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Production of Monocyte Chemoattractant Protein-1 and Macrophage Inflammatory Protein-1α by Inflammatory Granuloma Fibroblasts

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The formation of hepatic granulomas around persistently deposited* Schistosoma mansoni *eggs leads to parenchymal damage, ongoing fibrosis, and ultimate loss of liver function. In this study, the production of macrophage inflammatory protein-1α (MIP-1) and monocyte chemoattractant protein-1 (MCP-1) by granuloma fibroblasts was examined to establish the potential contribution of intragranuloma fibroblasts to the maintenance of the chronic inflammation. Isolated fibroblasts from dispersed acute infection hepatic granulomas were grown in tissue culture for 3 to 4 weeks and used on the third or fourth passage. We initially surveyed fibroblasts for production of MIP-1 and MCP-1 by reverse transcription-polymerase chain reaction (RT-PCR) after stimulation with interleukin (IL)-1, tumor necrosis factor, interferon (IFN)-γ, IL-4, or IL-10: cytokines found within the granuloma. These studies demonstrated constitutive expression of MCP-1 and differential up-regulation of MIP-1 on cytokine stimulation. Protein expression was then verified by immunohistochemical localization of MIP-1 and MCP-1 in paraformaldehyde-fixed fibroblasts and by direct quantitation of MIP-1 and MCP-1 in culture supernatants by specific ELISAs. These studies demonstrated constitutive expression of MCP-1 in unstimulated and cytokine-stimulated granuloma fibroblasts. In contrast, IL-1 (0.1 to 2.5 ng/ml), IFN-γ (10 μg/ml), and IL-10 (2.5 to 10 ng/ml) were able to induce the significant production of MIP-1 by the granuloma fibroblasts. Interestingly, normal noninflammatory fibroblasts from uninfected mice showed no significant production of MIP-1 or MCP-1 in response to these cytokines. These results suggest that granuloma fibroblasts may be phenotypically altered compared with normal fibroblasts and have a significant role in leukocyte recruitment, granuloma growth, and maintenance of the egg-induced lesion. (Am J Pathol 1994, 144:711–718)

Fibroblast proliferation in circumoval granulomas in the livers of *Schistosoma mansoni*-infected hosts is a complication that leads to severe hepatic fibrosis resulting in morbidity and mortality affecting hundreds of thousands of patients in third world countries. The role of the fibroblast in mediating the overall inflammation of the granuloma has not been elucidated. However, a recent study from our laboratory has demonstrated an inflammatory role for the fibroblast in human chronic inflammatory states.[1] This study was undertaken to further explore the inflammatory role of the fibroblast in the schistosome egg-elicited granuloma[2–5] by examining the capacity of granuloma-derived fibroblasts to either constitutively produce or generate chemotactic proteins in response to known cytokines found in the granuloma lesions. Specifically, we examined the production of macrophage inflammatory protein-1α (MIP-1α) and monocyte chemotactic protein-1 (MCP-1) (JE), both members of the C-C family of chemokines. These

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chemokines are primarily associated with chemotactic activity for mononuclear cells *in vitro* and *in vivo*.[6,7] Both MIP-1 (8) and MCP-1 (manuscript in preparation) appear to have a significant role in the development of the *S. mansoni* egg-induced granulomas, although there is no direct evidence that fibroblasts produce these chemokines in granuloma. The development of the granuloma is regulated by T lymphocytes through the production of inflammatory cytokines such as interleukin (IL)-1, tumor necrosis factor (TNF), interferon-γ (IFN-γ), IL-4, and IL-10.[9–15] We hypothesized that these cytokines may network with stromal cell populations, such as fibroblasts, within the granuloma to induce chemokines and thus promote recruitment of mononuclear cells to sites of inflammation.

