**Mycobacterium**-Mediated Chemokine Expression in Pleural Mesothelial Cells: Role of C-C Chemokines in Tuberculous Pleurisy

Kamal A. Mohammed, Najmunnisa Nasreen, Melissa J. Ward, Kamal K. Mubarak, Francisco Rodriguez-Panadero, and Veena B. Antony

Pulmonary tuberculosis is characterized by granulomatous inflammation with an extensive infiltration of mononuclear phagocytes, but the mechanisms of phagocyte recruitment to the pleural space is unknown. In this study, pleural fluid from patients with tuberculosis contained significantly \((P < .001)\) more biologically active MIP-1\(\alpha\) and MCP-1 (C-C cytokines) than did effusions from patients with congestive heart failure. Antigenic MIP-1\(\alpha\) and MCP-1 was detected by immunocytochemistry in pleural biopsy sections of patients with tuberculous pleurisy. In vitro, pleural mesothelial cells stimulated with bacille Calmette-Guérin (BCG) or interferon (IFN)-\(\gamma\) produced MIP-1\(\alpha\) and MCP-1. Reverse transcription–polymerase chain reaction studies confirmed that both BCG and IFN-\(\gamma\) induced MIP-1\(\alpha\) and MCP-1 expression in mesothelial cells, demonstrating that mesothelial cell–derived C-C chemokines play a biologically important role in the recruitment of mononuclear cells to the pleural space.

Tuberculosis (TB), a chronic mycobacterial infection caused by *Mycobacterium tuberculosis* (MTB), is the leading infectious cause of mortality in the world, and approximately one-third of the world’s population is infected with the organism [1]. In many areas, TB remains the most common cause of pleural effusions. MTB infection results in chronic granulomatous inflammation that is characterized by the presence of lymphocytes [2, 3] and mononuclear phagocytes at the site of infection [4, 5]. MIP-1\(\alpha\) and MCP-1 (C-C chemokines) are chemotactic for mononuclear phagocytic cells [6, 7]. MIP-1\(\alpha\) is a low-molecular-mass heparin-binding protein known to exert chemotactic and activating effects on phagocytic mononuclear cells [8, 9]. In addition, MIP-1\(\alpha\) is expressed in both acute and chronic inflammatory disease states [10, 11]. MCP-1 is an 8.7-kDa protein and has specific chemoattractant and activating activity for monocytes in acute inflammatory conditions [12]. Chemokine synthesis is induced in various cells by inflammatory stimuli. Mesothelial cells have been observed to produce C-C chemokine upon stimulation by inflammatory mediators [13]; however, their role in MTB-mediated pleurisy is undefined.

Mesothelial cells are metabolically active and continuously line the pleura in a monolayer. They are the first cell type to encounter pathogens that invade pleural space and thus are likely candidates to initiate and propagate an inflammatory reaction. Recent investigations suggest that mesothelial cells initiate pleural inflammation in response to various agonists at least in part by production and release of C-X-C and C-C chemokines [14–16]. Rapid recruitment of mononuclear phagocytic cells to the inflammatory site is key to containment of the infection; however, the mechanisms of their recruitment into the pleural space remain unclear. In this study, we investigated the role of pleural mesothelial cells in the production of MIP-1\(\alpha\) and MCP-1.

**Materials and Methods**

*Antigenic MIP-1\(\alpha\) and MCP-1 analysis in pleural fluid.* Sixteen patients with pleural effusions were studied. Pleural fluids were obtained via thoracentesis from patients with TB \((n = 7)\) and congestive heart failure (CHF) \((n = 9)\) [17]. Patients with TB were defined as those whose pleural fluid or pleural biopsy was culture-positive for *M. tuberculosis*. The presence of caseating granulomas in a patient with a positive purified-protein derivative was also considered diagnostic of pleural TB. A CHF effusion was defined as a transudative effusion in a patient with clinical stigmata of CHF. After thoracentesis, the pleural fluid was centrifuged at 1000 \(g\) and stored at \(-70^\circ C\) for \(<6\) weeks. MIP-1\(\alpha\) was estimated by sandwich ELISA (Biotrak; Amersham Life Sciences, Amersham, UK). MCP-1 was estimated by ELISA (Quantikine; R&D Systems, Minneapolis) in accordance with the manufacturer’s instructions. The lower detection limit was 2.0 pg/mL for cell culture supernatant and 3.0 pg/mL for serum or plasma samples.

