Tumor Necrosis Factor Production in Patients with Leprosy

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The spectrum of host responses to Mycobacterium leprae provides a model for investigating the role of cytokines in the pathogenesis of mycobacterial disease. Of particular interest is tumor necrosis factor (TNF), a cytokine which may have both antimycobacterial and immunopathologic effects. To evaluate the potential role of TNF in leprosy, we measured TNF production in response to M. leprae and its defined constituents by peripheral blood mononuclear cells from patients across the spectrum of disease. The levels of TNF induced through the stimulation of cells with M. leprae or its dominant "lipopolysaccharide," lipoarabinomannan, were higher in patients with the tuberculoid form of the disease than in those with the lepromatous form. In patients with erythema nodosum lepromatosis (ENL), a reactional state of lepromatous leprosy, the levels of TNF release by peripheral blood mononuclear cells were higher than in any other form of the disease. Treatment of ENL patients with thalidomide reduced TNF secretion by more than 90%. The mycolylarabinogalactan-peptidoglycan complex of Mycobacterium species, the protein-peptidoglycan complex, and muramyl dipeptide all elicited significant TNF release. Therefore, TNF release appears to be triggered by at least two major cell wall structural constituents of M. leprae, lipoarabinomannan and segments of the cell wall skeleton. The prominent TNF release in patients with the paucibacillary tuberculoid form of the disease compared with that in patients with the multibacillary lepromatous form suggests that this cytokine contributes to a resistant immune response to mycobacterial infection. However, the marked TNF release in patients with ENL indicates that TNF may also mediate immunopathologic effects, such as fever and tissue damage.

The spectrum of host responses to Mycobacterium leprae provides an opportunity to evaluate and correlate immune responses to a foreign pathogen with clinical manifestations of disease. At one pole of the spectrum, patients with tuberculoid leprosy have skin lesions, in which bacilli can rarely be identified, whereas at the opposite pole, patients with lepromatous leprosy have diffuse infiltration of skin and nerves with bacillus-laden macrophages (29, 30). Patients with tuberculoid leprosy have a strong cell-mediated immune response to M. leprae, manifested by positive skin tests (Mitsuda reaction) and marked lymphocyte proliferative responses to M. leprae in vitro. In contrast, patients with lepromatous leprosy have a depressed cell-mediated immune response to M. leprae, characterized by negative skin tests and diminished or absent lymphocyte proliferation.

Imposed upon this spectrum are the so-called "reactional states": reversal reaction and erythema nodosum lepromatosis (ENL). The reversal reaction appears to be a naturally occurring delayed-type hypersensitivity response to M. lepraef. Clinically, it is characterized by "upgrading" of the clinical picture towards the tuberculoid pole, including a reduction in the bacillary load. Immunologically, it is characterized by the development of strong skin test reactivity as well as lymphocyte responsiveness (5, 9, 17, 27, 42). The pathogenesis of ENL is thought to be related to the deposition of immune complexes in the lesions (8, 14, 43). Although there is some evidence indicating cell-mediated immunity (26, 37), significant clearance of bacilli from lesions does not occur.

Of the cytokines which regulate the immune response to infection, tumor necrosis factor (TNF) is of particular interest in the pathogenesis of mycobacterial disease. On the one hand, TNF may participate in protective immunity by mediating granuloma formation (20) and inhibiting mycobacterial growth in vitro (6). On the other hand, the effects of TNF may result in immunopathology, such as nerve damage (34) and tissue necrosis (31). In the present study, we evaluated the potential role of TNF in leprosy by measuring its production in response to M. leprae by peripheral blood mononuclear cells (PBMC) from patients across the spectrum of disease.

MATERIALS AND METHODS

Patients. Patients with leprosy were classified on the basis of the clinicopathologic criteria of Ridley and Jopling (28-30). Blood was obtained from the following patient groups: (i) tuberculoid leprosy; (ii) lepromatous leprosy without a reaction; (iii) lepromatous leprosy with active ENL; (iv) lepromatous leprosy with inactive ENL (after treatment with thalidomide); and (v) reversal reaction. All patients had received less than 5 years of chemotherapy.

Mycobacterial preparations. Lipoarabinomannan (LAM) was isolated and purified from M. leprae as described for the M. tuberculosis product (11). The purified LAM was redissolved in sterile pyrogen-free water, passed through a 2-ml DetoxiGel column (Pierce Chemical, Rockford, Ill.), and filtered through a 0.2-µm-pore-size sterile filter, and the filtrate was collected in a sterile pyrogen-free vial and dried. This procedure was necessary to remove any residual contaminating lipopolysaccharide (LPS).

The cell wall components, protein-peptidoglycan complex

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(mAGP) (19) and mycolylarabinogalactan-peptidoglycan (mAGP) complex (12, 21), were prepared as described earlier. To remove any residual proteins from the mAGP complex (12, 21), we further treated it with proteases as follows. mAGP isolated from M. leprae (10 mg) was suspended in 1.5 ml of pronase solution, and the suspension was incubated at 37°C overnight (pronase E; type XIV; Sigma, St. Louis, Mo.; 2 mg/ml in 2% sodium dodecyl sulfate [SDS] in phosphate-buffered saline [PBS]; pH 7.8). The cell wall suspension was centrifuged for 20 min and washed extensively with 2% SDS buffer. The pronase-treated cell wall was treated further with proteinase K solution (type XXVIII; Sigma) as described above. The protein-free cell wall was then boiled in SDS-PBS buffer for 10 min. The SDS was removed by further washing with PBS, distilled water, 80% acetone, and sterile pyrogen-free water. Sonicated M. leprae, unfractionated, was prepared as previously described (19).