Indeed, this study extends our initial findings and identifies granuloma fibroblasts as cellular sources of MIP-1 and MCP-1 within granulomas and thus may contribute to inflammation. In addition, this study indicates that IL-10, previously suggested to be immunosuppressive (13), is capable of inducing chemokine production necessary for the recruitment of leukocytes and maintenance of granuloma formation. Finally, comparison of normal fibroblasts from naive mice demonstrate that the inflammatory fibroblasts from the *S. mansoni* egg granulomas have the ability to produce both chemokines, whereas normal fibroblasts do not, indicating heterogeneity in fibroblast populations.

Materials and Methods

Animals and Infection

Female CBA/J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were maintained under standard pathogen-free conditions.

Five-to 8-week-old mice were infected by percutaneous tail infection with 25–30 Puerto Rican strain of *S. mansoni* cercariae.

Isolation, Expansion, and Stimulation of Granuloma Fibroblasts

Isolated hepatic granuloma cells were obtained from livers of 8 week-infected mice, as previously described (16). The cells were incubated overnight to allow adherent cells to attach at a concentration of 4×10^6/ml in a 25-mm^2 culture flask. The cells were incubated in Dulbecco's minimum essential medium supplemented with 1 mM L-glutamine, 10 mM HEPES, antibiotics, and 10% fetal calf serum and changed every other day. The fibroblasts were allowed to grow until near confluency when they were trypsinized (0.25%) off the flask and transferred to a 150-mm^2 culture flask. Once the cells again grew to confluency they were removed by trypsin then counted and cultured at a concentration of 1×10^5 cells/ml in 6-well plates. Fibroblasts were allowed to adhere overnight and were then stimulated with varying concentrations (0.1 to 10 ng/ml) of either murine IL-1, TNF, IFN-γ, IL-4, or IL-10, all of which play a role in granuloma regulation. The expansion process required 20 to 28 days, and little contamination (<1%) of passenger cells (ie, macrophages) was observed by the end as examined by nonspecific esterase stain. In addition, great care was taken to use the fibroblasts before the time their growth characteristics changed. Fibroblasts were isolated from lungs of uninfected normal mice and expanded in the same manner as isolated granuloma fibroblasts. Attempts to isolate and expand fibroblasts from livers of uninfected mice were unsuccessful.

Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Amplification of Granuloma Fibroblast mRNA

Differentially activated granuloma fibroblast cultures were homogenized in a solution containing 25 mM Tris, pH 8.0, 4.2 M guanidine isothiocyanate, 0.5% Sarkosyl, and 0.1 M 2-mercaptoethanol. After homogenation, the suspension was added to a solution containing an equal volume of 100 mM Tris, pH 8.0, 10 mM EDTA, and 1.0% sodium dodecyl sulfate. The mixture was then extracted two times each with phenol-chloroform and chloroform-isoamyl alcohol. The RNA was alcohol precipitated and the pellet dissolved in DEPC water. Total RNA was determined by spectrometric analysis at 260 nm wavelength. Two micrograms of total RNA was reversed transcribed into cDNA using a BRL reverse transcription kit (Bethesda Research Laboratories, Bethesda, MD) and oligo (dT)12–18 primers. The cDNA was then amplified using specific primers for β-actin as a control, murine JE (MCP-1), and murine MIP-1α. Primers used were designed using a PCR simulation program (Amplify, Bill Engels, Madison, WI) and the primers were found to be specific for the chemokine tested and not any other chemokine family member.

The primers used were 5′-GTG-GGG-CGC-CCC-AGG-CAC-CA-3′ (sense) and 5′-GCT-CGG-CCG-TGG-TGG-TGA-AGC-3′ (anti-sense) for β-actin, 5′-CTC-ACC-TGC-TGC-TAC-TCA-TTC-3′ (sense)

