The Role of Macrophage Inflammatory Protein 1α in Schistosoma mansoni Egg-induced Granulomatous Inflammation

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Summary

Macrophage inflammatory protein 1 α (MIP-1α) is a 6–8-kD, lipopolysaccharide-inducible monocyte and neutrophil chemotactic protein that may be important in acute and chronic inflammation. The present study determined the sequential production, source, and in vivo contribution of murine MIP-1α in synchronized Schistosoma mansoni egg pulmonary granuloma formation. Granulomas were examined under conditions of primary, secondary vigorous, and secondary immunomodulated immunity. Secreted MIP-1α was measured in 24-h supernatants from intact granulomas (700/ml) cultured with or without soluble egg antigen (SEA). Primary granulomas isolated from naive mice over a 16-d period showed low spontaneous MIP-1α production (<1 ng/ml). However, when primary granulomas were challenged with SEA, significant MIP-1α production was observed beginning at day 4 and peaking at day 16. Intact vigorous (isolated from 8-wk-infected mice) and modulated (isolated from 20-wk-infected mice) secondary pulmonary granulomas demonstrated comparable spontaneous MIP-1α production. Addition of SEA to vigorous stage granulomas augmented expression of MIP-1α at all time points, whereas stimulated modulated stage granulomas did not increase production. The latter observation is likely related to endogenous immunoregulatory mechanisms reported for modulated stage animals. Immunohistochemical localization of MIP-1α in granuloma sections and cytospin preparations from vigorous lesions localized MIP-1α production to macrophages within granulomas. Treatment of mice with rabbit anti-mouse MIP-1α antibodies significantly decreased 8-d primary granuloma formation (>40%) when compared with control mice. Anti-MIP-1α sera also decreased vigorous (>20%), but not modulated granuloma formation. These findings demonstrate that MIP-1α contributes to cellular recruitment during schistosome egg granuloma formation.

The recruitment of cells into an area of inflammation is a crucial step in the development of delayed-type hypersensitivity responses. Presumably, the secretion of chemokines contributes to the inflammatory response and is pertinent to the elicitation of cells into the affected area. One cytokine that may be responsible for the recruitment of both neutrophils and monocytes/macrophages to sites of inflammation is macrophage inflammatory protein-1 (MIP-1) (1, 2). Native MIP-1 is an LPS-inducible, heparin-binding protein made up of two 8-kD peptides, designated MIP-1α and -1β, and is a member of the C-C chemokine supergene family (1–3). In vivo, the injection of the doublet can induce an acute inflammatory response and prostaglandin-independent fever (2, 4).

(1) Abbreviations used in this paper: MIP-1α, macrophage inflammatory protein 1α; SEA, soluble egg antigen.

It is interesting that MIP-1 has been found to induce the production of other inflammatory cytokines, TNF-α, IL-1, and IL-6 (5).

