

# Macrophage Inflammatory Protein-1 $\alpha$

## A Novel Chemotactic Cytokine for Macrophages in Rheumatoid Arthritis

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### Abstract

We have shown that human macrophages (m $\phi$ s) play an important role in the elaboration of chemotactic cytokines in rheumatoid arthritis (RA) (Koch, A. E., S. L. Kunkel, J. C. Burrows, H. L. Evanoff, G. K. Haines, R. M. Pope, and R. M. Strieter. 1991. *J. Immunol.* 147:2187; Koch, A. E., S. L. Kunkel, L. A. Harlow, B. Johnson, H. L. Evanoff, G. K. Haines, M. D. Burdick, R. M. Pope, and R. M. Strieter. 1992. *J. Clin. Invest.* 90:772; Koch, A. E., P. J. Polverini, S. L. Kunkel, L. A. Harlow, L. A. DiPietro, V. M. Elner, S. G. Elner, and R. M. Strieter. 1992. *Science (Wash. DC)*. 258:1798). Recently, m $\phi$  inflammatory protein-1 (MIP-1 $\alpha$ ), a cytokine with chemotactic activity for m $\phi$ s and neutrophils (PMNs), has been described. We have examined the production of MIP-1 $\alpha$  using sera, synovial fluid (SF), and synovial tissue (ST) from 63 arthritic patients. MIP-1 $\alpha$  was higher in RA SF (mean, 29 $\pm$ 8 ng/ml [SE]) compared with other forms of arthritis (2.8 $\pm$ 1.7), or osteoarthritis (0.7 $\pm$ 0.4;  $P < 0.05$ ). RA SF MIP-1 $\alpha$  was greater than that found in either RA or normal peripheral blood (PB) ( $P < 0.05$ ). Anti-MIP-1 $\alpha$  neutralized 36 $\pm$ 3% (mean $\pm$ SE) of the chemotactic activity for m $\phi$ s, but not PMNs, found in RA SFs. RA SF and PB mononuclear cells produced antigenic MIP-1 $\alpha$ . Mononuclear cell MIP-1 $\alpha$  production was augmented with phytohemagglutinin or LPS. Isolated RA ST fibroblast production of antigenic MIP-1 $\alpha$  was augmented upon incubation of cells with LPS, and to a lesser extent with tumor necrosis factor- $\alpha$ . Isolated RA ST m $\phi$ s expressed constitutive MIP-1 $\alpha$  mRNA and antigenic MIP-1 $\alpha$ . Using ST immunohistochemistry, MIP-1 $\alpha$ <sup>+</sup> cells from RA compared with normal were predominantly m $\phi$ s and lining cells ( $P < 0.05$ ). These results suggest that MIP-1 $\alpha$  plays a role in the selective recruitment of m $\phi$ s in synovial inflammation associated with RA. (*J. Clin. Invest.* 1994. 93:921–928.) **Key words:** macrophage • synovium • synovial fluid • inflammation • chemotaxis

### Introduction

Rheumatoid arthritis (RA)<sup>1</sup> is characterized by persistent synovial inflammation, destruction of bone and cartilage, and nu-

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1. **Abbreviations used in this paper:** MCP-1, monocyte chemoattractant protein-1; m $\phi$ , macrophage; OA, osteoarthritis; PB, peripheral blood; RA, rheumatoid arthritis; rh, recombinant human; SF, synovial fluid; ST, synovial tissue.

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merous systemic manifestations (1). Chronic inflammatory cells, including macrophages (m $\phi$ s) and lymphocytes, are present in the synovial tissues (STs) (1). Cytokines are produced by cells within the inflamed STs, perhaps accounting for a number of the pathological and clinical manifestations of RA (2). These cytokines are predominantly products of synovial m $\phi$ s and fibroblasts, including IL-1, -6, and -8, TNF- $\alpha$ , colony stimulating factor-1 (CSF-1), GM-CSF, IL-1 receptor antagonist protein, monocyte chemoattractant protein-1 (MCP-1), and leukemia inhibitory factor (3–18). These cytokines alone or in concert serve to orchestrate the destructive phase of RA synovitis.

Recently, a novel chemotactic cytokine, MIP-1 $\alpha$ , has been described (19–23). MIP-1 $\alpha$  is an 8-kD protein produced by LPS-activated m $\phi$ s, other hematopoietic cells such as B and T lymphocytes, PMNs, as well as fibroblasts under some conditions (19, 24). MIP-1 $\alpha$ , a member of the chemokine gene superfamily, is chemokinetic for PMNs as well as chemotactic for m $\phi$ s and T cells, and hence may play a role in the recruitment of inflammatory leukocytes into the inflamed joint (19, 25, 26). The role of this cytokine in RA has not, as yet, been defined.

In this study, we found significantly greater levels of MIP-1 $\alpha$  in synovial fluids (SFs) from patients with RA as compared with osteoarthritis (OA) or other inflammatory and noninflammatory arthritis. MIP-1 $\alpha$  levels found in RA SFs were significantly greater than the quantities found in normal or RA peripheral blood (PB). RA SF chemotactic activity for normal PB m $\phi$ s, but not PMNs, was inhibited 36 $\pm$ 3% (mean $\pm$ SE) upon incubation with anti-MIP-1 $\alpha$ . A variety of cells, including ST fibroblasts and m $\phi$ s, as well as SF mononuclear cells, produced MIP-1 $\alpha$ . RA ST m $\phi$ s constitutively produced both MIP-1 $\alpha$  mRNA and protein. In arthritic as well as normal STs, we identified the predominant MIP-1 $\alpha$ <sup>+</sup> cells as the synovial lining cells and subsynovial m $\phi$ s. The numbers of MIP-1 $\alpha$ <sup>+</sup> cells found in the RA synovium were significantly greater than those found in the ST of normal individuals.