and 5′-GCA-TGA-GGT-GGT-TGT-GAA-AAA-3′ (anti-sense) for murine JE (MCP-1), and 5′-GCC-CTT-GCT-GTT-CTT-CTC-TGT-3′ (sense) and 5′-GGC-AAT-CAG-TTC-CAG-GTC-AGT-3′ (anti-sense) for murine MIP-1α, giving amplified products of approximately 550, 350, and 300 base pair, respectively. The amplification buffer contained 50 mM KCl, 10 mM Tris-HCL (pH 8.3), and 2.5 mM MgCl. Specific oligonucleotide primer was added (200 ng/sample) to the buffer, along with 1 μl of the reverse transcribed cDNA samples. The cDNA was amplified after determining the optimal number of cycles. The mixture was first incubated for 5 minutes at 94 C then was cycled 28 times at 95 C for 30 seconds, 58 C for 45 seconds, and elongated at 72 C for 75 seconds. This format allowed optimal amplification with little or no nonspecific amplification of contaminating DNA. After amplification the sample (20 μl) was separated on a 2% agarose gel containing 0.3 μg/ml (0.003%) of ethidium bromide and bands visualized and photographed using ultraviolet transillumination.

Production of Anti-Chemokine Antibodies

Rabbit anti-murine MIP-1α and MCP-1 antibodies were prepared by multiple site immunization of New Zealand white rabbits with recombinant murine MIP-1α (R & D Systems, Minneapolis, MN) or recombinant MCP-1 (Dupont, Wilmington, DE) in complete Freund's adjuvant. Polyclonal antibodies were titered by direct ELISA and specificity verified by the failure to cross-react with mIL-1α, mIL-2, mTNF, IL-6, mJE (or MIP-1α), mMIP-1β, hMCP-1, hIL-8, hRANTES, hMIP-1α, and hMIP-1β. Furthermore, the IgG fraction of the serum was purified over a protein A column and used in a sandwich ELISA as described below.

Chemokine Assays

Extracellular immunoreactive MCP-1 and MIP-1α was quantitated using a modification of a double ligand ELISA method as previously described.[17] Briefly, flat-bottomed 96-well microtiter plates (Nunc Immuno-Plate I 96-F, Denmark, Netherlands) were coated with 50 μl/well of rabbit anti-MIP-1α antibody (1 ng/μl in 0.6 M NaCl, 0.26 M H_3B0_4, and 0.08 N NaOH, pH 9.6) for 16 hours at 4 C and then washed with phosphate-buffered saline (PBS), pH 7.5, 0.05% Tween 20 (wash buffer). Microtiter plate nonspecific binding sites were blocked with 2% bovine serum albumin in PBS and incubated for 90 minutes at 37 C. Plates were rinsed four times with wash buffer and diluted (1:2 and 1:10) cell-free supernatants in duplicate were added, followed by incubation for 1 hour at 37 C. Plates were washed four times, followed by the addition of 50 μl/well biotinylated rabbit antichemokine antibody (3.5 ng/μl in PBS, pH 7.5, 0.05% Tween 20, and 2% fetal calf serum) and plates incubated for 30 minutes at 37 C. Plates were washed four times, streptavidin-peroxidase conjugate (Bio-Rad Laboratories, Richmond, CA) added, and the plates incubated for 30 minutes at 37 C. Plates were again washed four times and chromogen substrate (Bio-Rad Laboratories) added. The plates were then incubated at room temperature to the desired extinction and the reaction terminated with 50 μl/well of 3 M H_2SO_4 solution. Plates were read at 490 nm in an ELISA reader. Standards were 1/2 log dilutions of recombinant MCP-1 or MIP-1α from 1 pg/ml to 100 ng/ml. This ELISA method consistently detected concentrations above 10 pg/ml.

Immunohistochemical Localization of MCP-1 and MIP-1α in Cultured Fibroblasts

All fixed cells were blocked with normal goat serum for 30 minutes. The cells were covered with the rabbit antimurine MCP-1 or MIP-1α serum diluted in PBS (1:250) for 30 minutes at 37 C. After rinsing three times with PBS the cells were overlaid for 20 minutes with biotinylated goat anti-rabbit IgG (Biogenex, San Ramon, CA; supersensitive reagent 1:10). After rinsing three times with PBS cells were incubated for 20 minutes with streptavidin-peroxidase (Biogenex; 1:20) at 37 C. The slides were rinsed with PBS and overlaid with AEC until color development was observed (15 to 30 minutes). Sections were counterstained with Mayer's hemotoxylin.

