Expression and Regulation of Human Neutrophil-derived Macrophage Inflammatory Protein 1α

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Summary

Neutrophil (polymorphonuclear leukocyte [PMN]) sequestration is one of the histologic hallmarks of an acute inflammatory response. During the natural evolution of an inflammatory response, PMNs are often replaced by mononuclear cells. This shift in the elicitation of specific leukocyte populations usually occurs as the inflammatory lesion enters either the repair/resolution stage or progresses to a chronic inflammation. To elucidate a potential mechanism for the temporal change from predominantly PMN recruitment to the presence of monocytes, we postulated that PMNs could be a rich source of monocyte chemotactic factors. In our studies, we have identified a dose-dependent induction of monocyte chemotactic activity by PMNs treated with lipopolysaccharide (LPS; 1–100 ng/ml). Interestingly, this monocyte chemotactic activity was significantly attenuated in the presence of neutralizing anti-human macrophage inflammatory protein 1α (MIP-1α) antibodies. Moreover, immunolocalization studies demonstrated the expression of MIP-1α by stimulated PMNs. These findings showed that a significant amount of PMN-derived monocyte chemotactic activity was attributable to MIP-1α. Subsequent characterization of MIP-1α steady-state mRNA and antigen expression demonstrated both a dose- and time-dependent production by LPS-treated PMNs. Granulocyte/macrophage colony–stimulating factor (GM-CSF), a potent PMN activator, failed to induce the expression of MIP-1α over a wide range of concentrations. However, PMNs stimulated in the presence of both LPS and GM-CSF resulted in a synergistic expression pattern for MIP-1α. PMNs stimulated in the presence of both GM-CSF and LPS demonstrated an enhanced and prolonged expression for both MIP-1α mRNA and antigen, as compared with LPS alone. Messenger RNA stabilization analyses demonstrated that MIP-1α mRNA isolated from PMNs stimulated in the presence of GM-CSF and LPS had a prolonged mRNA $t_{1/2}$, as compared with LPS alone. These findings support the notion that PMNs are capable of producing MIP-1α in the presence of LPS, and that GM-CSF can influence this production through prolongation of MIP-1α mRNA $t_{1/2}$. The production of PMN-derived MIP-1α, in association with the expression of appropriate adhesion molecules at a site of inflammation, may be one of the central events that contributes to the temporal shift from predominantly PMNs to monocytes during the evolution of inflammation.

Human peripheral PMNs are the predominant leukocyte population at sites of acute inflammatory reactions (1). These leukocytes produce a number of inflammatory mediators, including reactive oxygen intermediates, arachidonic acid derivatives, and enzymes. Recently, a number of investigators have reported that PMNs can produce several polypeptide mediators of inflammation. Activated PMNs have been shown to produce specific cytokines, such as IL-1 (2–5), IL-6 (6), IL-8 (7–10), and TNF (11, 12). Since PMNs may comprise up to 80% of the circulating leukocyte pool in humans, this biosynthetically active leukocyte population must be considered an important source of cytokines during the early phases of inflammation. Previous reports have identified PMNs as a source of IL-8, a chemotactic cytokine for neutrophils and lymphocytes, at nanomolar and picomolar concentrations, respectively. Thus, PMN-derived cytokines may play an important role in the early initiation phase of an inflammatory response by the induction of the early response cytokines,

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IL-1 and TNF, and the expression of the chemotactic cytokine, IL-8. Whereas this latter cytokine will likely promote the continued recruitment of neutrophils to the inflammatory lesion, other recruitment factors must be expressed in order for the responses to progress to one characterized by the accumulation of monocytes/macrophages.

Recent investigations have demonstrated that the chemotactic cytokine, macrophage inflammatory protein 1 (MIP-1)[1], possesses biological activity for activating both neutrophils and monocytes/macrophages. Native MIP-1 is comprised of two 8-kD polypeptides termed MIP-1α and MIP-1β. This chemotactic cytokine is a member of the C-C chemokine supergene family and is an LPS-inducible, heparin-binding polypeptide first isolated from LPS-treated murine RAW 264.7 cells (13–16). MIP-1α induces neutrophil chemokinesis, and activates neutrophils to generate hydrogen peroxide (14). In vivo, MIP-1α can induce a pyrogenic response by acting as a prostaglandin-independent endogenous pyrogen (17). Recently, it has been reported that MIP-1α–treated murine peritoneal exudate macrophages exhibited enhanced antibody-independent macrophage cytotoxicity for tumor targets, in addition to stimulating the secretion of TNF, IL-1, and IL-6 from this macrophage population (18). These data indicate that MIP-1α may play a crucial role in the biology of inflammation and the pathogenesis of inflammatory diseases.

In the present studies, we present data demonstrating that PMNs may play an important role in altering the composition of leukocytes recruited to an area of inflammation via their ability to generate monocyte chemotactic factors. Furthermore, one of the major neutrophil-derived monocyte chemotactic factors is MIP-1α. PMNs challenged with LPS demonstrated a time- and dose-dependent increase in both steady-state MIP-1α mRNA expression, and antigen and chemotactic activity. The latter activity was suppressed by the addition of neutralizing MIP-1α antibody. Furthermore, the expression of MIP-1α mRNA by PMNs stimulated with LPS was augmented by GM-CSF, which resulted in the prolongation of the $t_{1/2}$ for MIP-1α mRNA. These investigations suggest that PMNs possess multifunctional activities during the initiation and maintenance of an inflammatory reaction and are an important source of this monocyte chemotactic cytokine.