Granuloma formation during murine schistosomiasis mansoni can be studied under conditions of primary, secondary vigorous, and secondary modulated immunity (6). During initial egg deposition (5–6 wk of infection), a primary granuloma forms around eggs in response to the foreign body and released soluble egg antigens (SEA) (6, 7). The continuous deposition of eggs sensitizes the host, and, subsequently, there is a more rapid secondary (vigorous) response which sequesters the deposited egg. Vigorous granuloma formation occurs at 8 wk of infection and accompanies peak production of inflammatory cytokines (8–10). As the infection progresses, the intensity of the response wanes and there is onset of modulated granuloma formation associated with impaired cytokine production by 20 wk (8–15). The formation of schistosome
egg-induced granulomas involves several stages of development (8, 16). First, there is an early recruitment stage followed by a maintenance or sustenance stage, which presumably continues until the destruction of the egg nidus. Finally, there is a resolution stage manifested by healing and fibrosis. The early cellular recruitment stage is critical for the development of the granuloma and likely requires the generation of chemokines for the elicitation of the leukocytic infiltrate. The maintenance phase would also require the generation of chemokines, necessary for the continued recruitment of cells and sustenance of the granuloma. The cytokines produced during early stages of granuloma formation may be important in the initiation of granuloma development and likely requires the generation of chemokines for the elicitation of the leukocytic infiltrate. The maintenance phase would also require the generation of chemokines, necessary for the continued recruitment of cells and sustenance of the granuloma. The cytokines produced during early stages of granuloma formation may be important in the initiation of granuloma development and likely requires the generation of chemokines for the elicitation of the leukocytic infiltrate. The maintenance phase would also require the generation of chemokines, necessary for the continued recruitment of cells and sustenance of the granuloma. The cytokines produced during early stages of granuloma formation may be important in the initiation of granuloma development and likely requires the generation of chemokines for the elicitation of the leukocytic infiltrate. The maintenance phase would also require the generation of chemokines, necessary for the continued recruitment of cells and sustenance of the granuloma. The cytokines produced during early stages of granuloma formation may be important in the initiation of granuloma development.
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incubated for 20 min with streptavidin-atkaline phosphatase (1:20; CA). After rinsing three times with PBS, the lung sections were normal goat serum for 30 rain. The sections were covered with ELISA method consistently detected MIP-la concentrations above dilutions of recombinant MIP-1α from 1 pg/ml to 100 ng/ml. This ELISA method consistently detected MIP-1α concentrations above 10 pg/ml.

**Immunohistochemical Localization of MIP-1α in Synchronous Granulomas.** Paraffin-embedded tissue sections mounted on poly-l-lysine slides were deparaffinized with xylene followed by stepwise rehydration in 100, 95, 70, and 50% ethanol followed by 10 min incubation in PBS. Cytospin preparations of dispersed granulomas were fixed with 4% paraformaldehyde and treated in a similar fashion to the tissue sections. All sections and fixed cells were blocked with normal goat serum for 30 min. The sections were covered with the rabbit anti-murine MIP-1α serum diluted in PBS (1:250) for 30 min at 37°C. After rinsing three times with PBS, the sections were overlaid for 20 min with biotinylated goat anti–rabbit IgG (supersensitive reagent 1:10; BioGenex Laboratories, San Ramon, CA). After rinsing three times with PBS, the lung sections were incubated for 20 min with streptavidin-alkaline phosphatase (1:20; BioGenex) at 37°C. The slides were rinsed with PBS and overlaid with Fast red (BioGenex) until color development was observed (15–30 min). Sections were rinsed and counterstained with Mayer’s hemotoxylin (Mayer & Myles Laboratories, Coopersburg, PA).

**Inhibition of In Vivo Granuloma Formation with Anti-MIP-1α Serum.** Pulmonary granuloma formation was observed after challenge with *S. mansoni* eggs in naive, 8-wk and 20-wk-infected mice after treatment with rabbit anti–murine MIP-1α or normal rabbit serum. Mice were pretreated and then treated every other day with 0.5 ml of serum intraperitoneally for 8 d with the primary and 4 d with vigorous and modulated secondary granuloma formation. After 8 or 4 d, respectively, the lungs were inflated and preserved in 10% buffered formalin. Histological sections were stained and granulomas were measured with computer-assisted morphometric analysis. Each group contained five to six mice, and 20 granulomas from each lung were measured.

**Statistics.** Statistical significance was determined by Student’s *t* test and significance was determined with *P* values <0.05.

**Results**

**RT-PCR Amplification of MIP-1α from Pulmonary Granulomas.** We first screened granulomatous lungs for MIP-1α mRNA by isolating whole RNA at different stages of granuloma development and performing RT followed by primer-directed PCR amplification. Fig. 1 illustrates that MIP-1α mRNA can be detected at most points of primary and secondary granuloma formation. Low signal levels were even detected in normal lungs. During primary granuloma formation, MIP-1α mRNA levels were equal to or slightly greater than that of the normal control lungs throughout the time frame studied (days 1–16). During secondary vigorous granuloma formation, significantly more mRNA was detected throughout the development period and as early as 12 h post-egg embolization. MIP-1α mRNA during secondary modulated granuloma formation was also upregulated by 12 h, but decreased at 1, 2, and 4 d with mRNA appearing again at days 8 and 16. These results suggested that MIP-1α is differentially expressed during the primary, secondary vigorous, and secondary modulated response.