### Methods

**Reagent preparation.** Human recombinant IL-1 $\beta$  with a specific activity of 5  $\times$  10<sup>8</sup> U/mg was purchased from R & D Systems (Minneapolis, MN). Human recombinant TNF- $\alpha$  with a specific activity of 1.3  $\times$  10<sup>7</sup> U/mg was a gift from Upjohn Co. (Kalamazoo, MI). Human recombinant MIP-1 $\alpha$  was purchased from R & D Systems. LPS (*Escherichia coli*, 0111:B4) was obtained from Sigma Chemical Co. (St. Louis, MO). PHA was obtained from Burrows Wellcome (Research Triangle Park, NC). Polyclonal antihuman MIP-1 $\alpha$  was produced by immunization of rabbits with recombinant MIP-1 $\alpha$  with CFA. This antibody does not cross-react with the following cytokines: TNF- $\alpha$ , IL-1, IL-2, IL-4, IL-6, or IFN- $\gamma$ . In addition, the antibody does not cross-react with members of the C-C chemokine family, including MCP-1, RANTES, and macrophage inflammatory protein 1 $\beta$  (MIP-1 $\beta$ ), or

members of the C-X-C chemokine family, including GRO- $\alpha$ , epithelial neutrophil-activating peptide-78, neutrophil-activating peptide-2, and IP-10 (24, 27, 28). mAb Leu-M5 (anti-CD11c, p150,95, CR4 receptor) detects m $\phi$ s (Becton Dickinson & Co., Mountain View, CA). mAb FVIII detects factor VIII-related antigen present on endothelial cells (Dakopatts, Carpinteria, CA).

**Patient population.** SFs were isolated from patients with either RA, OA, or other forms of arthritis during therapeutic arthrocentesis. PB specimens were obtained from some of the RA patients as well as from healthy volunteers. SFs and PB were used for isolation of mononuclear cells. ST was obtained from patients undergoing total joint replacements who met the American College of Rheumatology criteria for RA or OA (29, 30). Normal STs were obtained from fresh autopsies. STs from these patients were snap frozen in OCT (Miles Laboratories, Elkhart, IN). Alternatively, fresh RA tissues were used for isolation of m $\phi$ s or fibroblasts (see below). All specimens were obtained with Institutional Review Board approval.

**Isolation of human RA SF and blood mononuclear cells and PMNs.** Mononuclear cells from heparinized RA SFs were isolated by gradient centrifugation using Histopaque-1077 (Sigma Diagnostics, St. Louis, MO), as previously described (31). Mononuclear cells were isolated from the PB of normal volunteers, using Sepacell-MN (Sepratech Corp., Oklahoma City, OK), as described. PMNs were isolated using Ficoll-Hypaque density centrifugation (Accurate Chem. & Sci. Corp., Westbury, NY). Mononuclear cells or isolated lymphocytes or m $\phi$ s ( $2 \times 10^6$  cells/ml) were incubated with or without PHA (1  $\mu$ g/ml) or LPS (5  $\mu$ g/ml) and cultured in 24-well plates for 90 h at 37°C in RPMI + 10% FCS.

**Isolation of human RA ST fibroblasts and m $\phi$ s and preparation of conditioned media.** Fresh STs were minced and digested in a solution of dispase, collagenase, and DNase, as previously described (32, 33). Synovial fibroblast cells were cultured in RPMI + 10% FCS + gentamicin in 75-mm tissue culture flasks (Costar, Cambridge, MA). Upon reaching confluence, the cells were passaged by brief trypsinization (32). The cells were used at passage 4 or older, at which time they were a homogenous population of fibroblasts. The cells were plated at a concentration of  $3.1\text{--}4 \times 10^5$  cells/well in 24-well plates (Costar) in 1 ml serum-free RPMI. Various concentrations of IL-1 $\beta$ , TNF- $\alpha$ , or LPS in RPMI were added, and conditioned media harvested after 24 h.

For isolation of m $\phi$ s, the STs were minced and digested as described above. The resultant single-cell suspensions were fractionated into density-defined subpopulations by isopyknic centrifugation through continuous preformed Percoll gradients (Pharmacia Fine Chemicals, Piscataway, NJ). m $\phi$ s were enriched by adherence to fibronectin-coated collagen gels and selective trypsinization (incubation with trypsin/EDTA for 5–10 min) (32, 33). m $\phi$ s were harvested from the collagen gels by treatment with clostridial collagenase and found to be  $\geq 90\%$  pure, as assessed by Fc receptor-mediated phagocytosis of IgG opsonized sheep red blood cells, esterase staining, and staining with commercial anti-m $\phi$  mAbs (32, 33).

**MIP-1 $\alpha$  ELISA.** Antigenic MIP-1 $\alpha$  was measured using a modification of a double ligand method as described (24, 27, 28). In brief, 96-well plates (Nunc, Kamstrup, Denmark) were coated with 50  $\mu$ l/well rabbit anti-MIP-1 $\alpha$  (3.2  $\mu$ g/ml in 0.6 M NaCl, 0.26 M H<sub>3</sub>BO<sub>4</sub>, and 0.08 N NaOH, pH 9.6) for 16 h at 4°C and then washed in PBS, pH 7.5, 0.05% Tween-20 (wash buffer). Nonspecific binding sites were blocked with 2% BSA in PBS (200  $\mu$ l), and the plates incubated for 90 min at 37°C. Plates were rinsed three times with wash buffer, and diluted (neat, 1:5, and 1:10) test sample (50  $\mu$ l) in duplicate was added, followed by incubation for 1 h at 37°C. Plates were washed four times, and 50  $\mu$ l/well biotinylated rabbit anti-MIP-1 $\alpha$  (6  $\mu$ g/ml in PBS, pH 7.5, 0.05% Tween-20, 2% FCS) was added for 45 min at 37°C. Plates were washed four times, streptavidin-peroxidase conjugate (100  $\mu$ g/ml) (Dakopatts, Carpinteria, CA) was added, and the plates were incubated for 30 min at 37°C. The plates were washed three times, and 100  $\mu$ l chromogen substrate (0.67 mg/ml orthophenylenediamine dichloride) (Dakopatts) was added. The plates were incubated at 25°C for 6 min, and the reaction was terminated with 50  $\mu$ l/well of 3 M H<sub>2</sub>SO<sub>4</sub>