Statistical Analysis

Data are expressed as means ± SE. Data that appeared statistically significant were compared by analysis of variance for comparing the means of multiple groups and considered significant if *P* values were less than 0.05.

Results

RT-PCR Amplification of MIP-1 and MCP-1 mRNA from Granuloma Fibroblasts

We first screened granuloma fibroblasts for MCP-1 and MIP-1 mRNA expression by isolating whole RNA from the differentially stimulated cultured fibroblasts

and performing reverse transcription (RT) followed by primer-directed PCR amplification. Figure 1 illustrates that MCP-1 and MIP-1 mRNA could be detected in all stimulated fibroblast cultures. Constitutive expression of MCP-1 mRNA was detected in unstimulated control cultures of the granuloma fibroblasts and little up-regulation of mRNA could be detected with cytokine stimulation. In contrast, there was a lower background level of MIP-1 mRNA in unstimulated cells and the steady-state mRNA signal was up-regulated when fibroblasts were exposed to the various cytokines, especially with IL-1 and IL-10. These results suggested that MCP-1 mRNA was constitutively present, whereas MIP-1 was inducible in the presence of various cytokines. In contrast, fibroblasts grown from normal mice demonstrated low level or no amplified product (data not shown).

Immunohistochemical Localization of MCP-1 and MIP-1 Protein in Isolated Granuloma Fibroblasts

To identify the expression of MCP-1 and MIP-1 protein in the granuloma fibroblasts, paraformaldehyde-fixed cytokine-stimulated cultures were stained for the chemokines by immunohistochemistry. As illustrated the fibroblasts expressed protein for both MCP-1 (Figure 2) and MIP-1 (Figure 3). The MCP-1 was constitutively expressed in unstimulated control cultures and was not noticeably up-regulated by cytokine stimulation. In contrast, MIP-1 was also detected in unstimulated cultures; however, this chemokine was up-regulated by cytokine stimulation. These results correlated with the RT-PCR amplification results above (Figure 1) and verified the expression of MCP-1 and MIP-1 protein in granuloma fibroblasts.

MCP-1 and MIP-1 Production from Cultured Granuloma Fibroblasts

We next directly measured production of MCP-1 and MIP-1 protein from differentially stimulated fibroblast cultures. Fibroblasts were cultured for 24 hours in the presence or absence of various levels of cytokines normally found within the developing granuloma. These cytokines included IL-1, TNF, IFN-γ, IL-4, and IL-10. Figure 4 illustrates MCP-1 production in differentially stimulated fibroblasts. Control cultures demonstrated consistently higher background levels of MCP-1 (Figure 4). Addition of IL-1 (0.1 and 2.5 ng/ml), TNF (10 ng/ml), IFN-γ (2.5 and 10 ng/ml), IL-4 (2.5 and 10 ng/ml), or IL-10 (0.1 and 2.5 ng/ml) tended to increase MCP-1 production but this was not statistically significant. In contrast, the augmentation of MIP-1 production was significant in response to IL-1 (0.1 to 10 ng/ml), IFN-γ (10 μg/ml), and IL-10 (2.5 and 10 ng/ml) (Figure 5). These data indicated that MIP-1 was more specifically regulated than MCP-1 and overall correlated with the findings of RT-PCR, which demonstrated constitutive JE and selective up-regulation of MIP-1 mRNA expression. Interestingly, the examination of fibroblasts isolated and cultured from naive mice demonstrated a lower production of either MCP-1 or MIP-1 protein in response to the cytokines tested in this study (Figures 4 and 5). These results indicate that the fibroblasts within the inflammatory granuloma were activated and possibly phenotypically altered in such a manner to allow the increased production of the C-C family chemokines.