Materials and Methods

Reagent Preparation. Human recombinant GM-CSF (sp act, 4 × 10^7 U/mg protein); G-CSF (sp act, 2 × 10^8 U/mg protein); IL-3 (sp act, 0.1–0.4 ng/ml); and MIP-1α (sp act, ED_{50}, 25 ng/ml) were purchased from R & D Systems, Inc. (Minneapolis, MN). Cycloheximide (CHX) was purchased from Sigma Chemical Co. (St. Louis, MO). Stock LPS (*Escherichia coli* 0111:B4; Sigma Chemical Co.) and actinomycin D (Ac-D) (Sigma Chemical Co.) were prepared at concentrations of 200 μg/ml in complete medium and 5 mg/ml in DMSO (Sigma Chemical Co.), respectively. Complete medium consisted of RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 2 mM L-glutamine, 25 mM Hepes, 100 U/ml penicillin, 100 ng/ml streptomycin (Hazelton Research Products, Lenexa, KS) and 5% heat-inactivated FCS (Gibco Laboratories). Antisera to MIP-1α were raised in rabbits immunized with recombinant human MIP-1α using procedures established in our laboratory. The antisera to human MIP-1α did not cross-react with other known members of this chemoattractant cytokine family, including RANTES, monocyte chemoattractant protein 1 (MCP-1), or MIP-1β. All reagents, except LPS, were checked for endotoxin contamination using a limulus amebocyte lysate test kit (QCL-1000; Whittaker Bioproducts, Inc., Walkersville, MD). The concentration of endotoxin was consistently below 0.01 endotoxin units (EU)/ml in all reagents.

Isolation and Culture Conditions of PMNs and Monocytes. Human peripheral blood PMNs were obtained from heparinized venous blood of healthy volunteers by Ficoll-Hypaque (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) centrifugation and sedimentation in 5% dextran/0.9% saline. PMNs were subsequently separated from erythrocytes by lysis of erythrocytes in a solution of 0.15 M NH_4Cl, 0.01 M $NaHCO_3$, and 0.01 M tetra EDTA. The recovered PMNs were washed three times and resuspended at a density of 5 × 10^6 cells/ml in complete medium. The final cell preparation contained more than 99% PMN by morphology and nonspecific esterase staining, and viability was more than 98% by trypan blue dye exclusion. After Ficoll-Hypaque centrifugation, monocytes were purified by counterflow centrifugal elutriation from PBMC (19), and resuspended in complete medium at 10^4–10^6 cells/ml. PMNs and monocytes were incubated with various concentrations of LPS, and/or GM-CSF. After various periods of time, cell-free supernatants of PMN cultures and total PMN RNA were collected and stored at −20°C. In some experiments, cell lysates of PMN were resuspended and stored in the same volume of complete medium.

Assay of Monocyte Chemotactic Activity. Monocyte chemotaxis was performed as previously described (20). For monocyte chemotaxis, either 150 μl of supernatant specimen, 10^{-8} M FMLP (Sigma Chemical Co.), MIP-1α (1–100 ng/ml), or HBSS (Gibco Laboratories) was placed in duplicate bottomed wells of a modified Boyden chemotaxis chamber (Neuro Probe Inc., Cabin John, MD). A 5-μm pore size polycarbonate filter (polyvinylpyrrolidone-free, Nucleopore Corp., Pleasanton, CA) was placed in the assembly and 250 μl of mononuclear cell suspension (4 × 10^6/ml in HBSS) placed in each of the top wells. Chemotaxis chamber assemblies were incubated at 37°C in humidified 95% air/5% CO_2 for 75 min and the filters removed, fixed in absolute methanol, and stained with 2% toluidine blue (Sigma Chemical Co.). Monocytes that had migrated through to the bottom of the filter were counted in 10 high power fields using the following system: a Javelin chromachip camera (Javelin Electronics, Torrance, CA) attached to a Olympus BH-2 microscope, which was interfaced to a computer (Macintosh® II; Apple Computer, Inc., Cupertino, CA) with a frame grabber (Image Capture 1000; Scion Corp., Walkersville, MD) and Image 1.40 software (National Institutes of Health [NIH] Public Software, Bethesda, MD). Chemotactic Bioactivity was represented by the count of migrated monocytes per high power field. In neutralization experiments, sample supernatants were preincubated with 1:1,000 dilution of rabbit preimmune serum or neutralizing rabbit anti-human MIP-1α antiserum for 30 min at 37°C and then assayed for monocyte chemotactic bioactivity.

Antigen-specific ELISA. PMN-derived antigenic MIP-1α quantified using a modification of a double ligand method, as previ-

[1] *Abbreviations used in this paper:* Ac-D, actinomycin D; CHX, cycloheximide; hpf, high power field; MCP-1, monocyte chemoattractant protein 1; MIP-1α, macrophage inflammatory protein 1α.

ously described (21). Briefly, flat-bottomed 96-well microtiter plates were coated with 50 μl/well of rabbit anti-MIP-1α antibody (1 μg/ml in 0.6 mol/liter NaCl, 0.26 mol/liter H_3BO_4, and 0.08 N NaOH, pH 9.6) for 16 h at 4°C, and then washed with PBS, pH 7.5, 0.05% Tween 20 (wash buffer). Nonspecific binding sites on microtiter plates were blocked with 2% BSA in PBS and incubated for 90 min at 37°C. Plates were rinsed four times with wash buffer, and diluted neutrophil-derived conditioned media (50 μl) were added, followed by incubation for 1 h at 37°C. Plates were washed four times with wash buffer, then 50 μl/well of biotinylated rabbit anti-MIP-1α was added and incubated for 30 min at 37°C. After washing of plates, chromogen substrate was added. The plates were incubated at room temperature to the desired extinction, and the reaction terminated with 50 μl/well of 3 M H_3SO_4 solution, and were read at 490 nm in an ELISA reader. This ELISA method consistently had a sensitivity limit of ~50 pg/ml.

