Cytokine Production at the Site of Disease in Human Tuberculosis

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Clinical and immunologic evidence suggests that tuberculous pleuritis provides a model to understand protective immune mechanisms against Mycobacterium tuberculosis. We therefore evaluated the pattern of cytokine mRNA expression and cytokine production in pleural fluid and blood of patients with tuberculous pleuritis. RNA was extracted from mononuclear cells, reverse transcribed to cDNA, and amplified by polymerase chain reaction (PCR). After normalization for T-cell cDNA, cDNA from pleural fluid cells and peripheral blood mononuclear cells (PBMC) was amplified with cytokine-specific primers. PCR product was quantified by Southern blot. For the Th1 cytokines gamma interferon (IFN-γ) and interleukin-2 (IL-2), PCR product was greater in pleural fluid than in blood, whereas PCR product for the Th2 cytokine IL-4 was decreased in pleural fluid compared with blood. Concentrations of IFN-γ were elevated in pleural fluid compared with serum, but IL-2, IL-4, and IL-5 were not detectable. Mean concentrations of IFN-γ and IL-2 in supernatants of M. tuberculosis-stimulated pleural fluid cells were significantly greater than corresponding concentrations in supernatants of stimulated PBMC. In situ hybridization showed that increased IFN-γ production by pleural fluid cells was associated with a 20- to 60-fold increase in the frequency of antigen-reactive IFN-γ mRNA-expressing cells. Because IL-10 can be produced by T cells and macrophages, pleural fluid cells and PBMC were normalized for β-actin cDNA content and then amplified by PCR with IL-10-specific primers. IL-10 mRNA was greater in pleural fluid cells than in PBMC and was expressed predominantly by macrophages. IL-10 concentrations were elevated in pleural fluid versus serum. These data provide strong evidence for compartmentalization of Th1 cytokines and IL-10 at the site of disease in humans with a resistant immune response to mycobacterial infection.

Tuberculosis, long regarded as a declining disease soon to be relegated to the pages of medical history, is back with a vengeance. The worldwide epidemic of human immunodeficiency virus infection has created a large population with extraordinary susceptibility to Mycobacterium tuberculosis, and tuberculosis case rates have risen dramatically in regions where human immunodeficiency virus infection is widespread, including many parts of the United States (3, 6). Recent outbreaks of multidrug-resistant disease (13) underscore the need for novel approaches to treat and prevent tuberculosis, including the use of immunotherapeutic modalities that enhance normal antimycobacterial defenses. Development of such strategies requires a more detailed understanding of the human immune response to M. tuberculosis, including definition of the T-cell subpopulations and cytokines that mediate resistance to tuberculous infection.

Immunologic resistance to mycobacterial infection is thought to be mediated by cooperative interaction between T lymphocytes and macrophages. This interaction is dependent upon the interplay of cytokines produced by a variety of mononuclear cells, but predominantly by T cells. The specific cytokines that mediate immunologic resistance to mycobacteria in humans remain undefined. Although most evidence favors a dominant role for the Th1 cytokines gamma interferon (IFN-γ) and interleukin-2 (IL-2) (16, 25, 43, 49), other data suggest that a broad spectrum of cytokines may contribute to antimycobacterial immune defenses (5, 12).

Clinical and immunologic evidence suggests that tuberculous pleuritis provides a model to understand immune mechanisms potentially protective against M. tuberculosis. Unlike the case in most other forms of tuberculosis, pleuritis usually resolves without chemotherapy (42), suggesting that local cell-mediated immune responses result in effective clearance of bacilli. CD4+ T cells are selectively concentrated in the pleural space of patients with tuberculous pleuritis, and these T cells proliferate and secrete IFN-γ specifically in response to M. tuberculosis (10, 11, 47). To gain insight into the patterns of cytokine secretion that contribute to antimycobacterial defenses in vivo, we evaluated cytokine production in patients with tuberculous pleuritis.