solution in wash buffer plus 2% FCS. Plates were read at 490 nm in an ELISA reader. Standards were  $\frac{1}{2}$  log dilutions of recombinant MIP-1 $\alpha$  from 100 ng/ml to 1 pg/ml (50  $\mu$ l/well). The ELISA consistently detected MIP-1 $\alpha$  concentrations > 50 pg/ml.

**Northern blot analysis.** Total cellular RNA was obtained from  $2.5 \times 10^6$  RA ST m $\phi$ s using a modification of our previously described method (3, 16). Briefly, cells were scraped into a solution containing 25 mM Tris, pH 8.0, 4.2 M guanidine isothiocyanate, 0.5% Sarkosyl, and 0.1 M 2-mercaptoethanol. After homogenization, the suspension was added to a solution containing an equal volume of 100 mM Tris, pH 8.0, 10 mM EDTA, and 1% SDS. The mixture was then extracted with chloroform-phenol and chloroform-isoamyl alcohol. The RNA was alcohol precipitated and the pellet dissolved in diethylpyrocarbonate-treated H<sub>2</sub>O. Total RNA was separated by Northern analysis using formaldehyde, 1% agarose gels, transblotted onto nitrocellulose, baked, prehybridized, and hybridized with a <sup>32</sup>P-5' end-labeled oligonucleotide probe. A 30-mer oligonucleotide probe was synthesized using the published cDNA sequence for human-derived MIP-1 $\alpha$  (34). The probe was complementary to nucleotides 105–134 and had the sequence 5'-GAG-AGC-CAT-GGT-GCA-GAG-GAG-GAC-AGC-AAG-3'. Equivalent amounts of total RNA per gel were assessed by monitoring 28s and 18s rRNA.

**Bioassay for chemotactic activity for PMNs or m $\phi$ s.** Normal human PMNs or m $\phi$ s were obtained from PB by Ficoll-Hypaque density gradient centrifugation. PMNs were separated from erythrocytes by hypotonic lysis and then suspended in HBSS with calcium and magnesium (GIBCO BRL, Bethesda, MD) at  $2 \times 10^6$  cells/ml. m $\phi$ s were suspended in HBSS at  $3 \times 10^6$  cells/ml. All cells were > 95% viable by trypan blue exclusion. Chemotaxis was performed as previously described (3, 16). In brief, 150  $\mu$ l of SF that was diluted 1:1 with HBSS,  $10^{-7}$  M FMLP for PMNs, or  $10^{-8}$  M FMLP for m $\phi$ s (SIGMA BRL), or HBSS alone was placed in duplicate bottom wells of a blind-well chemotaxis chamber. A 3- $\mu$ m-pore-size polyvinylpyrrolidone-free polycarbonate filter for PMN chemotaxis or a 5- $\mu$ m-pore-size filter for m $\phi$  chemotaxis (Nucleopore Corp, Pleasanton, CA) was placed in the assembly and 250  $\mu$ l of cell suspension placed in each of the top wells. Chemotaxis chambers were incubated at 37°C in humidified 95% air/5% CO<sub>2</sub> for 1 h for PMN chemotaxis and 2 h for m $\phi$  chemotaxis. The filters were removed, fixed in absolute methanol, and stained with 2% toluidine blue (Sigma Chemical Co.). PMNs or m $\phi$ s that had migrated through to the bottom of the filter were counted in 10 high power fields ( $\times 1,000$ ). Positive control PB m $\phi$  maximal recruitment in response to recombinant human (rh) MIP-1 $\alpha$  occurred at 100 ng/ml (28). At 1:1,000 anti-MIP-1 $\alpha$  antiserum was capable of inhibiting 60–68% of the m $\phi$  chemotactic activity induced by 1–100 ng/ml rhMIP-1 $\alpha$  (28). In neutralization studies, SF was incubated with a 1:1,000 dilution of either control rabbit preimmune serum or anti-MIP-1 $\alpha$  antiserum for 30 min at 37°C before the chemotactic assay.

**Immunoperoxidase staining.** Frozen ST sections (4  $\mu$ m) were cut, and immunoperoxidase was stained using an avidin-biotin technique (Vector Laboratories, Burlingame, CA) (35–37). Slides, air dried for 2–16 h, were fixed in cold acetone for 20 min. All subsequent incubations were performed for 15 min at 37°C in a moist chamber. STs were pretreated with 50  $\mu$ l diluted normal horse serum (135  $\mu$ l horse serum in 10 ml 1% PBS-BSA, incubated with either rabbit anti-human MIP-1 $\alpha$ , preimmune rabbit serum, mAb Leu-M5, or control mAb, and washed twice. The slides were incubated with a 1:400 dilution of anti-rabbit biotinylated antibody in PBS-BSA, washed twice with PBS, incubated with avidin-conjugated horseradish peroxidase complex, and washed with PBS twice. Slides were then stained with diaminobenzidine tetrahydrochloride substrate for 5 min at room temperature, rinsed in tap water for 2 min, counterstained with Harris' hematoxylin, and dipped in saturated lithium carbonate solution for bluing. Serial tissue sections were examined to determine the percentage of each cell type expressing antigenic MIP-1 $\alpha$ .