Immunohistochemistry. PMNs (5.0×10^6/ml) were incubated with LPS (100 ng/ml) and/or GM-CSF (10 U/ml) for 24 h, and deposited on a glass slide by using a Cytospin II (Shandon Southern Instruments, Inc., Sewickley, PA). After air-drying, slides were fixed by 4% paraformaldehyde in PBS for 10 min. Before staining, the slides were again fixed for 30 min in ice-cold acetone. Endogenous peroxidase activity was quenched by incubating the slides for 30 min in absolute methanol and 3% hydrogen peroxide. After rinsing in PBS, the slides were blocked with a 1:50 dilution of normal goat serum for 30 min at 37°C, then treated for 2 h at 37°C with a 1:800 dilution of rabbit anti-human MIP-1α serum or the same dilution of preimmune rabbit serum. After incubation, preparations were rinsed three times with PBS, overlaid with biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories Inc., Burlingame, CA), incubated 20 min, and rinsed three times with PBS. The slides were then treated with streptavidin conjugated to peroxidase for 15 min at 37°C, rinsed three time with PBS, overlaid with 100 μl of equal vol of 3-amino-9-ethylcarbazole 40 mg/ml in *N,N*-dimethylformamide, (Sigma Chemical Co.), and treated with 3% hydrogen peroxide in 0.1 M sodium acetate for 15 min at 37°C to allow color development. After rinsing with distilled water, the slides were stained with Mayer's hematoxylin. In competitive inhibition studies, to demonstrate antibody specificity, immunostaining for human MIP-1α showed 100% inhibition by the addition of exogenous recombinant MIP-1α.

Isolation of Total PMN RNA and Northern Blot Analysis. Total cellular RNA from PMN was isolated as previously described (8). Briefly, guanidine isothiocyanate solution containing 25 mM Tris, pH 8.0, 4.2 M guanidine isothiocyanate, 0.5% Sarkosyl, and 0.1 M 2-ME was added to PMN pellets. After homogenization, the above suspension was added to a solution containing an equal volume of 100 mM Tris, pH 8.0, 10 mM EDTA, and 1.0% SDS. The mixture was then extracted with chloroform-phenol and chloroform-isoamyl alcohol. The RNA was alcohol-precipitated and the pellet dissolved in diethyl pyrocarbonate-treated H_2O. Total RNA was separated by Northern blot analysis using formaldehyde, 1% agarose gels, transblotted to nitrocellulose, baked, prehybridized, and hybridized with a ^{32}P-5′ end-labeled oligonucleotide probe specific for human MIP-1α or β-actin. The 30-mer oligonucleotide probe for MIP-1α was complementary to nucleotides 105-134 (5′-GAG-AGC-CAT-GGT-GCA-GAG-GAG-GAC-AGC-AAG-3′) (22). The 42-mer oligonucleotide probe for β-actin was complementary to nucleotides 432-473 (5′-GGC-TGG-GGT-GTT-GAA-GGT-CTC-AAA-CAT-GAT-CTG-GGT-CAT-CTT-3′) (23). Blots were washed and exposed to X-ray film. Specific cytokine mRNA was quantified using imaging analysis with the Image Capture 1000 frame grabber and Image 1.40 software.

Statistical Analysis. Data were analyzed by Macintosh® II computer using a statistical software package (Statview II; Abacus Concepts, Inc., Berkeley, CA) and expressed as mean ± SEM. Groups of data were evaluated by analysis of variance. Data that appeared statistically significant were compared by Student's *t* test for comparing the means of multiple groups, and considered significant if *p* values were <0.05.

Results

Activity of Monocyte Chemotaxis in PMN-conditioned Medium. In initial studies, we examined whether LPS-stimulated PMNs were capable of producing chemotactic factors for monocytes. Conditioned media from PMNs (PMN-CM) stimulated with graded doses of LPS (1–100 ng/ml) for 24 h were shown to possess significant monocyte chemotactic activity (Table 1). PMN-CM challenged with 10 ng/ml LPS demonstrated a significant increase in monocyte chemotactic activity, as compared with control (CTR)-CM (11.9 vs 5.5 cells/high power field [hpf], respectively). PMNs challenged with the maximum concentration of LPS used in this study, 100 ng/ml, produced a monocyte chemotactic activity which was ~50% of the FMLP positive control, 14.0 vs 30.5 cells/hpf, respectively. To address the potential role of residual LPS in the PMN supernatant to alter monocyte chemotaxis, we assessed the monocyte chemotactic activity of recombinant human MIP-1α in the presence and absence of graded doses (1–100 ng/ml) of LPS. In these studies LPS, at various doses, did not alter in vitro monocyte chemotaxis to 10 ng/ml of MIP-1α (data not shown). We examined the specificity of the monocyte chemotactic activity produced by the PMNs by adding neutralizing antibody directed against MIP-1α to the PMN-CM before assessing chemotactic activity. The neutralizing antibody to MIP-1α reduced the PMN-derived chemotactic activity by ~60% (Table 1). hrMIP-1α also had the ability to dose dependently induce monocyte chemotaxis, which was suppressed by our antibody to MIP-1α. Antibodies from preimmune sera had no significant effect on the PMN-derived monocyte chemotactic activity. These initial studies demonstrated that part of the activity produced by LPS-stimulated PMNs was attributable to MIP-1α.

Production of Antigenic MIP-1α from PMNs. Based on the above findings, we began a series of investigations to assess the expression of PMN-derived antigenic MIP-1α. As shown in Fig. 1, PMNs produced MIP-1α over a wide concentration range of LPS (0.1–1,000 ng/ml). Significant levels of PMN-derived MIP-1α were produced in response to LPS concentrations in the nanogram per milliliter range. Since GM-CSF was previously shown to induce PMN cytokine expression (3, 6, 10, 24), we examined the effect of this polypeptide on PMN-derived MIP-1α (Fig. 1). In this study, GM-CSF was not an effective stimulus for the production of PMN-derived MIP-1α when assessed over a 5-log concentration range of GM-CSF. In addition, both IL-3 and G-CSF were also found to be ineffective in inducing MIP-1α expression

Table 1. *Monocyte Chemotactic Activity in Supernatants of PMN Stimulated with LPS*