MATERIALS AND METHODS

Patient population. Pleural fluid and blood were obtained from 12 patients with tuberculous pleuritis seen at the Los Angeles County-University of Southern California Medical Center. All patients had unilateral exudative effusions without clinical evidence of concomitant pulmonary tuberculosis or human immunodeficiency virus infection. All of nine patients tested had positive tuberculin skin tests. The diagnosis was confirmed by either pathologic demonstration of granulomatous pleuritis on closed pleural biopsy (nine cases) or growth of M. tuberculosis from pleural fluid or tissue (six

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cases. All patients responded to antituberculosis therapy. The experiments described were performed on 4 to 7 of these 12 patients.

**Mycobacterial antigens.** *M. tuberculosis* Erdman (Trudeau Mycobacterial Culture Collection strain 107) was grown in a glycerol-alanine-salts medium (9). Cultures were autoclaved at 80°C and 1 h and lyophilized. Cells were washed thoroughly with water and resuspended in RPMI 1640 (GIBCO, Grand Island, N.Y.).

**Isolation of RNA and cDNA synthesis.** Freshly isolated pleural fluid mononuclear cells and peripheral blood mononuclear cells (PBMC) were lysed with 4 M guanidinium isothiocyanate and stored at −20°C prior to preparation of RNA, which was isolated as described previously (7). In some experiments, pleural fluid cells were separated into CD3+ and CD3− populations, using immunomagnetic selection with anti-CD3 (Leu-4; Becton Dickinson Monoclonal Center, Mountain View, Calif.), magnetic beads coated with rabbit anti-mouse immunoglobulin G (Dynal, Mountain View, Calif.), and a magnetic cell separator. In other experiments, CD14+ cells were obtained from pleural fluid cells by positive selection with anti-CD14 (Leu-M3; Becton Dickinson). Positively and negatively selected cells were lysed with guanidinium isothiocyanate in the same manner as were freshly isolated pleural fluid cells.

cDNA was synthesized from RNA by priming approximately 1 µg of total RNA at 42°C for 1 h in a final volume of 20 µL containing 1 µg of an oligo(dT) primer (Pharmacia Fine Chemicals, Piscataway, N.J.), 200 nmol of each deoxyribonucleoside triphosphate (Pharmacia), and mouse mammary leukemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, Md.) at 200 U/µg of RNA.

**Amplification of cDNA by PCR.** Aliquots of cDNA were used as a template for amplification by polymerase chain reaction (PCR), using a Perkin-Elmer thermocycler running a program of 32 to 35 cycles (26 cycles in the cases of β-actin and CD3 δ chain) and oligonucleotide primers specific for β-actin, CD3 δ chain, IFN-γ, IL-2, lymphotoxin, IL-4, IL-5, and IL-10. The primer sequences and reaction conditions have been described previously (7,51). PCR product was subjected to electrophoresis on 2% agarose gels and visualized by staining with ethidium bromide.

**Stimulation of mononuclear cells with *M. tuberculosis*.** Pleural fluid mononuclear cells or PBMC, 2 × 106, were obtained by centrifugation over Percoll gradients (Pharmacia) and suspended in 2 mL wells, each containing 10 µg of *M. tuberculosis* per mL, 10% human serum, and RPMI (GIBCO) with penicillin and streptomycin (GIBCO). Control wells contained no antigen. Cells were maintained at 37°C and 7.5% CO2. After 24 h, mononuclear cells were collected, lysed with guanidinium isothiocyanate, and stored at −20°C prior to preparation of cDNA. Cytocentrifuge preparations were made in some cases by standard techniques (28).

Cell culture supernatants were harvested after 24 h and stored at −70°C for measurement of cytokine concentrations. The cytokine concentration induced by *M. tuberculosis* was calculated as that measured in the appropriate supernatant minus the cytokine concentration in control wells containing media alone.