Discussion

The results of this study demonstrate that fibroblasts isolated from inflammatory granulomas may participate in the recruitment of leukocytes to a site of granu-

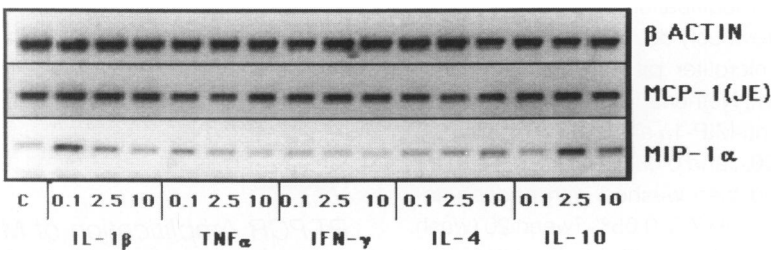

Figure 1. *RT-PCR amplification of C-C family chemokines MCP-1 and MIP-1α. Isolated granuloma fibroblasts were expanded and activated by various cytokines in 60-mm plates at a concentration of 1 × 10^5 fibroblasts/plate/ml. Total RNA was isolated, reverse transcribed into cDNA, and amplified with specific primers for B-actin, MCP-1, or MIP-1α. Data depicts a representative experiment using 28 amplification cycles each consisting of three steps: 30 seconds at 95 C, 45 seconds at 58 C, and 1 minute at 72 C. Dilutional studies of the B-actin demonstrated equal amounts relative to each sample. Repeat experiments gave similar results.*

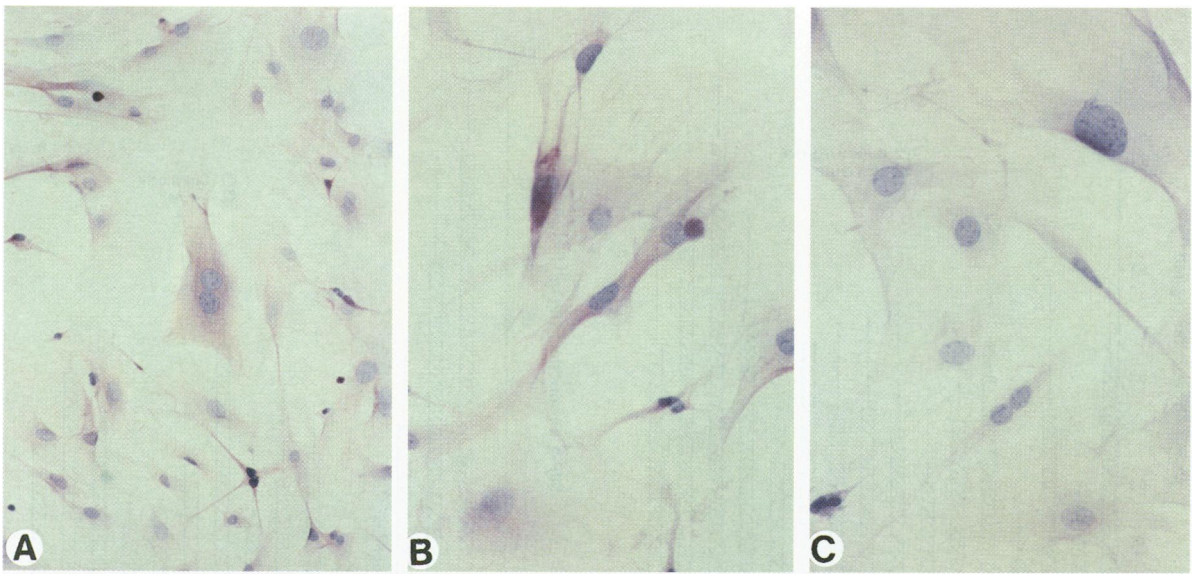

Figure 2. *Immunolocalization of MCP-1 in isolated granuloma fibroblasts. Rabbit antimurine MCP-1 serum* (A *and* B) *was used to localize MCP-1 in control* (A) *or cytokine (IL-1; 5 ng/ml)-activated* (B) *fibroblast cultures. Control serum demonstrated no nonspecific staining* (C). *Repeat experiments demonstrated similar staining patterns.*