Condition	Cells/hpf		
	CTR sera (1:1,000)	anti-MIP sera (1:1,000)	Percent suppression
FMLP (10^{-8} M)	30.5 ± 2.86*	31.2 ± 2.15	−0.02%‡
CTR-CM	5.5 ± 1.42	2.4 ± 0.82	56.4%
LPS (1 ng/ml)	6.6 ± 1.14	2.6 ± 0.71	60.6%
LPS (10 ng/ml)	11.9 ± 1.54	2.5 ± 1.00	79.0%
LPS (100 ng/ml)	14.0 ± 1.35	5.6 ± 1.14	60.0%
hrMIP-1α (1 ng/ml)	13.9 ± 2.09	4.9 ± 1.58	64.7%
hrMIP-1α (10 ng/ml)	25.7 ± 1.52	8.8 ± 1.14	65.8%
hrMIP-1α (100 ng/ml)	26.6 ± 3.34	9.8 ± 1.76	63.2%

* The results represent the mean ± SEM of migrated monocytes of 10 hpf done in duplicate wells. These values were subtracted mean values of HBSS plus CTR sera (8.7 cells/hpf), and of HBSS ± anti-MIP sera (7.7 cells/hpf), respectively.
‡ The percent suppression was calculated by subtracting the values of anti-MIP-1α serum (anti-MIP sera)-treated conditioned media from preimmune serum (CTR-sera)-treated conditioned media, and dividing by the values of anti-MIP-1α serum (anti-MIP sera)-treated conditioned media.

by PMNs (data not shown). However, GM-CSF was found to possess synergistic activity in the presence of LPS. As shown in Fig. 2, the addition of 10 U/ml of GM-CSF in the presence of graded doses of LPS was sufficient to increase the expression of PMN-derived MIP-1α. These results demonstrated that PMN-derived MIP-1α production was significantly increased in the presence of LPS (100 ng/ml) plus 10 U/ml GM-CSF (7.75 ± 1.48 ng/ml), as compared with 100 ng/ml of LPS alone (3.13 ± 0.92 ng/ml). We next determined the temporal expression pattern for PMN-derived MIP-1α in response to either LPS, GM-CSF, or LPS plus GM-CSF (Fig. 3 *A*). Antigenic MIP-1α production by LPS (100 ng/ml)-treated PMNs was detected first at 8 h and peaked at 36 h after stimulation. A similar kinetic pattern for the expression of antigenic MIP-1α was observed when PMNs were treated with LPS plus GM-CSF (10 U/ml). However, GM-CSF alone was not shown to induce MIP-1α expression over the 48-h time period. Since certain cytokines have been reported to remain cell-associated after stimulation, we determined the kinetics of MIP-1α levels in PMN cell lysates (Fig. 3 *B*). Whereas the kinetic pattern for the production of MIP-1α was similar between the supernatant and cell lysates, the latter had approximately threefold higher levels of MIP-1α. Interestingly, lysates of freshly isolated, and non-stimulated PMN contained detectable levels of antigenic MIP-1α. The constitutive expression of cell-associated MIP-1α may play an important role in the initiation and maintenance of inflammation by locally releasing a cytokine important to

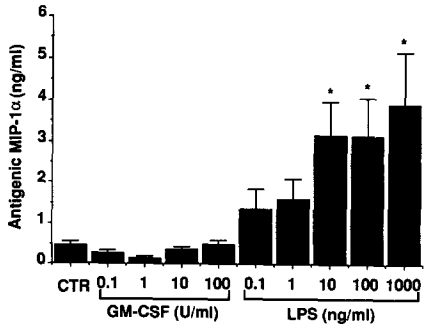

Figure 1. The concentration effect of neutrophil-derived antigenic MIP-1α production in response to a dose-dependent manner of LPS (0.1–1,000 ng/ml) or GM-CSF (0.1–100 U/ml). Supernatants were harvested 24 h after stimulation and assessed for antigenic MIP-1α by ELISA. Neutrophil-derived antigenic MIP-1α is expressed as the mean (ng/ml) ± SEM of supernatant. (*) p values < 0.05, as compared with CTR. (n = 6).

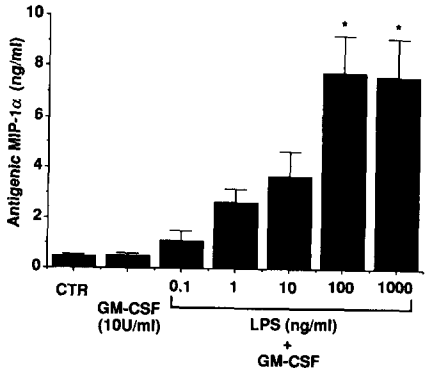

Figure 2. The concentration effect of neutrophil-derived antigenic MIP-1α production in response to a dose-dependent manner of LPS (0.1–1,000 ng/ml) plus 10 U/ml GM-CSF. The supernatants were harvested 24 h after stimulation. Neutrophil-derived antigenic MIP-1α is expressed as the mean (ng/ml) ± SEM of supernatant. (*) p values < 0.05, as compared with similar LPS concentration in Fig. 1. (n = 6).

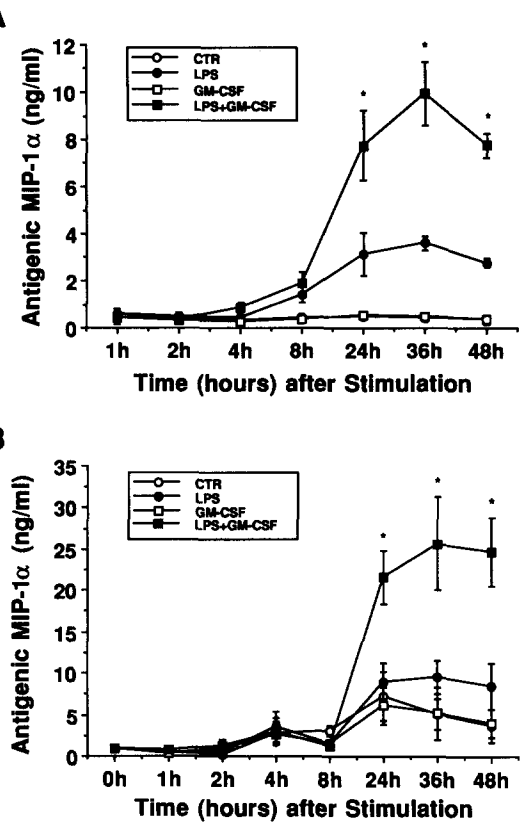

Figure 3. The time course for the production of PMN-derived MIP-1α production in response to either LPS, GM-CSF, or LPS plus GM-CSF. PMNs were stimulated at time 0 with either LPS (100 ng/ml) (—●—), GM-CSF (10 U/ml) (—□—), LPS plus GM-CSF (—■—), or CTR (—○—). (*A*) Supernatants or (*B*) lysates were collected at 1, 2, 4, 8, 24, 36, and 48 h after stimulation. Neutrophil-derived antigenic MIP-1α is expressed as the mean (ng/ml) ± SEM of supernatant. (*) p values < 0.05, as compared with LPS alone and CTR. (n = 5).

