MCP-1

Previous studies have demonstrated that murine MCP-1 produced in COS cells contains nearly 50% *O*-linked carbohydrate by apparent molecular weight (20). Human MCP-1 contains much less carbohydrate (22, 23). The C-terminal 49 amino acids of murine MCP-1 have no counterpart in human MCP-1, and this region is extremely rich in serine and threonine. Thus, we hypothesized that the *O*-linked carbohydrate of murine MCP-1 is added to the C-terminal region. To test this hypothesis, we constructed a murine MCP-1 cDNA that directs the synthesis of a truncated protein terminating at arginine-73 of processed MCP-1 (see *Materials and Methods*). Figure 4 shows that expression of truncated MCP-1 in COS cells led to the secretion of a nonmicroheterogeneous protein of $M_r \approx$ 14,000, suggesting that most of the carbohydrate is attached to amino acids in the C-terminal domain.

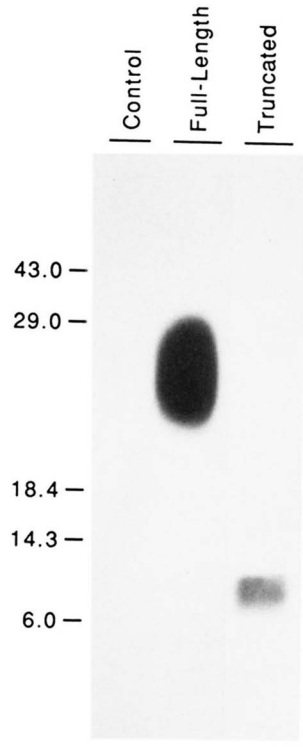

FIGURE 4. COS cells were electroporated using no DNA (Control), pXM-JE10 encoding full-length murine MCP-1 (full-length), or truncated murine MCP-1 in pXM (truncated) constructed as described in the text. Forty-eight hours after electroporation, cells were radiolabeled with [^{35}S]methionine for 4 h, and conditioned medium was analyzed by immune precipitation using anti-MCP-1 Abs. Immune precipitates were electrophoresed on a 15% polyacrylamide gel in SDS.

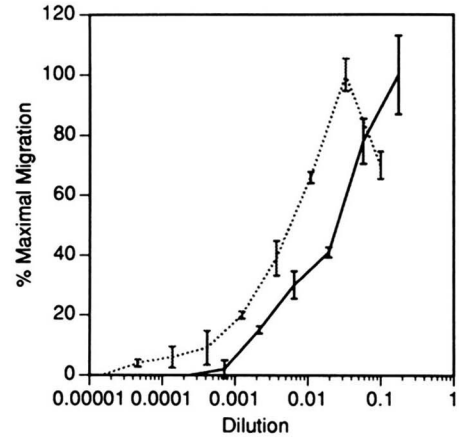

FIGURE 5. COS cells were electroporated as described in Figure 4, and plated in two 60-mm tissue culture dishes. One dish from each electroporation was radiolabeled with [^{35}S]methionine and processed for immune precipitation as in Figure 4. Laser densitometric analysis showed the presence of ≈5 times as much wild-type MCP-1 (encoded by pXM-JE10) as truncated MCP-1 in each unit volume of conditioned medium. Serum-free conditioned medium from the nonradiolabeled cultures was collected, and dilutions were tested for monocyte chemoattractant activity in vitro as described in *Materials and Methods*. The dilutions indicated in this figure were normalized for the fivefold difference in concentration of immunoreactive material. Solid line, wild-type MCP-1; dotted line, truncated MCP-1.

To test the chemoattractant potency of truncated MCP-1, we expressed wild-type and truncated MCP-1 transiently in COS cells, radiolabeled the proteins with [^{35}S]methionine, and subjected them to immune precipitation, as above. Both proteins have the same number of methionines, and rabbit anti-MCP-1 serum recognizes predominantly N-terminal epitopes. Thus, the immune precipitations were quantitative, and the relative amounts of truncated and wild-type MCP-1 were determined by laser densitometric analysis of x-ray films exposed to the immune precipitation products. Figure 5 shows an experiment in which dilutions of conditioned medium from wild-type and truncation mutant COS cell transfections were tested for monocyte chemoattractant activity after normalizing for relative amounts of each protein. The data show that within the limits of this quantitation, the truncation mutant retains all of the chemoattractant activity of wild-type MCP-1, with a possibly small increase in specific activity.