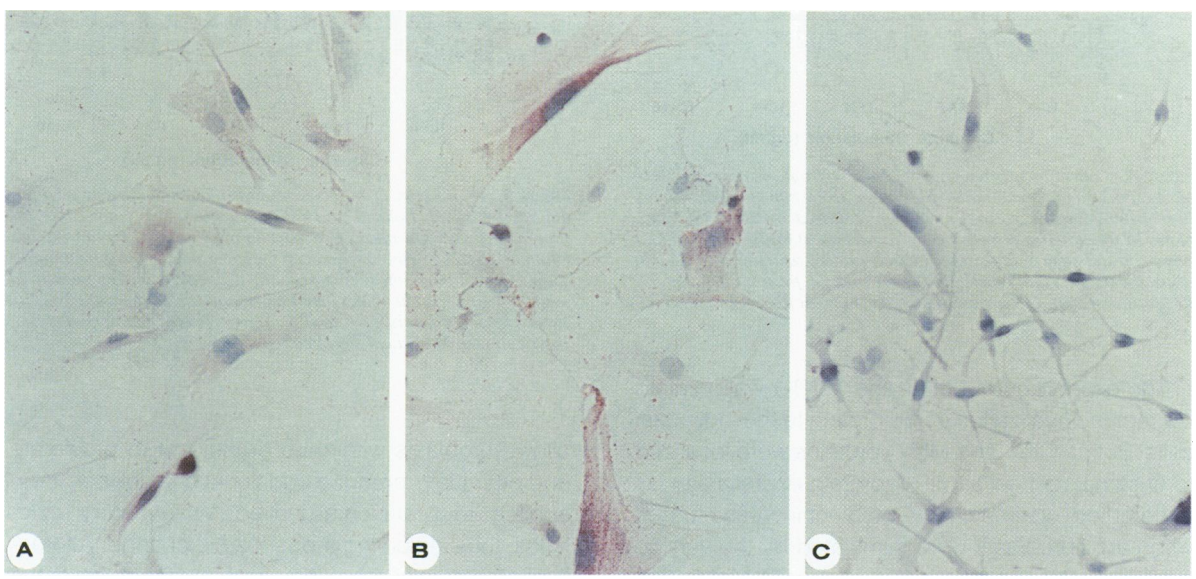

Figure 3. *Immunolocalization of MIP-1 in isolated granuloma fibroblasts. Rabbit anti-MIP-1 serum* (A *and* B) *was used to localize MIP-1 in control* (A) *or cytokine (IL-1; 5 ng/ml)-stimulated* (B) *fibroblast cultures. Control serum demonstrated no nonspecific staining* (C). *Repeat experiments demonstrated similar staining patterns.*

lomatous inflammation by producing both MIP-1 and MCP-1. These chemokines have been demonstrated to be produced during and participate in schistosome egg granuloma formation[23] (unpublished data). The stimuli used in our studies are present in significant concentrations during peak granuloma formation.[9–15] MCP-1 mRNA was constitutively expressed by the fibroblasts, whereas its production was not significantly up-regulated by any of the inflammatory cytokines tested including IL-1, TNF, IFN-γ, IL-4, and IL-10. In contrast, the MIP-1 mRNA expression was much lower in control cultures and the induction of MIP-1 expression was not highly constitutive, whereas IL-1 and IL-10 elicited significant production. These results concurred with the MIP-1 and MCP-1 RT-PCR data, suggesting constitutive expression of MCP-1 but differential induction of MIP-1 by the various cytokines.