cell elicitation upon PMN lysis in vivo. Thus, MIP-1α may provide a crucial mechanism for the local accumulation of MIP-1α and aid in further leukocyte recruitment. The viability of PMNs treated with either GM-CSF, LPS, or LPS plus GM-CSF was not found to be significantly altered during the study period as determined by trypan blue dye exclusion (data not shown).

Since the monocyte has been previously identified as a rich source of MIP-1α, it was important to rule out this cell as a major source of MIP-1α in these PMN studies. Although the PMN preparations were usually >99% PMNs, as assessed by morphology and nonspecific esterase staining, we further examined the potential contribution of monocyte-derived MIP-1α produced by different concentrations of monocytes. As shown in Table 2, LPS stimulated monocytes at 1–5 × 10^4 cells/ml (equivalent to 0.2–1% of PMN suspension) were able to produce only 0.26 ± 0.04 ng/ml of MIP-1α, whereas MIP-1α production from 5 × 10^5 cells/ml (equivalent to 10% of PMN) produced 4.20 ± 0.35 ng/ml. These data indicated that the levels of MIP-1α production in our PMN preparations were not based solely on monocyte contamination.

Immunocytochemical Demonstration of PMN-derived MIP-1α. To localize antigenic MIP-1α to stimulated PMNs, cell preparations were challenged with LPS (100 ng/ml) plus GM-CSF (10 U/ml) for 24 h and assessed by immunohistochemistry. As shown in Fig. 4, PMNs treated with LPS plus GM-CSF demonstrated a significant staining pattern for MIP-1α antigen (Fig. 4, *B* and *D*). However, PMNs treated in a similar fashion, but stained using preimmune serum (Fig. 4, *A* and *C*) demonstrated little nonspecific staining for MIP-1α. These studies demonstrate that MIP-1α is produced by stimulated PMNs and corroborate the above findings that MIP-1α is cell associated.

Kinetics of MIP-1α mRNA Expression in PMN. To determine whether increases in MIP-1α antigen are accompanied by similar increases in MIP-1α mRNA levels, we examined the expression of steady-state levels of PMN-derived MIP-1α mRNA. PMNs were incubated with either LPS (100 ng/ml), GM-CSF (10 U/ml), or LPS plus GM-CSF and total RNA isolated from cells at 1, 4, 8, 24, 36, and 48 h. As shown in Fig. 5, the expression of MIP-1α mRNA from PMNs stimulated with GM-CSF alone was essentially undetectable throughout the study period, whereas LPS was able to induce significant levels of steady-state MIP-1α mRNA. The maximum expression for MIP-1α occurred 24 h after LPS stimulation. Interestingly, the addition of GM-CSF plus LPS significantly augmented the steady-state levels of MIP-1α mRNA, as compared with LPS. Steady-state levels of MIP-1α mRNA were undetectable in unstimulated, freshly isolated PMNs or in PMNs incubated with medium alone (data not shown). To determine whether the expression of MIP-1α was dose dependent, concentration effect studies were conducted in the presence of graded doses of GM-CSF in the presence or absence of 10 ng/ml of LPS (Fig. 6). Steady-state levels of MIP-1α mRNA were not expressed over a wide concentration range of GM-CSF. However, the addition of 10 ng/ml of LPS plus graded doses of GM-CSF resulted in an increase in the expression of steady-state levels of MIP-1α mRNA. We next examined the effect of CHX and Ac-D on PMN-derived MIP-1α mRNA expression. As seen in Fig. 7, the addition of CHX augmented MIP-1α mRNA expression in PMNs stimulated with LPS and/or GM-CSF. Furthermore Ac-D completely inhibited MIP-1α expression by LPS and/or GM-CSF–stimulated PMNs. These studies suggest that MIP-1α expression by PMNs is expressed de novo after stimulation and that the PMN is susceptible to superinduction by CHX. Thus, the production of MIP-1α by PMNs is likely controlled by intracellular regulatory proteins, a phenomenon associated with the regulation of a variety of other cytokines.

Effect of GM-CSF on MIP-1α mRNA Stability. The above data suggested that GM-CSF could induce a synergistic effect on the expression of PMN-derived MIP-1α mRNA. To further investigate a possible mechanism for the effect of GM-CSF in this system, we performed the following MIP-1α mRNA stability experiments to assess the half-life of this PMN-derived cytokine. After 24-h incubation with LPS (100 ng/ml) and/or GM-CSF (10 U/ml), Ac-D (5 μg/ml) was added to PMN culture and total RNA isolated at specific time points. As shown in Fig. 8, MIP-1α mRNA from PMNs