**Measurement of cytokine concentrations.** Cytokine concentrations were measured in pleural fluid, serum, and cell culture supernatants. Pleural fluid and blood were centri-

<table>
<thead>
<tr>
<th>Patient</th>
<th>Pleural fluid mononuclear cells</th>
<th>PBMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1,626</td>
<td>1,626</td>
</tr>
<tr>
<td>2</td>
<td>2,945</td>
<td>3,029</td>
</tr>
<tr>
<td>3</td>
<td>1,997</td>
<td>1,983</td>
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<td>2,019</td>
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<tr>
<td>5</td>
<td>517</td>
<td>519</td>
</tr>
<tr>
<td>6</td>
<td>430</td>
<td>430</td>
</tr>
</tbody>
</table>

* Aliquots of cDNA from pleural fluid and PBMC were amplified by PCR with primers specific for CD3 δ chain and then hybridized to radiolabeled internal probes. Results for patients 1 through 4 were obtained in one experiment, and those for patients 5 and 6 were obtained in a second experiment in which the radioactivity of the probe was less intense.
were subjected (0) PBMC the probe, mRNA). and the mRNA) above. at hybridized from prior with the hybridized IFN-γ, as described previously (4), assay of the total number of positive cells were counted. They contained greater expression of the mRNA, as measured by enzyme-linked immunosorbent assay (ELISA) (4, 17).

In situ hybridization. Cytocentrifuge preparations of cells cultured with mycobacterial preparations or media alone were subjected to in situ hybridization to detect mRNA for IFN-γ, as described previously (8, 14). Each sample was hybridized with the sense probe (homologous to cellular mRNA) and the antisense probe (complementary to cellular mRNA). mRNA for IFN-γ hybridized with the antisense probe, while the sense probe was unhybridizable and served as a negative control. In each experiment, samples known from prior studies to be positive or negative for IFN-γ mRNA expression were used as controls. Cells were considered positive for expression of cytokine mRNA when they contained greater than twice the number of grains of the most positive cell on the slide of the same preparation to which the sense probe had been added. The number of positive cells on the slide was counted and expressed as a percentage of the total number of cells present on the slide.

Statistical analysis. Results obtained for pleural fluid mononuclear cells and PBMC were compared by a paired t test or by the Wilcoxon rank sum test when the data were not normally distributed.

RESULTS

Cytokine mRNA expression by pleural fluid mononuclear cells. To investigate the pattern of cytokines produced at the site of tuberculous infection, we compared cytokine mRNA expression in freshly isolated pleural fluid mononuclear cells and PBMC of patients with tuberculous pleuritis. Because the cytokines we evaluated were likely to be produced by T cells, we used equivalent amounts of T-cell cDNA from pleural fluid and blood samples of each patient. cDNA from both sources were serially diluted, and CD3 δ-chain cDNA was amplified by PCR and then hybridized to radiolabeled probe. Results of normalization for samples from six patients are shown in Table 1. PCR amplification and hybridization of serial dilutions of sample cDNA revealed a log-linear relationship between the amount of substrate cDNA and radioactivity incorporation (data not shown).

After normalization for T-cell cDNA, cDNA from pleural fluid cells and PBMC was amplified with primers specific for

FIG. 1. Expression of mRNA for the Th1 cytokines IFN-γ (A), IL-2 (B), and lymphotoxin (C). cDNA from pleural fluid cells (■) and PBMC (■) from six patients with tuberculous pleuritis were normalized for T-cell cDNA, amplified by PCR with cytokine-specific primers, and hybridized to radiolabeled probes. Radioactivity incorporation is expressed as counts per minute.