Glycosidase digestion of CHO-produced murine MCP-1

We next analyzed the structure of carbohydrate in murine MCP-1 produced by CHO cells. Figure 6 shows that digestion of MCP-1 with *N*-glycanase slightly decreased the highest molecular weight component of MCP-1, but did not greatly reduce the microheterogeneity or the size of most of the protein. This small amount of N-linked carbohydrate is consistent with the presence of a single consensus sequence for *N*-linked glycosylation in the core MCP-1 protein.

Digestion with neuraminidase collapsed the microheterogeneous $M_r \approx 25{,}000$ band to a less heterogeneous M_r 22,000 species. Digestion with neuraminidase followed by *O*-glycanase resulted in a band at $M_r \approx 12{,}000$, near the predicted size of the core, unglycosylated murine MCP-1 (Figure 6, *right*). Several attempts to digest the same sample of MCP-1 with *N*-glycanase, neuraminidase, and *O*-glycanase in series resulted in persistent microheterogeneity and incomplete loss of *O*-linked carbohydrate. The conditions necessary for *N*-glycanase treatment probably inhibited neuraminidase activity, and because *O*-glycanase can only digest desialylated carbohydrate, the *O*-glycanase treatment was incomplete.

Neuraminidase- and *O*-glycanase-digested MCP-1 was purified by HPLC and tested for monocyte chemoattractant activity in vitro. Figure 7 shows that deglycosylated MCP-1 retains full chemoattractant activity with a specific activity of 1,490,000 U/mg.

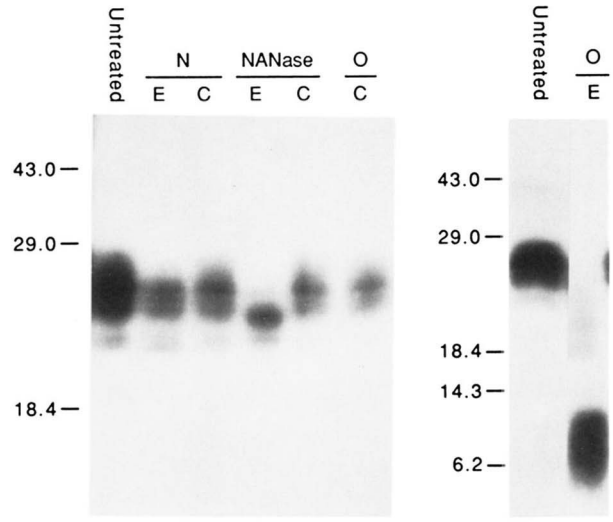

FIGURE 6. 10A-10 cells were radiolabeled with [^{35}S]methionine, and aliquots of conditioned medium were subjected to glycosidase treatment followed by immune precipitation as described in the text. Immune precipitates were analyzed on two different 15% polyacrylamide gels for optimal display. For both gels, E indicates experimental treatment and C indicates control treatment with water substituted for the indicated glycosidase. *Left gel*: N, *N*-glycanase; NANase, neuraminidase; O, *O*-glycanase. *Right gel*: O, *O*-glycanase.

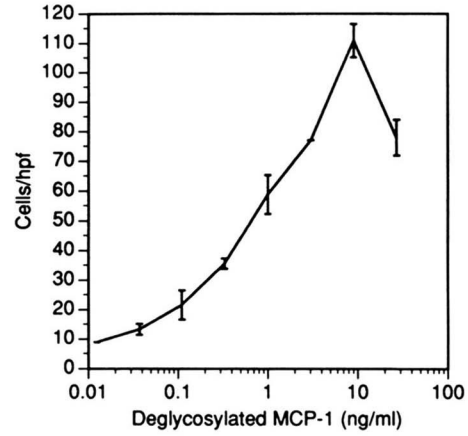

FIGURE 7. Pure MCP-1 was digested with neuraminidase and *O*-glycanase as described in *Materials and Methods*. An aliquot of digested material was analyzed by SDS-PAGE and Coomassie staining to document complete digestion. The remainder of the digest was purified by reversed-phase HPLC as described for undigested MCP-1. This material was tested for monocyte chemoattractant activity.

Sulfation of murine MCP-1

The presence of abundant *O*-linked carbohydrate in MCP-1 suggested the possibility that some of the polysaccharide might be in the form of glycosaminoglycans. Attempts to radiolabel CHO-produced MCP-1 with [^{35}S]sulfate never resulted in incorporation of detectable amounts of radiolabel (Fig. 8). However, COS-produced MCP-1 has evidence for minimally sulfated structures (Fig. 8). Because the sulfate does not appear in the M_r 14,000 component of MCP-1 or in the C-terminal truncation mutant, it is presumably part of the carbohydrate component. Attempts to radiolabel MCP-1 from 3T3 cells using [^{35}S]sulfate failed to demonstrate the presence of sulfated material, but the relatively small amounts of MCP-1 produced by these cells (compared to engineered cells) precludes a definitive interpretation.