*Histochemical immunostaining of pleural biopsy sections.* Pleural biopsy sections were obtained from patients with TB pleurisy who were undergoing biopsy during the process of disease diag-

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Patients and volunteers gave informed consent before collection of pleural fluids via thoracentesis. The protocol was approved by the Indiana University Institutional Review Board.

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1 Present affiliation: Thoracic Research Unit, Department of Medicine, Mayo Clinic, Rochester, Minnesota.

Reprint correspondence: Dr. Veena B. Antony, Veterans Affairs Medical Center, 1481 W. 10th St., 111-P, Indianapolis, IN 46202 (vantony@iuuiui).
nosis. The sections were immunostained with avidin-biotin conjugate and peroxidase as described previously [17].

Isolation and characterization of human pleural mesothelial cells. Pleural fluid was obtained via thoracentesis from patients with transudative pleural effusions secondary to CHF. Most had intractable CHF and symptomatic pleural effusions. None had evidence of an infectious etiology for the pleural effusion. Pleural fluid was placed in a heparinized container and centrifuged at 1000 g for 10 min, and the red blood cells in the cellular pellet were lysed by hemolytic Gey's solution. The cells were resuspended in Hams-199 culture medium (Gibco Laboratories, Grand Island, NY) containing 15% fetal bovine serum (Harlan Bioproducts, Indianapolis, IN) and plated in 75-mL culture flasks. The following day nonadherent cells were removed; medium was changed thrice weekly thereafter. The cells grew to confluence in 7–14 days. The mesothelial cells were characterized by the presence of classic cobblestone morphology [18], absence of factor VIII antigen, and presence of cytokeratin [19, 20]. Mesothelial cells were 99.4% pure, and there was no NK cell or T cell contamination as tested by fluoro- 

ence-activated cell sorting analysis (data not shown). All cells were utilized between the second and fourth passages.

Antigenic MIP-1α and MCP-1 production in vitro. Mesothelial cells (0.5 × 10^6/mL) were incubated in the presence of heat-killed bacille Calmette-Guérin (BCG; 10^6 cfu), recombinant human interferon (IFN)γ (500 U/mL; R&D Systems) or BCG + IFN-γ in serum-free medium at 37°C in 5% CO₂. The culture supernatant was collected at various time points (6, 12, 24, and 48 h), and MIP-1α and MCP-1 levels were measured by sandwich ELISA as described earlier. The cells were spared for RNA isolation by reverse transcription (RT)-polymerase chain reaction (PCR) and for Southern analysis.

Isolation of RNA and RT-PCR and Southern analysis. Total cellular RNA was isolated from human mesothelial cells by use of TRI-reagent [21]. Total RNA (1 μg) was reverse transcribed into cDNA with MuLV reverse transcriptase (Perkin-Elmer Cetus, Norwalk, CT). The cDNA was then amplified using specific primers described earlier. The PCR products run on an agarose gel were transferred to nylon filter by capillary blotting for Southern hybridization [22] and were detected with γ-32P-d-CTP-labeled oligonucleotide probe 5′-AGACCTGT- TTCACACATCTCTGCT-3′ (antisense) for β-actin; 5′-TAAC- CAAAGCGAGCCGGCAGG-3′ (sense) and 5′-GGTCACACGC- TATGTTCTCACAAGG-3′ (antisense) for MIP-1α; 5′-TGCTGC- TATAACTTCACCAATA-3′ (sense) and 5′-TGGGGAAAGCTG- TGCTGC-3′ (antisense) for MCP-1. Human β-actin served as control. After 30 cycles of amplification, PCR products were analyzed by agarose gel electrophoresis; their identities were initially confirmed following sequence determination. The PCR products run on an agarose gel were transferred to nylon filter by capillary blotting for Southern hybridization [22] and were detected with γ-32P-d-CTP-labeled oligonucleotide probe 5′-AGACCTGT- ACGCCACACAGTGCTGTCTGG-3′ (β-actin), 5′-GTGAG- GAGTGTTCCAGAATGTCCAGCGG-3′ (MIP-1α), and 5′- GAGATCTGTTGTCGGCCAAAAGCAGGTTG-3′ (MCP-1). The expected sizes of the PCR products are 302-, 173-, and 259-bp for β-actin, MIP-1α, and MCP-1, respectively.