**MIP-1α Production from In Vitro Cultured Granulomas.** We next directly measured sequential production of MIP-1α by egg granulomas isolated from the lungs of normal mice or mice at 8 (vigorous) and 20 (modulated) wk of infection. Granulomas were collected at 1, 2, 4, 8, and 16 d post-egg challenge and were incubated in culture in the presence or absence of SEA (5 µg/ml) for 24 h. The time course study showed a significant increase in antigen-elicited production of MIP-1α as the primary granuloma developed from 4 to 16 d (3.7 ± 0.6 to 25.9 ± 16 ng/ml, respectively) (Fig. 2). Antigen-elicited production of MIP-1α was not detected before day 4.

A different pattern of MIP-1α production was observed during the vigorous and modulated secondary response (Fig. 3). In the absence of SEA, both vigorous and modulated granulomas produced comparable background levels of MIP-1α ranging from 2.0 to 7.8 ng/ml. However, SEA stimulation revealed a clear difference between the groups. SEA augmented production of MIP-1α only in the vigorous granuloma cultures. The modulated granulomas failed to show augmentation of MIP-1α production with addition of SEA. Compared with the primary response, the vigorous granuloma MIP-1α production was accelerated peaking at day 2 (16 ± 4.6 ng/ml) and only partially declining at days 4 and 8 (10.5 ± 4.7 and 8.1 ± 2.5 ng/ml, respectively). Modulated granulomas also showed an accelerated onset of MIP-1α production, but levels were consistently lower than those for vigorous lesions. Thus, MIP-1α production showed a direct correlation with the rate and amplitude of granuloma formation.

**Immunohistochemical Localization of MIP-1α Production in Egg Granulomas.** To identify potential sites of MIP-1α production, sections of paraffin-embedded granulomatous tissue from normal and schistosome-infected mice were stained for MIP-1α immunohistochemically. As shown in Fig. 4, MIP-1α is localized to mononuclear cells in granulomas. These studies

![Figure 1. Detection of MIP-1α mRNA by RT-PCR amplification. Whole RNA was isolated from lungs at different time points of granuloma development and reverse transcribed utilizing oligo (dT)12-18 primers. Specific oligonucleotide primers were used to amplify MIP-1α and β-actin using 0.5 µg total RNA equivalents. The reaction mixture was amplified by 28 cycles each consisting of three steps; 30 s at 95°C, 45 s at 58°C, and 60 s at 72°C. Repeat experiments demonstrated similar results.](#)
Figure 2. MIP-1α production by isolated primary pulmonary granulomas. Isolated pulmonary granulomas (700/ml) from naive mice at various points of development were cultured for 24 h with or without SEA. Culture supernatants were collected and the level of MIP-1α was measured by ELISA. Data represents the mean ± SEM of three separate experiments using different mice and separate egg harvests.

demonstrated a greater number of cells stained positive in vigorous lesions (Fig. 4, A and B) than in either the primary or secondary modulated granulomas (data not shown). To further clarify the specific cell population that was producing MIP-1α, vigorous (8-wk) granulomas were isolated and dispersed. The dispersed whole or macrophage-depleted (by plastic adherence) granulomas cells were incubated with SEA. Immunohistochemical localization of MIP-1α was then performed on cytospin specimens of adherent and nonadherent cell populations. In all cases, the only cells that expressed MIP-1α was the macrophage population (Fig. 5, A–C). In addition, extracellular MIP-1α was detected in cultures of unseparated secondary vigorous granuloma cells but not macrophage-depleted cultures (1.2 ng/ml and <0.1 ng/ml.

Figure 3. MIP-1α production by isolated secondary vigorous and modulated pulmonary granulomas. Isolated pulmonary granulomas (700/ml) from S. mansoni-infected mice at various points of development were cultured for 24 h with or without SEA. Culture supernatants were collected and the level of MIP-1α was measured by ELISA. Data represents the mean ± SEM of three separate experiments using different mice and separate egg harvests.