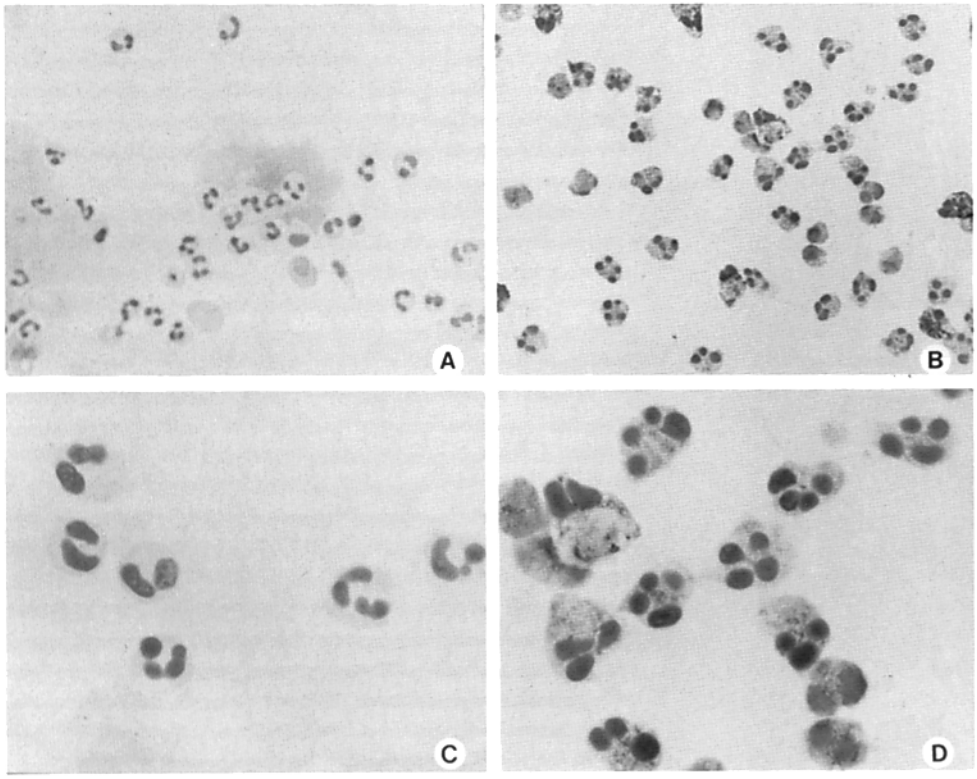

Figure 4. Low (×200)- and high (×400)-power photomicrographs of immunohistochemical localization of PMN-derived MIP-1α. PMNs stimulated with LPS plus GM-CSF for 24 h were deposited on a glass slide, and stained with either anti-MIP-1α antibody or preimmune sera. (*A* and *C*) Control staining by preimmune sera. (*B* and *D*) Positive staining by anti-MIP-1α sera.

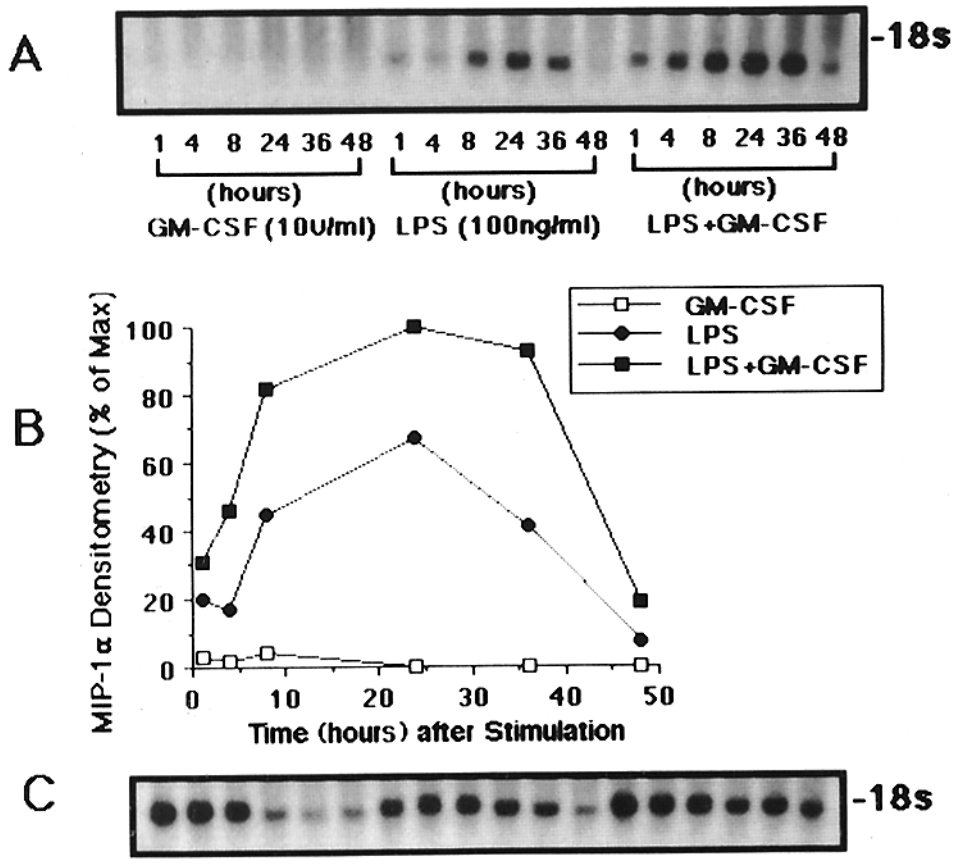

Figure 5. The time course of neutrophil-derived steady-state levels of MIP-1α mRNA in response to LPS, GM-CSF, and LPS plus GM-CSF. Neutrophils were stimulated at time 0 with either LPS (100 ng/ml), and/or GM-CSF (10 U/ml) and total RNA was isolated at 1, 4, 8, 24, 36, and 48 h after stimulation. (*A*) Representative Northern blots of neutrophil-derived steady-state MIP-1α mRNA. (*B*) Corresponding density of the Northern blots in *A*. (*C*) Equivalent loading of total RNA was determined by equal levels of steady-state β-actin mRNA level per lane.