Isolation of peripheral blood mononuclear cells (PBMC). Peripheral blood was collected from healthy volunteers by venipuncture. The blood was mixed with heparin and overlaid on an equal volume of Histopaque 1.077 (Sigma, St. Louis), and centrifuged at 1700 g for 20 min. The buffy coat between the two phases was aspirated and washed three times in Hanks' balanced salt solution (HBSS). Cell viability was checked by Trypan blue dye exclusion and kept at 4°C until use. PBMC viability remained >95%.

PBMC chemotaxis assay. The biologic activity of C-C chemokines, MIP-1α and MCP-1, produced in vitro by pleural mesothelial cells and in vivo by pleural fluid was estimated by PBMC chemotaxis using Boyden chamber assay (Transwell; Costar, Cambridge, Massachusetts) plates as reported earlier [17]. In brief, 10⁵ PBMC were introduced into the inner well. The outer well received HBSS, pleural fluid, 10⁻⁷ M FMLP (Sigma), or mesothelial cell culture supernatant with or without mouse anti-human MIP-1α antibody or MCP-1 antibody (PeproTech, Rocky Hill, NJ). PBMC that had migrated to the bottom of the filter were counted in 10 high-power fields under a microscope. Chemotactic bioactivity was expressed as chemotactic index (i.e., no. of cells per 10 high-power fields).

Statistical analysis. The significance of differences between experimental and control groups were tested by 2-tailed Student’s t test. Differences between group means were compared by Kruskal-Wallis 1-way analysis of variance on ranks. P < .05 was considered significant. All computations were done with the aid of computer software (SigmaStat; Jandel Scientific, Costa Mesa, CA).

Results

TB pleural effusions have higher C-C chemokine levels than do CHF effusions. MIP-1α and MCP-1 concentrations were elevated in TB effusions. Pleural fluid from patients with CHF had low levels of MIP-1α and MCP-1. This difference was highly significant (figure 1).

Figure 1. C-C chemokine levels in TB (n = 7) and congestive heart failure (CHF) pleural fluid (n = 9). Results are mean ± SE. TB pleurisy effusions differ significantly (P < .001) from CHF effusions (*).
Table 1. Neutralization of monocyte chemotactic activity of pleural fluid with neutralizing MIP-1α and MCP-1 antisera.

<table>
<thead>
<tr>
<th>Pleural fluid source</th>
<th>No antibody</th>
<th>Isotype antibody</th>
<th>MIP-1α antibody</th>
<th>MCP-1 antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breasts of heart failure patients</td>
<td>157.43 ± 8.35</td>
<td>148.75 ± 11.29 (5.51)</td>
<td>141.40 ± 9.86 (10.18)</td>
<td>136.46 ± 10.78 (13.32)</td>
</tr>
<tr>
<td>TB patients</td>
<td>336.28 ± 15.43</td>
<td>322.59 ± 12.72 (4.07)</td>
<td>194.90 ± 9.69 (42.04)*</td>
<td>236.57 ± 13.85 (29.65)*</td>
</tr>
</tbody>
</table>

NOTE. Monocyte chemotactic index of pleural fluid in absence or presence of antibodies for MIP-1α, MCP-1, or mouse isotype. Pleural fluid was preincubated with excess of MIP-1α, MCP-1, or mouse isotype antibody for 30 min at 37°C for neutralization of these chemokines before addition into Boyden chambers. 10−7 M FMLP served as positive control. Chemotactic indices of Hanks’ balanced salt solution and FMLP are 53.45 ± 4.15 and 483.42 ± 21.65, respectively. Data are mean ± SE of 4 independent observations.