FIG. 2. Expression of mRNA for the Th2 cytokines IL-4 (A), IL-5 (B), and IL-10 (C). cDNA from pleural fluid cells (■) and PBMC (■) from six patients with tuberculous pleuritis were normalized for T-cell cDNA, amplified by PCR with cytokine-specific primers, and hybridized to radiolabeled probes. Radioactivity incorporation is expressed as counts per minute.
TABLE 2. Radioactivity incorporation by PCR product after amplification of cytokine cDNA

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Radioactivity incorporation (mean cpm)*</th>
<th>Pleural fluid mononuclear cells</th>
<th>PBMC</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>1,046 (251)</td>
<td>457 (68)</td>
<td></td>
<td>0.06</td>
</tr>
<tr>
<td>IL-2</td>
<td>3,704 (549)</td>
<td>2,138 (522)</td>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td>Lymphotoxin</td>
<td>5,496 (733)</td>
<td>5,303 (753)</td>
<td></td>
<td>0.74</td>
</tr>
<tr>
<td>IL-4</td>
<td>811 (298)</td>
<td>2,126 (318)</td>
<td></td>
<td>0.03</td>
</tr>
<tr>
<td>IL-5</td>
<td>67 (7)</td>
<td>67 (5)</td>
<td></td>
<td>0.98</td>
</tr>
<tr>
<td>IL-10</td>
<td>513 (138)</td>
<td>731 (280)</td>
<td></td>
<td>0.47</td>
</tr>
</tbody>
</table>

* Value in parentheses is standard error.

the cytokines IFN-γ, IL-2, lymphotoxin, IL-4, IL-5, and IL-10. Hybridization of PCR product to radiolabeled probes revealed significant differences between cytokine mRNA in pleural fluid mononuclear cells and in PBMC. For the cytokines characteristically produced by Th1 cells, radioactivity incorporation representative of mRNA for IFN-γ and IL-2 was greater in pleural fluid cells than in PBMC (Fig. 1A and B), whereas the amounts of mRNA for lymphotoxin were similar in pleural fluid cells and PBMC (Fig. 1C). Only patient 6 showed no increase expression of mRNA for IFN-γ or IL-2 mRNA in pleural fluid cells. This patient was not clinically distinguishable from the other five patients.

For cytokines characteristically produced by Th2 cells, radioactivity incorporation representative of mRNA for IL-4 was decreased in pleural fluid cells compared with PBMC (Fig. 2A). mRNA for IL-5 was barely detectable (Fig. 2B), and expression of IL-10 mRNA was variable in pleural fluid cells and PBMC (Fig. 2C). Mean values for radioactivity incorporation representative of mRNA for cytokines in pleural fluid cells and PBMC are shown in Table 2. All PCR amplification and hybridization experiments for a given cytokine were performed at the same time.

PCR amplification of cytokine cDNA in plasmids. To confirm that the differences observed in radioactivity incorporation by PCR product represented quantitative differences in substrate cDNA, we compared results of PCR amplification and hybridization of patient samples with parallel results obtained by amplification of plasmids containing known copy numbers of cDNA for IFN-γ, IL-2, and IL-10. Hybridization of PCR product for the samples and plasmids was performed at the same time. For plasmids containing IFN-γ, the relationship between copy number of IFN-γ cDNA and radioactivity incorporation was log-linear for amplification of 10 to 10³ copies (Fig. 3). The values obtained for radioactivity incorporation by IFN-γ cDNA PCR product from the samples ranged from 230 to 2,259 cpm, which was within the linear range of PCR amplification. These results indicate that the greater radioactivity incorporation representative of IFN-γ mRNA in pleural fluid versus blood reflected differences in substrate IFN-γ mRNA in these compartments. Similar experiments with plasmids containing IL-2 and IL-10 cDNA indicated that radioactivity incorporation by PCR product from the samples was within the linear range of PCR amplification (data not shown).

Cytokine concentrations in pleural fluid and serum. To determine whether the pattern of selective cytokine mRNA concentration in pleural fluid cells reflected cytokine production at the site of disease, we measured concentrations of IFN-γ, IL-2, IL-4, IL-5, and IL-10 in pleural fluid and serum of seven patients. In confirmation of previously published results, IFN-γ concentrations were markedly elevated in pleural fluid (mean, 1,178 pg/ml), but not detectable in serum (P < 0.01; Figure 4A). Concentrations of IL-10 were also elevated in pleural fluid compared with serum (mean, 155 versus 16 pg/m; P < 0.05; Fig. 4B). IL-2 was barely detectable (22 pg/ml) in serum of two patients but not in pleural fluid. IL-5 was detectable in one pleural fluid sample but not in serum, and IL-4 was not detectable in either pleural fluid or serum.