Discussion

As a first step toward the rational use of mouse models for the investigation of chemokine physiology, the present study was designed to analyze the structure and activities of murine MCP-1. We produced recombinant murine MCP-1 in CHO cells, purified it to homogeneity, and found that it attracted monocytes but not neutrophils in vitro. The specific activity of murine MCP-1 for attracting human monocytes in vitro was similar to that of human MCP-1 (15, 21). This observation, along with reports that human MCP-1 attracts rodent monocytes in vivo (29, 30), suggests that MCP-1 activity may not be limited by rodent/human species barriers. It should be mentioned, however, that at least one group of investigators has been unable to demonstrate binding of human MCP-1 to rat mononuclear cells, although there was no mention of whether these cells were still chemotactically responsive to rat MCP-1 (31). Radioiodinated biologically active murine MCP-1 bound specifically to human monocytes with a K_d of 0.77 nM, and identified ≈3000 binding sites per cell. These values are comparable to those obtained for human MCP-1 binding to human monocytes (31–33), and the K_d is in the same range as the EC_{50} for chemoattraction.

O-linked carbohydrate comprised nearly 50% of the apparent mass of CHO-produced murine MCP-1, similar to natural MCP-1 secreted by fibroblasts (22). Based on its high content of serine and threonine, the C-terminal domain of murine MCP-1 was predicted to be the region most likely to contain *O*-linked carbohydrate. This was confirmed by expressing a truncated murine MCP-1 mutant, lacking 52 C-terminal amino acids and demonstrating secretion of (1) a protein whose size was consistent with the absence of significant amounts of carbohydrate and (2) a protein without the microheterogeneity of full-length MCP-1's *O*-linked carbohydrate. The serine- and threonine-rich C-terminal portion of MCP-1 has no counterpart in human MCP-1, and its absence would explain the relative paucity of *O*-linked carbohydrate in human MCP-1 (23). This mutational analysis further demonstrated that all of MCP-1's chemoattractant activity resides in the first 73 amino acids of the protein. Although the data of Figure 5 suggest that truncated MCP-1 might have a slightly higher specific activity than wild-type, the difference is modest and may only be the result of difficulties in the indirect

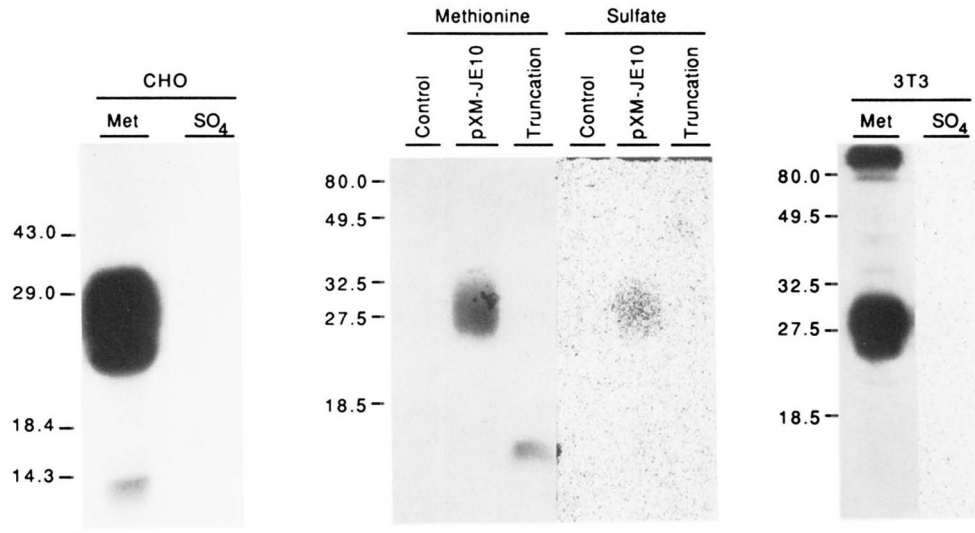

FIGURE 8. CHO cells expressing murine MCP-1, COS cells transfected with pmt21 (control plasmid), pXM-JE10 (wild-type MCP-1), or a plasmid encoding the C-terminal truncation mutant, or 3T3 cells treated with PDGF were radiolabeled with [^{35}S]methionine or [^{35}S]sulfate as described in the text. Medium conditioned by these cells was analyzed by immune precipitation and electrophoresis through 15% polyacrylamide gels. Methionine-labeled material was exposed to film for 24 h, sulfate-labeled material was exposed to film for 20 days (CHO cell material) or analyzed by PhosphorImager (transfected COS cell or 3T3 cell material). (COS cell-produced MCP-1 had low but detectable [^{35}S]sulfate incorporation after exposure to x-ray film for 10 days.)

quantitation method used in this experiment. Murine MCP-1 thus appears to have two distinct domains, an amino-terminal domain responsible for monocyte chemoattraction and a carboxyl-terminal domain with an as yet undetermined function.