MIP-1α and MCP-1 contribute monocyte chemotactic activity in TB effusions. Pleural fluid from patients with TB pleurisy had significantly (P < .001) higher chemotactic activity for PBMC than did pleural fluid from patients with CHF (table 1). When neutralizing antibody to MIP-1α and MCP-1 was added, a maximum suppression of 42.04% and 29.65% chemotactic activity was noticed in TB effusion. The effusions due to CHF showed a suppression of 10.18% and 13.32%, respectively, with MIP-1α and MCP-1 neutralizing antibody.

Immunocytochemical detection of antigenic C-C chemokines in pleural biopsies. To immunolocalize the cellular expression of antigenic MIP-1α and MCP-1 in patients with TB pleurisy, pleural biopsy sections were stained with MIP-1α and MCP-1 antibodies. The mesothelial monolayer in pleural biopsy sections showed positive peroxidase staining with MIP-1α and MCP-1 antibodies but not with an irrelevant isotype antibody (figure 2).

Activated pleural mesothelial cells release C-C chemokines in vitro. When pleural mesothelial cells were stimulated in the presence of BCG, IFN-γ, or BCG + IFN-γ in serum-free medium, they released MIP-1α and MCP-1 antigenic protein in a time-dependent manner (figure 3). At all time points (6–48 h), stimulated mesothelial cells released significantly more (P < .001) MIP-1α and MCP-1 than did unstimulated control cultures. The maximal response occurred at 24 h and plateaued thereafter. The combination of IFN-γ and BCG induced higher levels of chemokine than either alone.

Pleural mesothelial cell culture supernatant mediates PBMC chemotaxis. Stimulated culture supernatants were tested for mononuclear cell chemotactic bioactivity. BCG and IFN-
Figure 3. Bacille Calmette-Guérin (BCG)-mediated C-C chemokine production in pleural mesothelial cells. A and B, MIP-1α and MCP-1, respectively. Data at each time point are mean ± SE of 6 independent experiments. * P < .001 vs. serum-free medium. IFN, interferon.

γ-activated conditioned media also induced chemotaxis. BCG + IFN-γ–activated culture supernatants showed greater chemotaxis than did BCG-stimulated supernatant (table 2). Neutralization of MIP-1α and MCP-1 by excess of respective antibody resulted in 43.38% and 34.42% inhibition of chemotactic activity, respectively. Recombinant MIP-1α and MCP-1 (1000 pg/mL)–induced chemotaxis was neutralized completely when the respective antibody was added (data not shown).

BCG mediates C-C chemokine mRNA expression in pleural mesothelial cells. Figure 4 shows the expression of MIP-1α and MCP-1 mRNA in stimulated pleural mesothelial cells as demonstrated by RT-PCR. BCG, IFN-γ and BCG + IFN-γ induced MIP-1α and MCP-1 mRNA expression. The accuracy of MIP-1α– and MCP-1–specific message amplified by RT-PCR was confirmed by Southern hybridization (figure 5).

Discussion

Granulomatous inflammation, such as TB, is a specific type of inflammation characterized by the accumulation of mononuclear phagocytes. Several locally generated chemokines are responsible for the movement of inflammatory cells from the vascular compartment into the pleural space [23, 24]. MIP-1α and MCP-1 are chemotactic for mononuclear phagocytes [8, 9]. While lymphocytes are the predominant cell population in TB effusions [2, 3], significant proportions of mononuclear phagocytes are also found in TB effusions [25, 26]. Therefore, we probed MIP-1α and MCP-1 levels in pleural fluid from patients with TB and CHF. CHF pleural fluid was chosen as a control because there is absence of pleural involvement or inflammation. In CHF, the pleural fluid accumulates primarily because of abnormal hydrostatic forces. We found that MIP-1α and MCP-1 concentrations were high in TB pleural effusions compared with effusions due to CHF.