**FIG. 3.** PCR amplification and hybridization of plasmid containing IFN-γ cDNA. Plasmid containing known copy numbers of IFN-γ cDNA was amplified by PCR with primers specific for IFN-γ and hybridized to radiolabeled probe. Radioactivity incorporation is shown on the y axis as counts per minute.
with fluid, but produced by Because measured by fluid (U) Radioactivity incorporation we blood cells. pleural a and blood is stimulated in cytokine selective amplification, PCR to determine the able in (mean, Cytokine IL-2, IL-4, mRNA was higher in pleural fluid than in serum. Mean concentrations of IFN-γ produced by pleural fluid cells were significantly higher than those produced by PBMC (mean, 9,180 versus 1,537 pg/ml; P = 0.002) (Fig. 6A). Similar findings were noted for concentrations of IL-2 (mean, 688 versus 16 pg/ml; P = 0.001) (Fig. 6B). In five of six cases, PBMC produced more IL-10 than did pleural fluid cells (Fig. 6C), but this difference did not achieve statistical significance (P = 0.07). IL-4 and IL-5 were not detectable in any of the supernatants tested.

**Frequency of cells that produce IFN-γ.** To determine whether the higher concentration of IFN-γ in stimulated pleural fluid cells was due to a higher frequency of antigen-reactive cytokine-producing cells, in situ hybridization was performed on cytocentrifuge preparations of pleural fluid cells and PBMC cocultured with *M. tuberculosis* Erdman. The frequency of cytokine mRNA-expressing cells was 20- to 60-fold higher in pleural fluid than in blood (P = 0.0007) (Fig. 7).

**DISCUSSION**

The data presented in this report provide strong evidence for compartmentalization of Th1 cytokines and IL-10 at the site of disease in humans with a resistant immune response to *M. tuberculosis*. Expression of mRNA for IFN-γ and IL-2 was greater in pleural fluid than in blood of patients with tuberculous pleuritis, and concentrations of IFN-γ were 15-fold higher in pleural fluid than in serum. Pleural fluid lymphocytes stimulated with *M. tuberculosis* produced substantially higher concentrations of IFN-γ and IL-2 than did peripheral blood lymphocytes, and the frequency of cells expressing IFN-γ mRNA was 20- to 60-fold higher in pleural fluid than in blood. IL-10 produced by macrophages was also selectively concentrated in the pleural space and may serve to downregulate the local immune response.

Immunologic resistance and susceptibility to intracellular pathogens in mice are thought to be mediated by CD4+ T cells with specific patterns of cytokine secretion. Th1 cells that produce IFN-γ confer resistance to infection with *Leishmania* sp. (26, 43), *Candida* sp. (41), and *Schistosoma mansoni* (20, 39, 40), whereas Th2 cells that produce IL-4 may mediate protective immunity against *Trichinella spiralis* and *Trichuris muris* (19, 24). Immunologic resistance to mycobacterial infection is probably mediated by T cells that
product IFN-γ and IL-2, but not IL-4. Lymphocytes from mice vaccinated with mycobacteria (48, 50) and those showing resistance to rechallenge with M. bovis (29) produce high concentrations of IFN-γ and IL-2 but low levels of IL-4. In contrast, lymphocytes from mice without resistance to rechallenge with mycobacteria produce high levels of IL-4 and low levels of IFN-γ and IL-2 (29).

Published data on cytokine production in the human immune response to mycobacterial infection are less definitive than those in murine infection. Some CD4+ M. leprae-reactive and M. tuberculosis-reactive human T-cell clones are Th1-like, producing high concentrations of IFN-γ but low concentrations of IL-4 and IL-5 (16, 25, 43). In contrast, we and others have found that most M. tuberculosis-reactive T-cell clones from healthy tuberculin reactors secrete a less restricted pattern of cytokines that includes IFN-γ, IL-2, IL-4, IL-5, and IL-10 (5, 12). The pattern of cytokines secreted by human T-cell clones depends in part on differences in culture conditions. For example, addition of IL-4 favors development of Th0 and Th2 clones, whereas absence of exogenous IL-4 favors development of Th1 clones (1, 33).