Murine MCP-1's microheterogeneity was demonstrated to be the result of terminal sialic acid residues, because treatment with neuraminidase eliminated microheterogeneity. Thus, the number of serines or threonines substituted with carbohydrate is the same for each protein in this population of MCP-1 molecules, whereas the number of sialic acids in each *O*-linked carbohydrate group is variable. It is unknown if the degree of sialic acid substitution has any functional significance. In fact, the functional significance of any of murine MCP-1's carbohydrate is unclear. Carbohydrate is not necessary for monocyte chemoattraction in vitro, because the deglycosylated protein had the same specific activity as the fully glycosylated form (Fig. 7). This observation might have been predicted on the basis of the fact that the underglycosylated M_r 14,000 species of murine MCP-1, the truncated unglycosylated murine MCP-1 mutant, and unglycosylated human MCP-1β all attract monocytes in vitro with similarly high specific activities.

We did not examine the structure of the *O*-linked carbohydrate of murine MCP-1 in detail, although the substrate specificity of *O*-glycanase implies that the reducing terminal sugar is *N*-acetyl galactosamine. The extremely low levels of sulfation in COS cell-produced MCP-1 suggest that the carbohydrate is probably not glycosaminoglycan-like, but rather is a simple oligosaccharide with sialic acid added directly to *N*-acetyl galactosamine or through a single branch to galactose. We cannot exclude the possibility that the carbohydrate may be undersulfated glycosaminoglycan, although this is unlikely because 3T3 cells make fully sulfated glycosaminoglycan (34) and yet 3T3 cell-produced MCP-1 had no detectable sulfate. Alternatively, the sulfate may be added to the lone predicted N-linked oligosaccharide.

One cannot infer any specific functions on the basis of these carbohydrate structures. Such substitutions have been suggested to protect proteins from proteolysis, prolonging their effective half-lives (35, 36). A more intriguing possibility would be that the carbohydrate is involved in specific recognition events in vivo. For example, macrophage inflammatory protein-1β, another C-C chemokine, has been shown to interact with chondroitin sulfate on the surface of endothelial cells (37). The carbohydrate portion of murine MCP-1 may be involved in similar recognition events, although the absence of extensive glycosylation in human MCP-1 suggests that whatever its function, its presence in a bifunctional MCP-1 protein has not been conserved through evolution. Alternatively, as the number of identified chemokines continues to expand, another human chemokine may be described that has carbohydrate like that of murine MCP-1. As of now, however, no chemokine in any species, other than rodent MCP-1, contains a large amount of carbohydrate. The amino acid sequences of rat, guinea pig, and rabbit MCP-1 suggest that they may be similarly substituted, and their apparent molecular size and microheterogeneity (when secreted

from eukaryotic cells) are consistent with extensive *O*-linked glycosylation (38).

We also described a striking disparity between murine MCP-1's chemoattractant potency in vivo vs in vitro. Although some investigators have described extensive monocytic infiltration in response to human MCP-1 injected into rodents (29, 30), we have been unable to do so. This suggests that monocyte chemotaxis is a complex process in vivo with multiple components, a concept that is obvious from first principles. At the very least, monocyte infiltration would require the expression of adhesion molecules on the endothelial cell surface, and it has not been demonstrated that MCP-1 injection alone can accomplish this.

Two further lines of evidence suggest that other factors in addition to MCP-1 are required for monocytic infiltration in vivo. First, although we saw no overwhelming monocytic infiltrates in response to MCP-1 protein injected into mouse skin, we did demonstrate such infiltrates after injecting CHO cells expressing murine MCP-1 into nude mice (24). Presumably, the CHO cells themselves were supplying costimuli for monocyte attraction. Second, we have constructed transgenic mice expressing high levels of biologically active MCP-1 in a variety of organs, none of which show any evidence for monocytic infiltration.[4] Perhaps these mice are missing another stimulus required for MCP-1 to attract monocytes.

Chemokine physiology in vivo continues to be difficult to understand. With the development of mouse models for chemokine overexpression or loss, some of the more puzzling issues may become clearer. The availability of large quantities of eukaryotically produced, fully glycosylated murine MCP-1 should help this process.

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