Cytospin preparations of isolated RA ST m $\phi$ s were made using  $10^5$  cells per slide in a cytospin (Shandon I; Shandon, Swickley, PA). Slides were stained using immunohistochemistry as described above.

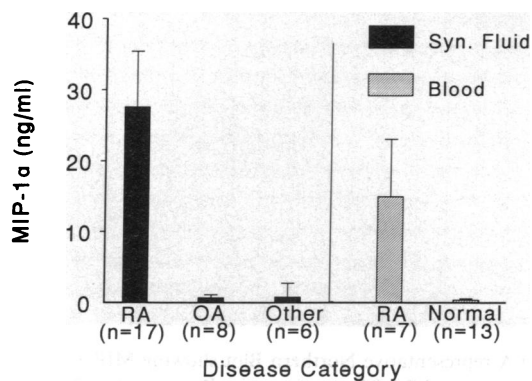
**Statistical analysis.** Statistical analysis was performed using an analysis of variance (38). Correlations were performed using a Pearson correlation. *P* values < 0.05 were considered significant.

## Results

**Antigenic MIP-1 $\alpha$  is elevated in SF from RA compared with SF from OA or other arthritis.** MIP-1 $\alpha$  was measured by ELISA using samples obtained from 31 patients (Fig. 1). RA SFs contained the largest quantities of MIP-1 $\alpha$  (29 $\pm$ 8 ng/ml [mean $\pm$ SE]). RA SF levels of MIP-1 $\alpha$  were significantly greater than levels found in OA (0.7 $\pm$ 0.4 ng/ml) or SFs from patients with other forms of arthritis (2.8 $\pm$ 1.7 ng/ml; *P* < 0.05). Patients with other forms of arthritis included one with chronic myelogenous leukemia, one with mixed connective tissue disease, one with polymyositis, one with acute rheumatic fever, and two with Reiter's syndrome. In addition, RA SF MIP-1 $\alpha$  levels were greater than that found in RA or normal PB.

**RA SF MIP-1 $\alpha$  is chemotactic for normal PB m $\phi$ s but not PMNs.** To determine whether RA SF MIP-1 $\alpha$  was biologically active, we determined the relative contribution of SF MIP-1 $\alpha$  to chemotaxis for normal PB cellular constituents. In murine systems MIP-1 $\alpha$  is chemokinetic for PMNs in vitro as well as causes a rapid influx of PMNs when injected into the footpad of mice or intercosternally in rabbits (19). The effects of rhMIP-1 $\alpha$  on PMN chemotaxis are less clear, with some reports indicating that this cytokine is not chemotactic for PMNs (39, 40). We therefore determined the effect of neutralizing anti-MIP-1 $\alpha$  on RA SF chemotactic activity for normal human PB PMNs. While chemotactic activity for PMNs was present in the RA SFs, no diminution of the chemotactic response was found upon incubation of SFs with neutralizing anti-MIP-1 $\alpha$  (data not shown).

Recently, we have found that rhMIP-1 $\alpha$  in vitro is chemotactic for monocytes (28). We therefore examined the contribution of RA SF MIP-1 $\alpha$  to the chemotactic activity for normal PB m $\phi$ s found in these SFs. SFs were incubated with either nonimmune or anti-MIP-1 $\alpha$  antiserum (Table I). Chemotaxis was expressed as the mean cells per high power field ( $\times$ 400). Incubation with either antibody did not alter the response to FMLP-induced chemotaxis. In contrast, incubation with anti-MIP-1 $\alpha$  resulted in 31–49% (mean, 36 $\pm$ 3% [SE]) suppression



**Figure 1.** Antigenic MIP-1 $\alpha$  levels in SF from various arthritides as well as RA and normal PB. Results represent the mean $\pm$ SE. Six replicate determinations per patient were performed. (N) Number of patients examined.

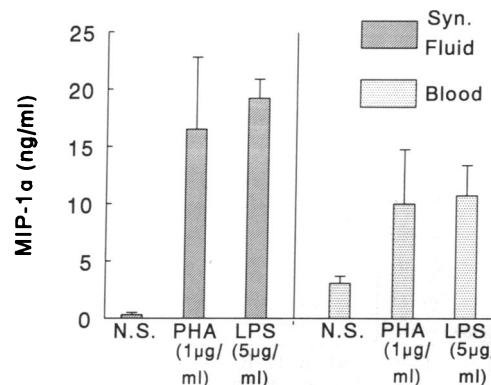
**Table I.** Chemotaxis of Normal Human PB m $\phi$ s in Response to RA SFs Incubated in the Presence and Absence of Anti-MIP-1 $\alpha$

Patient	Mean cells/high power field ( $\times$ 400)		Percent suppression*	MIP-1 $\alpha$ <sup>‡</sup> ng/ml
	Nonimmune serum	Anti-MIP-1 $\alpha$		
1	161	82	49	2.5
2	101	65	36	10.2
3	98	62	38	16.8
4	75	54	29	3.1
5	80	56	31	3.2
6	85	56	34	0.9

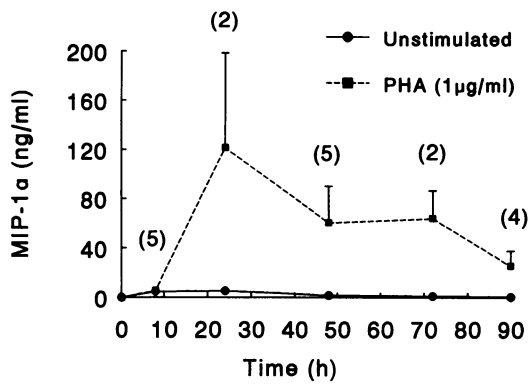
RA SFs were assayed for their ability to induce chemotaxis of normal PB m $\phi$ s. The results represent the analysis of 10 high power fields per sample. The ability of anti-MIP-1 $\alpha$  to neutralize chemotactic activity of m $\phi$ s was determined. Positive control migration in response to FMLP (10<sup>-8</sup> M) was a mean of 129 cells per high power field. Negative control migration in response to HBSS was a mean of eight cells per high power field. \* For percent suppression in all patient samples assayed, *P* < 0.05. ‡ MIP-1 $\alpha$  levels were determined by ELISA.

of chemotactic activity for m $\phi$ s of the SFs obtained from six RA patients.