Figure 4. Immunolocalization of MIP-1α in vigorous granulomas from 8-wk S. mansoni-infected mice. Paraffin-embedded granulomatous tissue was sectioned and stained using MIP-1α specific polyclonal rabbit antisera (B) or normal rabbit serum (A) as a control. Repeat experiments demonstrated similar patterns of staining.
Figure 5. Immunolocalization of MIP-1α in dispersed granuloma cells of vigorous granulomas from 8-wk S. mansoni-infected mice. Granulomas were collagenase dispersed and cultured at a concentration of 10⁶/ml and rechallenged with SEA. After 24 h, adherent cells and nonadherent cells were cytospin fixed with 4% paraformaldehyde. Fixed cells were immunohistochemically stained using normal rabbit serum (A) or MIP-1α-specific polyclonal rabbit antiserum (B and C). Repeat experiments demonstrated that only macrophages (B) and Giant cells (C) stained positive for MIP-1α.
respectively). Taken together, these studies indicate that the macrophage was the primary cellular source of MIP-1α in the granuloma.

**Inhibition of Pulmonary Egg Granulomas by Anti-MIP-1α Antisera.** To determine whether or not MIP-1α contributed to granuloma development, we performed in vivo neutralization studies with polyclonal rabbit anti-mouse MIP-1α serum. Primary granulomas were initiated in naive (8-d) and infected mice (4-d). Groups of mice at each stage of infection were treated with anti-MIP-1α or control serum every other day. At 8 d, mice were killed and the lungs were examined histologically. As shown in Fig. 6, morphometric assessment of granuloma development demonstrated that anti-MIP-1α treatment attenuated granuloma formation by more than 40% (P < 0.001) as compared with controls (8,156 ± 587 μm² and 13,461 ± 832 μm², respectively). A representative photomicrograph (Fig. 7, A and B) demonstrates the abrogation due to in vivo neutralization of MIP-1α in primary granuloma formation. In many lesions, nearly all of the cellular infiltration was inhibited by the anti-MIP-1α treatment.

We next determined whether anti-MIP-1α serum treatment could affect secondary vigorous and modulated granuloma formation. In these studies, animals were treated with anti-MIP-1α serum at days 0 and 2, then killed at day 4. Depletion of MIP-1α significantly (P < 0.01) attenuated vigorous granuloma area by >20% as compared with controls (24,574 ± 1,249 μm² and 30,660 ± 932 μm², respectively) (Fig. 6). In contrast, neutralization of MIP-1α in modulated granuloma formation failed to have a significant effect on granuloma size as compared with infected control mice (17,997 ± 910 μm² and 18,709 ± 932 μm², respectively). Fig. 7 illustrates that the degree of abrogated recruitment with vigorous lesions (C and D) was not as great as in primary granuloma formation, and no decrease was evident at the modulated stage of infection (E and F).

**Discussion**

In the present study, we have demonstrated the temporal expression, cellular source, and the in vivo contribution of MIP-1α in the development of pulmonary *S. mansoni* egg-induced granulomas. The expression of MIP-1α during the course of primary and secondary granuloma formation corresponded to the immune status of the host. The primary granuloma, which peaks in size at 8 to 16 d after embolization (6, 16), began expressing detectable MIP-1α at day 4 and continued to increase through day 16. The constitutive levels of MIP-1α were much higher in the secondary vigorous and modulated granulomas as compared with the primary granulomas. The constitutive levels of MIP-1α in the secondary response may reflect a primed environment for the rapid expression of MIP-1α and recruitment of cells for accelerated granuloma formation around the deposited egg. The in vitro expression of MIP-1α by isolated vigorous granulomas was accelerated in response to SEA, detectable at day 1, and peaking at day 2, with levels largely sustained at days 4 and 8. It is interesting that the modulated granulomas expressed background levels of MIP-1α comparable with the vigorous stage granulomas, however, upon stimulation with SEA, there was increased population of MIP-1α only at day 1. In general, the in vitro production of SEA-specific MIP-1α corresponded directly to the level of MIP-1α-specific mRNA as determined by RT-PCR. The decreased production of MIP-1α at the modulated stage of infection corresponds to previous reports demonstrating decreased T cell function and lymphokine production (6, 8, 11-15). These findings, along with the antigen-specific increases in MIP-1α at the vigorous stages in the present study would suggest that this cytokine is regulated in conjunction with or by T lymphocytes and their products. Lack of antigen-elicted MIP-1α production by intact modulated secondary granulomas may reflect local chemokine regulation within the granuloma that would allow initial early sequestration of the egg, yet impair further elicitation of leukocytes to the developing granulomas.