Table 2. *MIP-1α Production from Monocytes*

Condition	MIP-1α				
	1 × 10⁴* (0.2%)‡	5 × 10⁴ (1%)	1 × 10⁵ (2%)	5 × 10⁵ (10%)	1 × 10⁶ (20%)
CTR	ND§	0.07 ± 0.02	0.16 ± 0.03	0.36 ± 0.06	3.32 ± 0.56
GM-CSF (10 U/ml)	ND	0.05 ± 0.02	0.19 ± 0.04	0.51 ± 0.08	3.19 ± 0.37
LPS (100 ng/ml)	ND	0.26 ± 0.04	0.97 ± 0.13	4.20 ± 0.35	10.24 ± 1.20
LPS + GM-CSF	ND	0.26 ± 0.02	0.76 ± 0.07	3.92 ± 0.42	12.50 ± 1.73

* Monocytes (cells/ml) were resuspended in complete media at various concentrations, and incubated for 24 h.
‡ Percentage would be equivalent to that of monocytes potentially contaminating the PMN cultures.
§ ND, Not detected.

treated with GM-CSF plus LPS possessed a markedly prolonged $t_{1/2}$, as compared with LPS alone (LPS, 2 h vs LPS plus GM-CSF, 5 h) (Fig. 8 *B*). In these studies, the stability of β-actin mRNA isolated from PMNs treated with LPS or LPS plus GM-CSF did not appear to be altered. In total, the above studies demonstrate that GM-CSF may be an important polypeptide mediator for the continued maintenance of an inflammatory response via its ability to augment the expression of PMN-derived chemotactic cytokines.

Discussion

PMNs are usually recognized as an important leukocyte population involved in acute inflammatory responses. Historically, this cell population was not considered an important source of de novo polypeptide mediators, and its role in host defense was felt to occur only via its phagocytic activity and

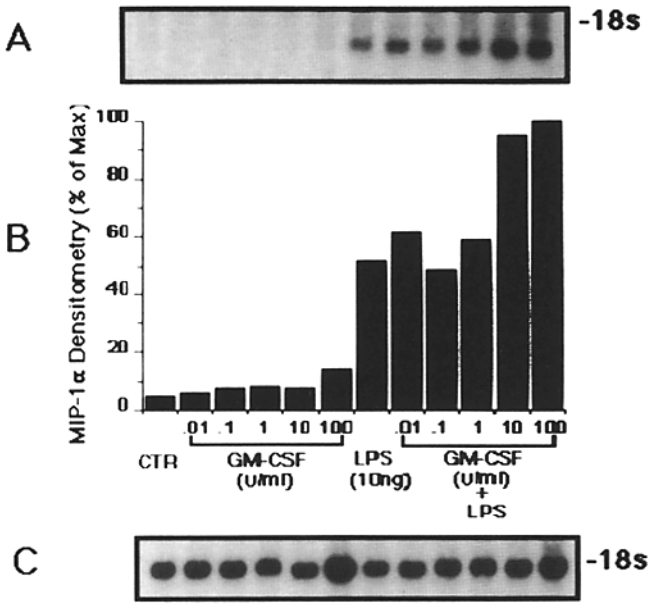

Figure 6. The concentration effect of neutrophil-derived steady-state levels of MIP-1α mRNA in response to a dose-dependent manner of GM-CSF in the absence or presence of 10 ng/ml of LPS. Neutrophils were stimulated with graded concentrations of GM-CSF (0.1–100 U/ml) with or without LPS (10 ng/ml), and total RNA was isolated at 8 h after stimulation. (*A*) Representative Northern blots of neutrophil-derived steady-state MIP-1α mRNA. (*B*) Corresponding density of the Northern blots as assessed by image analysis. (*C*) Equivalent loading of total RNA as determined by equal levels of steady-state β-actin mRNA level per lane.

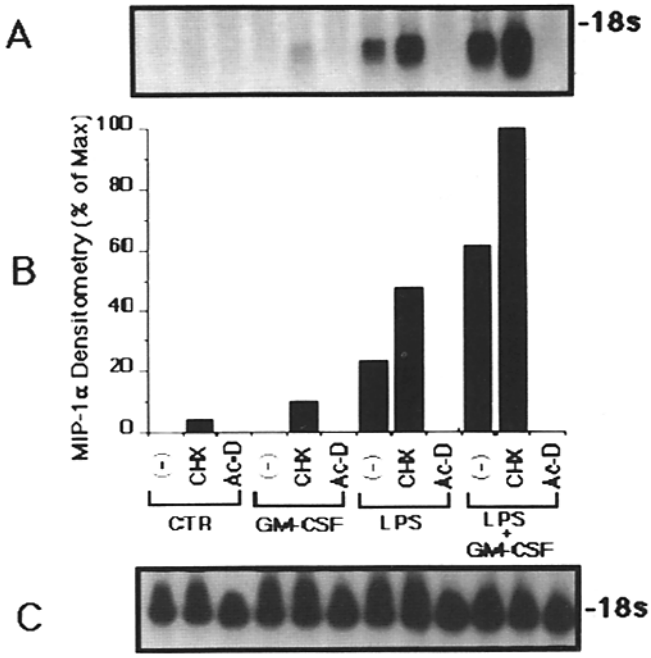

Figure 7. The effect of CHX, or Ac-D on neutrophil-derived steady-state levels of MIP-1α mRNA in response to LPS, GM-CSF, or LPS plus GM-CSF. Neutrophils were preincubated with CHX (5 μg/ml), or Ac-D (5 μg/ml) for 1 h and stimulated with LPS (100 ng/ml), GM-CSF (10 U/ml), or LPS plus GM-CSF. Total RNA was isolated at 4 h after stimulation. (*A*) Representative Northern blots of neutrophil-derived steady-state MIP-1α mRNA. (*B*) Corresponding density of imaging analysis. (*C*) Equivalent loading of total RNA was determined by equal levels of steady-state β-actin mRNA level per lane.