**RA mononuclear cells produce MIP-1 $\alpha$ : augmentation with LPS or PHA stimulation.** Since RA SFs contained MIP-1 $\alpha$ , we determined the SF cell types responsible for production of this cytokine. To determine whether RA mononuclear cells produced MIP-1 $\alpha$ , mononuclear cells were obtained from matched SF and PB from three patients. Cells (2  $\times$  10<sup>6</sup> cells/ml RPMI + 10% FCS) were cultured with or without LPS or PHA for 90 h and their supernatants assayed for antigenic MIP-1 $\alpha$  (Fig. 2). PB mononuclear cells released small quantities of MIP-1 $\alpha$  (2.8 $\pm$ 0.7 ng/ml [SE]). MIP-1 $\alpha$  levels were augmented over threefold upon incubation of PB mononuclear cells with PHA, or LPS. Matching mononuclear cells obtained from SF



**Figure 2.** Mononuclear cells were isolated from matching SF and PB obtained from three RA patients. Cells (2  $\times$  10<sup>6</sup> cells/ml RPMI + 10% FCS) were cultured for 90 h in the presence or absence of LPS or PHA and supernatants were collected. Supernatants were assayed for antigenic MIP-1 $\alpha$  by ELISA. Results represent the mean $\pm$ SE of six replicate determinations. (N.S.) Nonstimulated cells.



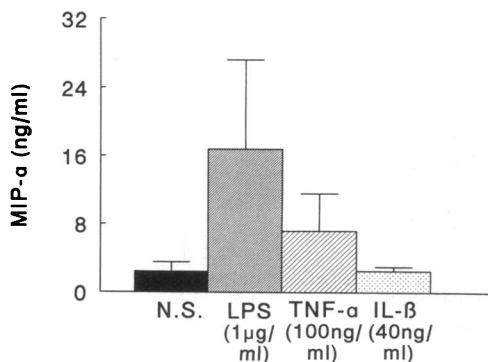
**Figure 3.** Time-dependent generation of SF mononuclear MIP-1 $\alpha$ . Mononuclear cells were isolated from RA SFs. Cells ( $2 \times 10^5$  cells/ml RPMI + 10% FCS) were cultured for various time periods in the presence or absence of PHA. Numbers in parentheses represent the number of patient samples available for a given time point. Results represent the mean  $\pm$  SE of six replicate determinations.

also produced small quantities of MIP-1 $\alpha$  ( $0.3 \pm 0.2$  ng/ml [SE]), though these levels were not significantly different from those obtained from PB cells. Incubation of these mononuclear cells with PHA or LPS augmented MIP-1 $\alpha$  released  $> 50$ -fold (Fig. 2).

To examine the time course of MIP-1 $\alpha$  secretion from RA SF mononuclear cells, cells were cultured in the presence or absence of PHA (Fig. 3). Nonstimulated cells released small quantities of MIP-1 $\alpha$  ( $4.0 \pm 2.1$  ng/ml [SE]). In contrast, PHA-stimulated MIP-1 $\alpha$  production peaked by 24 h of cell culture, declining steadily over the next 72 h of culture.

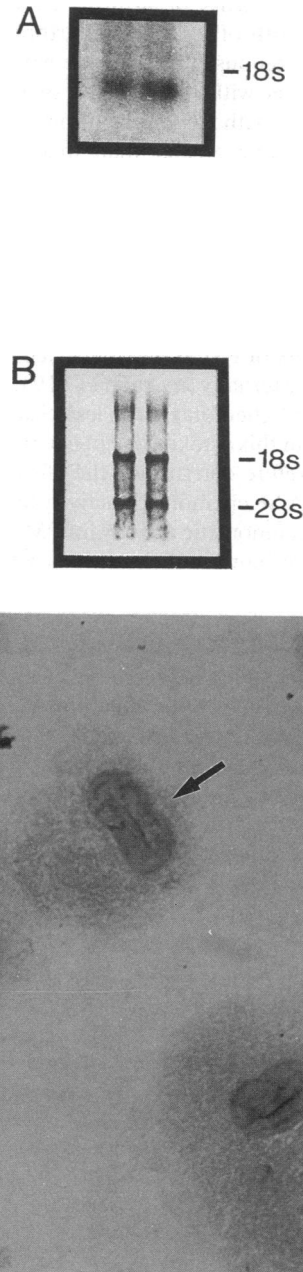
**RA ST fibroblast production of MIP-1 $\alpha$ : augmentation upon LPS or TNF- $\alpha$  stimulation.** We determined whether cells isolated from RA ST produced MIP-1 $\alpha$ . Isolated RA fibroblasts from four RA patients were cultured in the presence or absence of IL-1 $\beta$ , TNF- $\alpha$ , or LPS, and supernatants were collected after 24 h. While producing MIP-1 $\alpha$  without stimulation ( $2.4 \pm 1.1$  ng/ml [SE]), the production of fibroblast-derived MIP-1 $\alpha$  was augmented upon incubation of cells with LPS and to a lesser extent TNF- $\alpha$ , but not IL-1 $\beta$  (Fig. 4).