Immunohistochemical localization of MIP-1α from intact granulomas, as well as dispersed granuloma cells, has identified the macrophage as the primary cellular source of MIP-1α. This corroborates previous in vitro studies showing the macrophage as the predominant cellular source of MIP-1α (5). In addition, the dispersion of the vigorous granulomas demonstrated little detectable MIP-1α production by the nonadherent lymphocytes and eosinophils. The fact that MIP-1α could be detected by immunohistochemical localization within granulomas at all stages of infection indicates that this chemokine is involved to a greater or lesser degree in the development of both primary and secondary granulomas.

The contribution of MIP-1α to primary granuloma for-
Figure 7. Photomicrographs of granuloma formation in normal serum (A, C, and E) and anti-MIP-1α (B, D, and F) treated naive and infected mice. Illustrations are representative of the degree of inhibition in mice treated with serum in primary (A and B) and secondary vigorous (C and D) and modulated (E and F) granuloma formation. Photomicrographs are of granulomas in lungs taken from data in Fig. 6.
mation was demonstrated by a significant reduction (≥40%) of lesion area with anti-MIP-1α serum treatment. In vivo depletion of MIP-1α in 8-wk-infected, vigorous stage mice decreased granuloma area by more than 20%, demonstrating at least a partial role in secondary granuloma formation. The failure of anti-MIP-1α to impair modulated granulomas is consonant with their impaired MIP-1α production in response to SEA and supports the notion that MIP-1α is downregulated at the modulated stage.

There may be several reasons why the anti-MIP-1α treatment had less effect on the vigorous lesions. The vigorous granulomas produced significantly more MIP-1α than did the primary lesions, and there may have been insufficient neutralization of the MIP-1α at this stage. A second explanation may entail the production of other chemotactic factors during the development of secondary modulated granulomas which would allow the granulomatous response to continue at a reduced level. Chemokines, such as murine JE (18) and RANTES (19), which are both chemotactic for mononuclear cells, may be more prevalent during secondary granuloma formation. Finally, the type and level of chemokines produced during the various stages of granuloma formation may prove to be pertinent to the specific population of cells recruited to the granuloma. The primary granuloma is characterized by the presence of predominantly mononuclear cells. However, with the development of the secondary granuloma, there is a rapid shift in cellular constituents from mononuclear to predominantly eosinophils. The decreased presence of macrophages in the secondary vigorous granuloma may be indicative of the decreased role for MIP-1α in monocyte recruitment as reflected by the reduced inhibitory effect of the anti-MIP-1α antiserum treatment.

Our findings show that MIP-1α is produced in both the early recruitment and maintenance phases of primary and secondary granuloma formation and is therefore a likely contributor to cellular recruitment throughout the growth of the granuloma. The role of MIP-1α production in the granuloma may not only be limited to cellular recruitment, but may also involve the generation of other inflammatory cytokines, IL-1, IL-6, and TNF-α (5). The production of MIP-1α within the granuloma may result in increased cytokine production along with local macrophage proliferation and activation. TNF has recently been shown to restore granuloma formation in SCID mice (20). Therefore, the increased production of TNF in response to MIP-1α could promote granuloma formation during the maintenance phase when TNF production has previously been detected (8).

The present study provides the first evidence that MIP-1α has an important role in vivo during granuloma formation. The presence of MIP-1α within the granuloma may serve as a monocyte chemotaxin as well as an activating factor for infiltrating leukocytes. It is interesting that our study also suggests that the regulation of chemokines such as MIP-1α may be responsible for determining the degree and rate of granuloma formation.

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