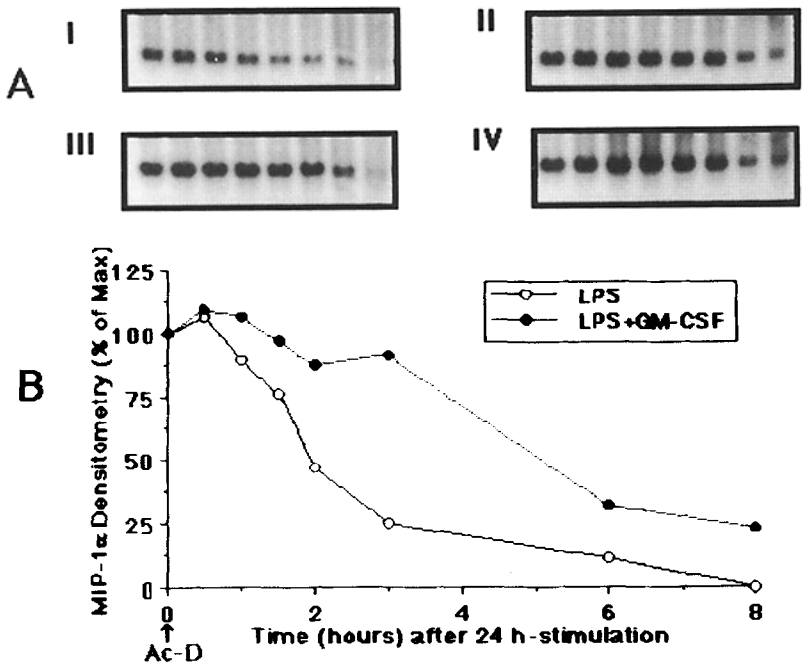

Figure 8. The half-life of neutrophil-derived steady-state levels of MIP-1α mRNA in response to LPS, or LPS plus GM-CSF. Neutrophils were stimulated with LPS (100 ng/ml) or LPS plus GM-CSF (10 U/ml). After 24-h incubation, Ac-D (5 μg/ml) was added and total RNA was isolated at various time points. (*A*) (*I* and *II*) Representative Northern blots of neutrophil-derived steady-state MIP-1α mRNA. (*III* and *IV*) Equivalent loading of total RNA was determined by equal levels of steady-state β-actin mRNA level per lane. (*B*) Corresponding density of the image analysis.

the production and release of enzymes and reactive oxygen species. However, recent data have demonstrated that PMNs are biosynthetically active and can produce a variety of cytokines known to play an important role in inflammation. These cytokines include: IL-1 α and β (2–5), IL-6 (6), IL-8 (7–10), TNF-α (11, 12), and IL-1 receptor antagonist (24). Since PMNs are one of the first cells to arrive at a site of inflammation, the cytokine secreting activity of these cells may indicate a role for PMNs during the maintenance of an inflammatory site.

The progression and maintenance of many inflammatory responses are often histologically defined by a significant PMN recruitment phase followed by the elicitation of mononuclear cells. This temporal appearance of specific leukocytes would suggest that the PMN may play a role in mononuclear cell recruitment. However, few studies have actually demonstrated that PMNs can generate a chemotactic factor(s) for monocytes. In the present study, we demonstrated that peripheral blood PMNs were a significant source of MIP-1α, which is a potent chemoattractant for monocyte. MIP-1α belongs to a supergene family of chemotactic cytokines which are identified by the location of four cysteine amino acids; two of the cysteines are found in juxtaposition to each other. This family contains other chemotactic cytokines, including MCP-1, RANTES, and MIP-1β. In addition, this group of chemotactic cytokines is distantly related to another supergene family of neutrophil chemotactic factors, which includes IL-8. These cytokines appear to play a key role in inflammation and immune responses by their chemotactic activities and their ability to activate PMNs, monocytes, T cells, eosinophils, and basophils (16, 25–27). MIP-1α has been shown to possess a variety of biological activities, including the induction of neutrophil chemokinesis and activation of neutrophils to generate hydrogen peroxide (14), serving as a prostaglandin-independent endogenous pyrogen (17), stimulating the secretion of TNF, IL-1α, and IL-6 from murine peritoneal macrophages (18), and inducing the chemotaxis of basophils and histamine release from mast cells (28).

In this study, LPS challenge (8–36 h) was identified as an important stimulus for the expression of PMN-derived MIP-1α. Interestingly, GM-CSF, an inflammatory mediator previously identified as a stimulus for PMN-derived cytokines, did not induce the expression of PMN-derived MIP-1α alone, but did cause a significant synergistic rise in the production of PMN-derived MIP-1α. The mechanism for the increase in MIP-1α production by GM-CSF plus LPS appeared to be due to the stabilization of MIP-1α mRNA, resulting in a prolonged mRNA $t_{1/2}$. Whereas mRNAs which code for various structural proteins are quite stable, recent studies have identified mRNAs that code certain inducible proteins, including cytokines, oncogenes, and some enzymes, are relatively unstable and possess half-lives on the order of 10–60 min (29). Furthermore, the "AUUUA" nucleotide motif in the 3' untranslated region has been identified as playing an important role in regulating the longevity of many cytokine mRNA species. This motif appears to have relevance for the rapid decay of specific cytokine mRNA (29, 30). Interestingly, MIP-1α does possess this motif in the 3' untranslated region of its mRNA (14, 15, 23, 31).

GM-CSF is likely to play an important role during the maintenance of an inflammatory lesion not only by increasing the production of a PMN-derived monocyte chemotactic cytokine, but also by increasing the longevity of PMNs themselves. Recent studies have demonstrated that GM-CSF can increase the survival of PMNs as they participate in inflammation and prolong the circulating half-life of PMNs in vivo

(32, 33). PMNs appear to be susceptible to GM-CSF stimulation as a number of cytokines, including IL-1, IL-1 receptor antagonist, IL-6, G-CSF, and M-CSF are produced by PMNs challenged with GM-CSF (3, 6, 10, 24). However, in the above studies we could not detect the expression of MIP-1α by PMNs stimulated with GM-CSF alone. The above findings demonstrate that PMNs are an important source of the monocyte chemotactic protein MIP-1α and the expression of this chemotactic cytokine is regulated by both exogenous and endogenous factors. The production of MIP-1α by stimulated PMNs could conceivably place this cell in a key position to orchestrate the switch in leukocyte infiltration from the acute PMN response to more chronic mononuclear cell recruitment.

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