**RA ST m $\phi$  production of MIP-1 $\alpha$ .** Since we and others have shown previously that RA ST m $\phi$ s are important cells in the

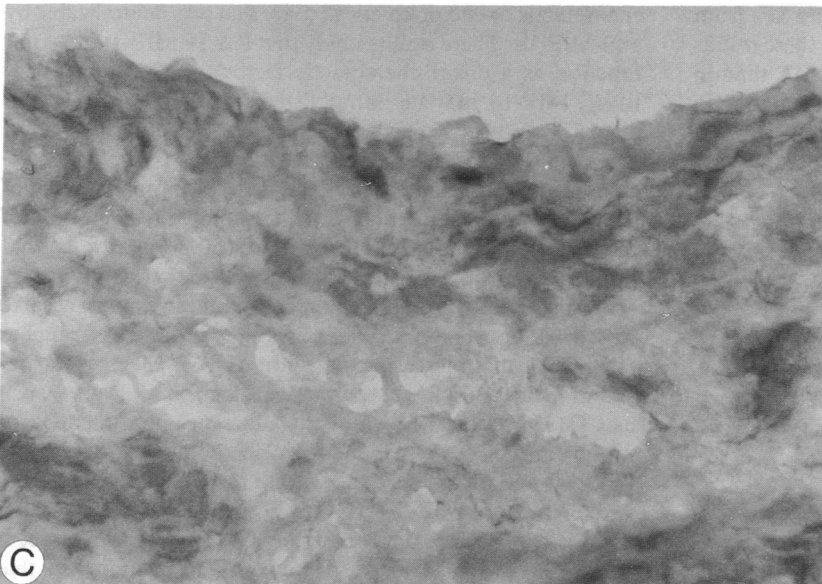
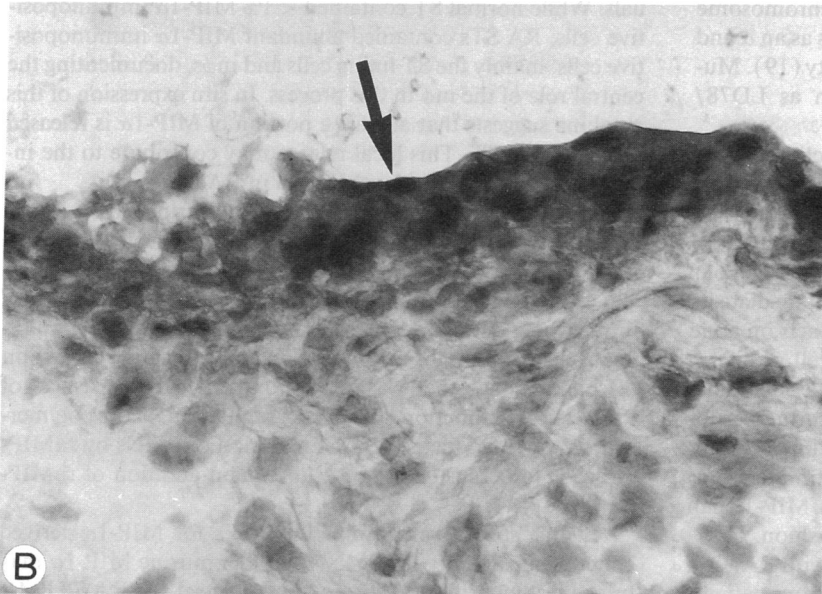
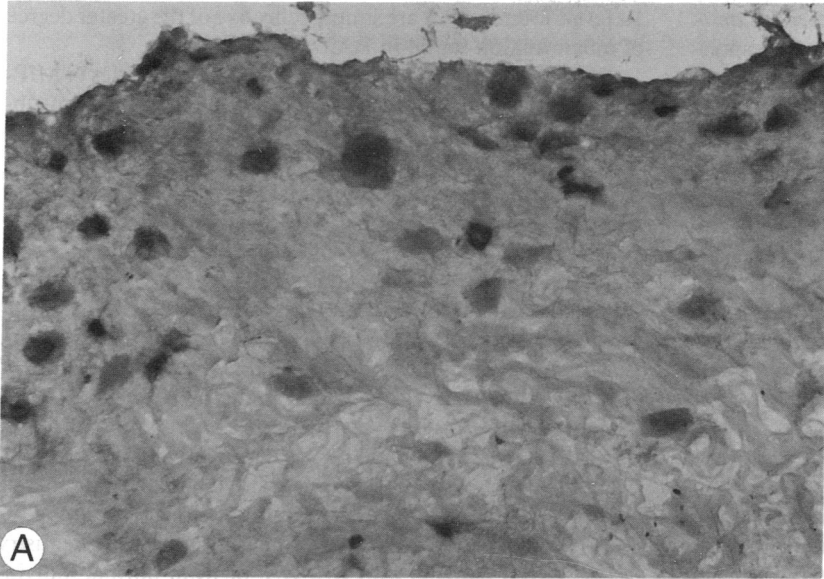


**Figure 4.** ST fibroblast MIP-1 $\alpha$  production by LPS or TNF- $\alpha$  stimulation. RA fibroblasts ( $3-4 \times 10^5$  cells/well) were cultured in serum-free RPMI for 24 h. Results represent the mean  $\pm$  SE from four patient samples. Six replicates of each sample were assayed. (N.S.) Nonstimulated cells.

production of cytokines in the RA joint, we assessed the levels of MIP-1 $\alpha$  mRNA from isolated RA m $\phi$ s. RA m $\phi$ s isolated in this fashion expressed constitutive steady-state MIP-1 $\alpha$  mRNA (Fig. 5). This compares to virtually absent steady state MIP-1 $\alpha$  mRNA levels obtained from resting normal human PB m $\phi$ s (data not shown) (27). Cytospin preparations of these same cells were analyzed for antigenic MIP-1 $\alpha$  by immunohistochemistry. These isolated m $\phi$ s were found to be positive for the expression of MIP-1 $\alpha$  antigen.