The pleural effusions from patients with TB demonstrated chemotactic activity for mononuclear phagocytes. The MIP-1α and MCP-1 contribution to the biologic chemotactic activity of TB pleural effusions was significantly higher than with CHF. In the TB pleural effusions, MIP-1α contributed 42.04% of the monocyte chemotactic activity and MCP-1 contributed 29.65%. The TB pleurisy biopsy specimens immunostained with MIP-1α and MCP-1 antibody were positive for these chemokines (figure 2), demonstrating that pleural mesothelial cells in response to M. tuberculosis produce C-C chemokines in vivo. These results suggest that in TB pleurisy, mycobacteria-stimulated pleural mesothelial cells release MIP-1α and MCP-1 in

![Graph showing chemotaxis](image)

Table 2. Neutralization of monocyte chemotactic activity of mesothelial cell culture media (24-h culture) with neutralizing MIP-1α and MCP-1 antisera.

<table>
<thead>
<tr>
<th>PMC culture media</th>
<th>No antibody</th>
<th>Isotype antibody (% suppression)</th>
<th>MIP-1α antibody (% suppression)</th>
<th>MCP-1 antibody (% suppression)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMC (control)</td>
<td>52.82 ± 3.61</td>
<td>51.54 ± 3.03 (2.42)</td>
<td>51.04 ± 4.1 (3.36)</td>
<td>51.36 ± 3.25 (2.76)</td>
</tr>
<tr>
<td>BCG</td>
<td>158.57 ± 8.32</td>
<td>153.64 ± 7.84 (3.10)</td>
<td>112.16 ± 6.36 (29.26a)</td>
<td>130.65 ± 9.52 (17.60a)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>173.63 ± 11.27</td>
<td>168.78 ± 12.06 (2.79)</td>
<td>117.44 ± 10.41 (32.36a)</td>
<td>124.38 ± 10.23 (28.36a)</td>
</tr>
<tr>
<td>BCG + IFN-γ</td>
<td>216.31 ± 12.75</td>
<td>209.25 ± 13.59 (3.26)</td>
<td>122.46 ± 12.68 (43.38a)</td>
<td>141.85 ± 14.72 (34.42a)</td>
</tr>
</tbody>
</table>

NOTE. Monocyte chemotactic index of pleural mesothelial cell (PMC) culture supernatant in absence or presence of antibodies for MIP-1α, MCP-1, or mouse isotype. Culture media were preincubated with excess of MIP-1α, MCP-1, or mouse isotype antibody for 30 min at 37°C for neutralization of these chemokines before addition into Boyden chambers. 10−7 M FMLP served as positive control. Chemotactic indices of Hanks’ balanced salt solution and FMLP are 48.69 ± 3.18 and 469.53 ± 16.37, respectively. Data are mean ± SE of 4 independent observations. BCG = bacille Calmette-Guérin, IFN = interferon.

* P < .001 vs. control.
the pleural space. We speculate that the chemokines released by pleural mesothelial cells are responsible in part for initiation of the inflammatory response to recruit mononuclear cells to the pleural space.

The precise mechanism of pleural involvement with pulmonary TB remains unknown. Several reports indicate that mesothelial cells can produce an array of products upon activation. When pleural mesothelial cells were challenged with BCG, they produced MIP-1α and MCP-1 protein in vitro (Figure 3). These observations were further supported by enhanced chemokine mRNA expression (Figure 4). In other studies, *M. tuberculosis* stimulated IL-8 [27] and MCP-1 [28] production in monocytic cell lines. C-C and C-X-C chemokine levels were elevated in bronchoalveolar lavage fluid lavage from persons with TB [29]. The infection of murine macrophages with various strains of *M. tuberculosis* found to induce chemokine gene expression in vitro [30] suggests that mycobacteria can induce chemokine expression.