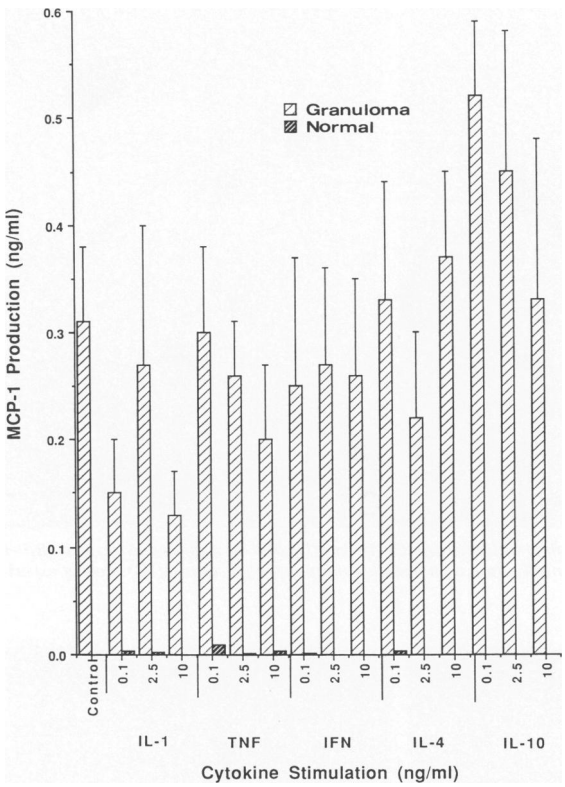

Figure 4. *Constitutive production of MCP-1 from granuloma fibroblasts. Isolated and expanded fibroblasts from either inflammatory liver granulomas or normal mice were cultured in 6-well (60-mm) plates in the absence or presence of a cytokine stimulus. Twenty-four hour culture supernatant was collected and measured for MCP-1 in a specific ELISA. Data represents the mean ± SE from six experiments for granuloma and four experiments for normal fibroblast cultures.*

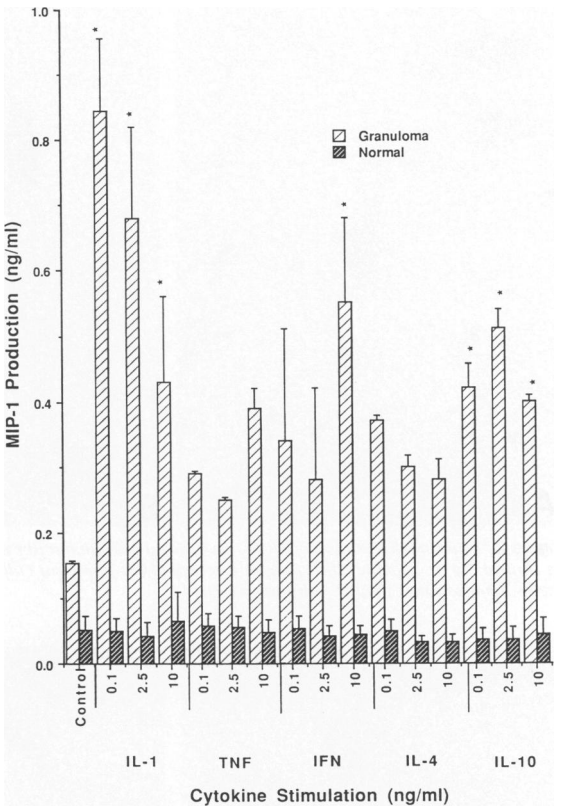

Figure 5. *Production of MIP-1 from granuloma fibroblasts is differentially activated. Isolated and expanded fibroblasts from either inflammatory liver granulomas or normal mice were cultured in 6-well (60-mm) plates in the absence or presence of a cytokine stimulus. Twenty-four hour culture supernatant was collected and measured for MIP-1 in a specific ELISA. Data represents the mean ± SE from six experiments for granuloma and four experiments for normal fibroblast cultures. *Statistical significance at the* $P < 0.05$ *level.*