Because the cytokine patterns observed for human T-cell clones cultured in vitro may not reflect events in vivo, it is critical to evaluate the cytokine response at the site of mycobacterial disease. Studies in leprosy indicate that mRNA for IFN-γ and IL-2 is highest in tuberculous leprosy patients with a relatively resistant immune response, whereas expression of mRNA for IL-4, IL-5, and IL-10 predominates in lepromatous leprosy patients with ineffective immunity (51). It remains uncertain from these studies whether the predominance of Th1 cytokines in tissue lesions of tuberculous leprosy patients reflects dominance of Th1 cells in blood of these patients or results from compartmentalization of Th1 cells in tissue lesions. Our current findings suggest that selective concentration of Th1 cells predominates at the site of disease in patients with a resistant immune response to mycobacterial infection.

Enhanced production of IFN-γ and IL-2 in the pleural space is likely to contribute to immune defenses against M. tuberculosis. IFN-γ enhances antimycobacterial activity of murine macrophages (22), probably through increased production of reactive nitrogen metabolites (23), and IFN-γ production is a functional marker of murine T cells that confer adoptive immunity against M. bovis and M. tuberculosis (30, 37). Although the capacity of IFN-γ to enhance antimycobacterial activity by human macrophages in vitro remains controversial (18, 46), administration of this cytokine reduced the bacillary burden in patients with lepromatous leprosy (36). IL-2 may also facilitate mycobacterial elimination through its activity as a potent T-cell growth and activation factor that further increases the local concentration of macrophage-activating factors such as IFN-γ.

Because IL-4 deactivates macrophages (2, 27, 31, 38) and blocks T-cell proliferation by downregulation of IL-2 receptor expression (34), the relative absence of IL-4 in the pleural space may enhance host immune defenses. Exclusion of IL-4 may also explain the relatively low concentration of immunoglobulin in pleural fluid despite the hyperglobulinemia in serum of tuberculosis patients (32).

In contrast to findings for IL-4, IL-10 was detectable in pleural fluid and IL-10 mRNA was expressed predominantly by macrophages, which may produce IL-10 in response to high local concentrations of mycobacterial antigens such as lipoarabinomannan (7). IL-10 may inhibit synthesis of cytokines such as tumor necrosis factor alpha by macrophages (17) and IFN-γ by T cells (21, 35), which may otherwise result in a marked inflammatory response and tissue damage.
Enhanced release of IL-10 by PBMC versus pleural fluid cells stimulated in vitro with Mycobacterium tuberculosis may reflect the relatively greater percentage of macrophages in PBMC compared with pleural fluid of patients with tuberculous pleuritis (4a).

The human immune response to infectious agents is often a double-edged sword that contributes to elimination of pathogens but may also be harmful to host cells. In pleural tuberculosis, the inflammatory response effectively eliminates mycobacteria but produces large pleural effusions, fever, chest pain, and shortness of breath. It is intriguing to speculate that IFN-γ, a prominent cytokine in the pleural space, contributes to cell-mediated immunity through macrophage activation, as well as to immunopathology through enhancement of production of tumor necrosis factor, which in turn causes fever and tissue necrosis. In support of this hypothesis, concentrations of tumor necrosis factor are elevated in pleural fluid of patients with tuberculous pleuritis (8), and administration of IFN-γ to lepromatous leprosy patients enhances mycobacterium-induced tumor necrosis factor production, resulting in fever, malaise, and weight loss (44). Further studies of the local immune response in human tuberculosis will enhance our understanding of the complex host-pathogen interaction and facilitate development of immunomodulatory therapy to reduce morbidity and mortality from this disease.

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