When pleural mesothelial cells were stimulated in vitro in the presence of IFN-γ, they produced MIP-1α and MCP-1 in a

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**Figure 4.** Bacille Calmette-Guérin (BCG)-mediated expression of MIP-1α and MCP-1 mRNA in pleural mesothelial cells on agarose gels stained with ethidium bromide. Results are representative of 3 independent experiments. A and B, MIP-1α– and MCP-1–specific amplification products, respectively. β-actin was positive control for reverse transcription–polymerase chain reaction. Lane 1, molecular weight (HaeIII) marker. Cells in other lanes: 2, unstimulated; 3, BCG-stimulated; 4, interferon (IFN)-γ-stimulated; 5, BCG + IFN-γ-stimulated.

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**Figure 5.** Southern analysis of bacille Calmette-Guérin (BCG)-mediated chemokine expression in pleural mesothelial cells (representative of 3 observations). Lanes 1, unstimulated; 2, BCG-stimulated; 3, interferon (IFN)-γ-stimulated; 4, BCG + IFN-γ-stimulated. β-actin was positive control for reverse transcription–polymerase chain reaction.
time-dependent manner (figure 3), suggesting that activated pleural endothelial cells contribute MIP-1α and MCP-1 in pleural fluid. The chemokine release was detectable as early as 6 h and continued to 48 h. RT-PCR and Southern analysis of MIP-1α and MCP-1 mRNA expression confirmed these findings (figures 4, 5). When IFN-γ was incubated with BCG, there was an additive effect in pleural mesothelial cell chemokine expression. At the site of M. tuberculosis infection, elicited blood monocytes and surrounding macrophages can become activated and synthesize a number of potent mediators with autocrine and paracrine effector activities [31]. M. tuberculosis stimulates IFN-γ production in human PBMC [32]. IFN-γ was detected in pleural fluid from patients with TB, and M. tuberculosis cell wall components enhanced IFN-γ production in pleural fluid PBMC [33].

Mycobacterial infection elicits a Th1 pattern of cytokine production [34]. Thus, released Th1 cytokines in response to mycobacterial infection may also amplify the production of mesothelial cell chemokine production in the pleural space and ultimately result in mononuclear phagocyte recruitment and granuloma formation. Our results demonstrate that both endogenous and exogenous agonists contribute to chemokine production in pleural mesothelial cells and that Th1 cytokines also play a role in pleural mesothelial cell expression of chemokines and thus in mononuclear cell recruitment in TB pleurisy.

C-C chemokines are recognized as important mediators in a variety of inflammatory states [6]. Cellular sources of MIP-1α include alveolar and peritoneal macrophages, monocytes, T lymphocytes, eosinophils, and neutrophils [35]. The cellular source of MCP-1 includes human PBMC [36]. TB pleural effusions are typically associated with several of these cell types [17], and these recruited cells may contribute to MIP-1α and MCP-1 in pleural fluid. Because the mesothelium anatomically occupies an important position in the pleural space, pleural mesothelial cells are the first cells to respond to invading organisms in this space. Since BCG induces MIP-1α and MCP-1 production in pleural mesothelial cells, the high levels of these chemokines in TB effusions may be secondary to activation of pleural mesothelial cells in response to the mycobacterium in the pleural space.

Our results indicate that in tuberculous pleuritis, mycobacteria induce chemokine release from the pleura, which initiate pleural inflammation and result in recruitment of mononuclear phagocytes to the pleural space. In vitro, human mesothelial cells release MIP-1α and MCP-1 when stimulated with BCG, and these chemokines may be critical in initiating and regulating sequential cellular response to mycobacterial infection in the pleural space.

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References