The expression of MIP-1 and MCP-1 was verified by immunolocalization of the protein to the individual cells. Both MCP-1 and MIP-1 proteins were localized to unstimulated cells. MIP-1 protein expression was augmented, whereas MCP-1 did not increase further when the fibroblasts were stimulated by the various cytokines. The constitutive induction of MCP-1 mRNA may reflect the activational status of fibroblasts isolated from granulomas.[18] Normal lung fibroblasts from naive mice showed very low or no background MCP-1 production and little up-regulation of chemokine protein levels after cytokine stimulation. These data indicate that fibroblasts within the inflammatory lesions compared with normal fibroblasts were activated to allow the up-regulation of chemokine gene expression. One possible mechanism of activation is by the signal activation route previously described involving a platelet-derived growth factor (PDGF) signal (19). PDGF caused the up-regulation of the JE gene (now known as MCP-1)[18] and can be found within sites of inflammation, such as the schistosome egg granuloma. Thus, this competence signal would allow fibroblasts within the granuloma to proliferate and potentially produce additional chemokines in response to granuloma-derived inflammatory cytokines, thus indicating a phenotypic change. Alternatively, the continuous exposure of the fibroblast to the multiple inflammatory mediators within the granuloma may have an altering effect on the fibroblast's phenotype that would persist throughout the *in vitro* expansion procedure and allow increased chemokine production. The overall contribution of the fibroblast to the recruitment of leukocytes in the developing granuloma is difficult to assess. Our studies indicate that 1×10^5 cells produced nearly 1 ng/ml of chemokine in culture, suggesting that they have potential for considerable chemokine production.

MCP-1 production can be induced by IL-1 and TNF in epithelial cells.[20] In addition, IL-4 was shown to induce MCP-1 expression in endothelial cell cultures[21] and pulmonary fibroblasts (unpublished observations). Likewise, renal cortical epithelial cells

have been shown to produce MCP-1.[22] MCP-1 primarily functions as a chemoattractant for monocytes but has recently been shown to induce the expression of leukocyte integrins CD11b (Mac-1 α-subunit) and CD11c (p150,95 α-subunit) on the surface of monocytes.[23] In addition, MCP-1 has been found to stimulate monocytes to produce IL-1 and IL-6 but not TNF.[23] MIP-1 has primarily been shown to be a product of macrophages and T lymphocytes.[18,19] MIP-1 can induce acute inflammation,[24] a prostaglandin-independent fever,[25] and the production of IL-1, IL-6, and TNF from macrophages.[26] Also, the *in vivo* neutralization of MIP-1 can alter granuloma growth and development.[8] The expression of MCP-1 and MIP-1 by granuloma-derived fibroblasts may prove very important in sustaining an inflammatory response.

Activation of chemokine production by T lymphocyte-derived lymphokines was not surprising because schistosome egg granuloma formation is T lymphocyte dependent.[27–30] IFN-γ is maximally produced during early (6 week) infection when granuloma development is less intense,[12] whereas IL-10 and IL-4 are both maximally produced during the peak of leukocyte recruitment and granuloma growth at the acute stage of *S. mansoni* infection.[13,31] IL-4 has recently been demonstrated to actively participate in granuloma formation in neutralization studies of this lymphokine *in vivo*, which demonstrated inhibited granuloma development.[9,14,15] However, IL-10 has primarily been perceived as an inhibitory cytokine that limits IFN-γ production from T helper 1-type cells and decreases the efficiency of antigen-presenting cells to present antigen.[32] The data from this study demonstrates that IL-10 can participate in the inflammatory process by up-regulating the production of leukocyte chemotactic proteins, MIP-1 and MCP-1, ultimately leading to the propagation of the granuloma. Ongoing studies in our laboratory have likewise detected up-regulation of MIP-1 in granuloma macrophages stimulated by IL-10 (unpublished data). Lymphokine and cytokine production and the overall T helper lymphocyte function at the modulated stage of *S. mansoni* infection are severely decreased.[28–30] Regulation of IL-1 and IL-10, as well as the other cytokines, is one mechanism of modulation at the chronic stage of infection that would lead to decreased chemokine production, leukocyte recruitment, and ultimately diminished granuloma formation.

The data presented in this study indicate that granuloma fibroblasts constitutively express MCP-1, whereas MIP-1 production can be significantly up-regulated in response to inflammatory cytokines produced within the granuloma, including IL-1, IFN-γ, and IL-10. In contrast, normal fibroblasts from naive mice produced little or no chemokine constitutively or after cytokine stimulation, suggesting a functional difference compared with granuloma-derived fibroblasts. Overall these results suggest that inflammatory granuloma fibroblasts can participate in the recruitment of leukocytes around the deposited egg and enhance granuloma formation.

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