**Figure 5.** (A) A representative Northern Blot showing MIP-1 $\alpha$  gene expression from isolated RA ST m $\phi$ s from two RA patients. (B) 18S and 28S rRNA demonstrating equivalent loading of total RNA in A. (C) Immunoperoxidase stained cytocentrifuge preparation of freshly isolated RA ST m $\phi$ s showing MIP-1 $\alpha$  antigen expression (arrows) ( $\times 870$ ).



**Figure 6.** Immunoperoxidase reactivity of anti-MIP-1 $\alpha$  with frozen human STs ( $\times 422$ ). (A) Lack of MIP- $\alpha$ -reactive cells in a normal ST. (B) Intense lining layer (arrow) MIP-1 $\alpha$  reactivity in an RA ST. (C) Lack of reactivity of RA ST with treated with nonimmune serum (control).

RA ST lining cells and *mφs* are immunopositive for antigenic MIP-1 $\alpha$ . To determine which cells in the STs were responsible for MIP-1 $\alpha$  production in vivo, we performed immunohistochemistry on frozen ST samples from 9 patients with RA, 10 patients with OA, and 4 normal individuals. RA ST *mφs* and lining cells were MIP-1 $\alpha$  immunopositive (Fig. 6). While 35 $\pm$ 10% (SE) of RA ST lining cells expressed antigenic MIP-1 $\alpha$ , significantly fewer normal lining cells were MIP-1 $\alpha$  immunopositive (0.3 $\pm$ 0.3 [SE];  $P < 0.05$ ) (Fig. 7). Similarly, while 24 $\pm$ 6% (SE) of RA ST *mφs* were MIP-1 $\alpha$  immunopositive, only 1 $\pm$ 0.7% (SE) of normal ST *mφs* were MIP-1 $\alpha$  immunopositive ( $P < 0.05$ ) (Fig. 7). OA lining cells and *mφs* were immunopositive for MIP-1 $\alpha$ , however, fewer cells were positive than were found in RA STs.

## Discussion

MIP-1 $\alpha$  is a recently described cytokine belonging to the C-C chemokine supergene family (39, 41). This family includes ACT-2, I-309, RANTES, TCA-3, and MCP-1 (40). The genes encoding this supergene family reside on human chromosome 17 (40). The murine MIP-1 $\alpha$  homologue migrates as an  $\alpha$  and  $\beta$  doublet with 60% peptide and 57% cDNA identity (19). Murine MIP-1 $\alpha$  and human MIP-1 $\alpha$  (also known as LD78/ pAt464) have 75% amino acid homology (19).

MIP-1 $\alpha$  has a variety of proinflammatory activities that suggest its possible importance in chronic inflammatory diseases like RA. Murine MIP-1 $\alpha$  is an endogenous pyrogen when given intravenously to rabbits or into the hypothalamic preoptic area of the rat (42, 43). However, unlike other endogenous pyrogens, such as IL-1 or TNF- $\alpha$ , MIP-1 $\alpha$  does not mediate its pyrogenic effect via prostaglandins (42). MIP-1 $\alpha$  acts on bone marrow colony-forming units to regulate stem cell proliferation (44, 45). MIP-1 $\alpha$  has recently been found to be chemotactic for T lymphocytes (26). MIP-1 $\alpha$  activates murine peritoneal *mφs* to become cytotoxic for tumors, and stimulates *mφ* proliferation. Murine MIP-1 $\alpha$ , but not MIP-1 $\beta$ , stimulates *mφ* secretion of IL-1 $\alpha$ , TNF- $\alpha$ , or IL-6 (46, 47). Thus, MIP-1 $\alpha$  can act directly on its cell of origin in an autocrine fashion.

In this study we examined SFs from patients with RA, OA, and other forms of arthritis. We found that SFs from patients with RA contained significantly more MIP-1 $\alpha$  than SFs from patients with OA or other forms of arthritis. While these results may imply a more important role for MIP-1 $\alpha$  in RA than in other forms of arthritis, it is also possible that the higher MIP-

1 $\alpha$  levels found in RA are simply reflective of the greater degree of inflammation found in RA.

Having found that RA SF contains large quantities of MIP-1 $\alpha$ , we determined which specific cell types in the SF contributed to production of this cytokine. RA SF and PB mononuclear cells produced small amounts of MIP-1 $\alpha$  constitutively. To determine the regulation of MIP-1 $\alpha$  production, we incubated SF cells with PHA or LPS. Mononuclear cell MIP-1 $\alpha$  production was augmented by LPS or PHA stimulation.

We next determined whether cells in RA STs produced antigenic MIP-1 $\alpha$ . RA ST fibroblasts produced MIP-1 $\alpha$ , particularly in response to TNF- $\alpha$  or LPS. Hence, RA ST fibroblasts respond to some of the same stimuli that induce production of IL-8 and MCP-1 in the inflamed joint (3, 16). RA ST *mφs* expressed both constitutive MIP-1 $\alpha$  mRNA and protein. Thus, MIP-1 $\alpha$ , like MCP-1 and IL-8, is constitutively produced by ST *mφs* in the context of chronic synovial inflammation.

To determine the contribution of other cell types to MIP-1 $\alpha$  production in vivo, we performed immunohistochemistry on STs from patients with RA, OA, or from normal individuals. While normal ST contained < 1% MIP-1 $\alpha$  immunopositive cells, RA STs contained abundant MIP-1 $\alpha$  immunopositive cells, mainly the ST lining cells and *mφs*, documenting the central role of the *mφ* in this process. In situ expression of this cytokine suggests that at least a portion of MIP-1 $\alpha$  is released from the RA ST. This local release may contribute to the increased serum MIP-1 $\alpha$  levels found in RA.

One of the main reported actions of murine MIP-1 $\alpha$  is to attract PMNs (48). Murine MIP-1 $\alpha$ , when injected intradermally in mice, elicits a potent PMN response (48). McColl et al. (40) have recently shown that rhMIP-1 $\alpha$  stimulates PMN intracellular calcium accumulation accompanied by a simultaneous change in right-angle light scatter, indicating induction of shape change that was not accompanied by measures of PMN effector function such as degranulation, actin polymerization, or chemotaxis (40). Stimulation of PMNs by rhMIP-1 $\alpha$  leads to desensitization to subsequent addition of rhMIP-1 $\alpha$  (40).

In this study, we examined RA SFs for MIP-1 $\alpha$ -derived chemotactic activity for PMNs. Though murine MIP-1 $\alpha$  may be chemokinetic for PMNs, we did not detect any RA SF PMN chemotactic or chemokinetic activity that was neutralizable by anti-MIP-1 $\alpha$ . These results imply that RA SF MIP-1 $\alpha$  does not function as a direct chemotactic factor for PMNs. Alternatively, MIP-1 $\alpha$  may be immobilized to ST endothelium and exert a chemotactic effect for PMNs in this fashion, as has been proposed for the related chemokine IL-8 (49). Rot (49) showed that IL-8 binds selectively to the luminal surface of small blood vessel endothelium, the site of transmigration of recruited cells. Similarly, Tanaka et al. (50, 51) proposed that proteoglycans on the luminal surface of endothelium are responsible for the immobilization of proadhesive cytokines like MIP-1 $\beta$ . Finally, it is possible, as has been suggested by Hughes et al. (52), that increasing concentrations of chemotaxins result in increases in PMN cell surface adhesion molecules, such as CD11b/CD18, with resultant adherence-dependent locomotion and subsequent increases in chemokine concentration. Since we and others have shown that the RA joints are replete with cells expressing CD11b, it is possible that such a mechanism is operative in the context of RA (53, 54). More recent studies by Wang et al. (55), however, have indicated that rhMIP-1 $\alpha$  is not chemotactic for human PB PMNs. Thus, in

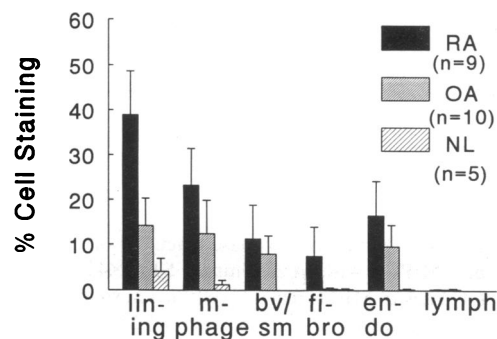


Figure 7. Immunohistochemical demonstration of MIP-1 $\alpha$  expression in STs. (*mphage*) Macrophage; (*bv/sm*) vascular smooth muscle; (*fibro*) fibroblast; (*endo*) endothelial cell; (*lymph*) lymphocyte.



accord with the studies reported by Wang et al. (55) for human PB PMNs, it appears that RA SF MIP-1 $\alpha$  is not a direct chemotactic factor for PMNs.

m $\phi$ s are key effector cells in the pathogenesis of RA. m $\phi$ s function as antigen-presenting cells as well as potent secretory cells, liberating cytokines such as IL-1, TNF- $\alpha$ , IL-8, and MCP-1 (3–11, 13, 14, 16–18, 56–58). Additionally, they release arachidonic acid metabolites, oxygen free radicals, and proteases such as collagenase and stromolysin (56). These cells are also important in the neovascularization, which accompanies the growth and proliferation of the hypertrophic ST (32, 33, 58). The mechanisms by which these important cells enter the synovial tissue have as yet not been fully defined.

One of the prominent effects of human MIP-1 $\alpha$  appears to be on the m $\phi$ . The effects of human MIP-1 $\alpha$  on human mononuclear phagocytes are beginning to be elucidated. We and others have recently shown that human MIP-1 $\alpha$  is a potent chemotaxin for human m $\phi$ s, being chemotactic but not chemokinetic for these cells (28, 55). Other m $\phi$  chemotactic cytokines present in the RA joint include transforming growth factor- $\beta$ , platelet-derived growth factor, GM-CSF, lymphocyte-derived chemotactic factor, and MCP-1 (3). Indeed, we found that anti-MIP-1 $\alpha$  resulted in suppression of over one-third of the chemotactic activity for m $\phi$ s found in RA SFs. Thus, MIP-1 $\alpha$  may function in conjunction with these other m $\phi$  chemotactic factors to recruit m $\phi$ s into the RA joint.

In summary, in this study we have shown that RA SFs contain significantly more MIP-1 $\alpha$  than SFs from OA or other forms of arthritis. Mononuclear cells found in RA SFs are capable of producing MIP-1 $\alpha$ , and both LPS and PHA are potent stimuli for their MIP-1 $\alpha$  production. Likewise, cells from the RA ST were found to produce MIP-1 $\alpha$ . RA ST fibroblasts produced MIP-1 $\alpha$ , particularly in response to LPS or TNF- $\alpha$  stimulation. RA ST m $\phi$ s constitutively produced MIP-1 $\alpha$ , and along with ST lining cells, were the main MIP-1 $\alpha$  immunopositive cells found in the ST. One of the main biological functions of MIP-1 $\alpha$  in the RA joint appears to be the direct recruitment of m $\phi$ s, with MIP-1 $\alpha$  accounting for over one-third of the chemotactic activity for m $\phi$ s found in RA SFs. Hence, MIP-1 $\alpha$  may be one of the major cytokines contributing to the chemoattraction and retention of RA m $\phi$ s in the inflamed joint.

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