Every effort was made to exclude endotoxin from all preparations, including the use of sterile techniques, pyrogen-free reagents, and DetoxiGel. Previous experiments with the Limulus amebocyte assay had shown that a parallel preparation of LAM from M. tuberculosis contained 0.0016 to 0.0034% endotoxin (35). Since the mAGP complex and the PPC are both insoluble, they could not be passed through the DetoxiGel to remove any possible residual endotoxin. Muramyl dipeptide (N-acetylmuramyl-L-alanyl-D-isoglutamin) was purchased from Sigma.

Stimulation of cells with mycobacterial preparations. PBMC were isolated by centrifugation over Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, N.J.). Mononuclear cells (10⁶) were suspended in 200-μl flat-bottomed wells (Falcon, Oxnard, Calif.) containing RPMI 1640 with gentamicin (Sigma), 10% heat-inactivated sterile filtered human AB serum, and various concentrations of mycobacterial preparations.

Cell culture supernatants were harvested after 24 h for the measurement of TNF concentrations. The TNF concentration induced by a given mycobacterial preparation was calculated as that measured in the appropriate supernatant minus the cytokine concentration in control wells lacking the mycobacterial stimulus. All supernatants were stored at −70°C prior to the determination of TNF levels.

Measurement of cytokine concentrations. Concentrations of TNF in cell supernatants were measured by a radioimmunoassay (Centocor, Malvern, Pa.), an enzyme-linked immunosorbent assay (ELISA) (Genzyme, Boston, Mass.), or a bioassay with WEHI 164 clone 13 cells (15). We previously reported that there is excellent agreement among results obtained by these three assays (3). In the present study, the mean TNF concentrations measured in each patient group by the bioassay versus the immunoassays were not significantly different. The trends were consistent across different patient groups for each assay. Therefore, results are reported as the mean values for all patients studied.

The Centocor radioimmunoassay for TNF is a solid-phase radioimmunoassay which detects as little as 10 pg of TNF per ml. Samples were added to polystyrene beads coated with murine monoclonal antibodies specific for human TNF. The amount of TNF adherent to the beads was determined by measurement of the degree of radioactivity incorporated upon the addition of 125I-labeled anti-TNF monoclonal antibodies. The cytokine concentrations in the samples were determined by comparison with radioactivity incorporated by specimens containing known TNF concentrations.

The Genzyme TNF ELISA is based on the multiple-antibody sandwich principle and can detect 12 pg of TNF per ml. Samples were incubated in microtiter plate wells coated with a mouse monoclonal antibody specific for human TNF. A rabbit anti-human TNF polyclonal antibody was added, and then biotin-conjugated goat anti-rabbit immunoglobulin G was added. Streptavidin-conjugated peroxidase, which binds to biotin, was added. Peroxide substrate and OPD chromogen were added to yield increased A₄₉₂ values, indicating the presence of TNF.

The bioassay for detecting TNF was based on target cell line WEHI 164 clone 13, which is extremely sensitive to the cytotoxic effects of TNF. WEHI 164 clone 13 cells were a kind gift of Genentech, Inc., South San Francisco, Calif. The assay was performed as previously described (15). In brief, samples were incubated with the WEHI cells for 22 h. The percentage of dead cells, which provides a measure of the concentration of TNF in the sample, was calculated by measuring the optical density of the culture after the addition of MTT tetrazolium as described by Mosmann (24). The concentrations of TNF were determined by comparison with a standard curve generated with specimens containing known TNF concentrations.

Statistical analysis. For data that were normally distributed, the t test was used to test for statistical significance. For data that were not normally distributed, the Wilcoxon rank sum test was used.

RESULTS

Production of TNF across the spectrum of leprosy. To investigate the potential role of TNF in mediating the clinical manifestations of leprosy, we measured the capacity of PBMC from patients across the spectrum of disease to produce TNF in response to M. leprae. TNF release was highest in patients with ENL, intermediate in those with tuberculoid leprosy, and lowest in patients with lepromatous leprosy. TNF release was significantly higher in patients with tuberculoid leprosy than in those with lepromatous leprosy (P = 0.01) and in those with active ENL than in those with inactive ENL (P = 0.01). The difference between TNF release in patients with ENL and those with tuberculoid leprosy was not statistically significant.
Because TNF release was markedly elevated in patients with active ENL, we evaluated the dynamics of TNF release by PBMC from these patients. TNF release fell by more than 90% (from $4,756 \pm 1,853$ pg/ml to $456 \pm 85$ pg/ml; $P < 0.01$) as the episode of ENL subsided during treatment with thalidomide at 100 to 200 mg/day for 5 to 46 days (Fig. 2).

**TNF release by M. leprae constituents.** The availability of several constituents of *M. leprae* in highly purified forms has enabled us to identify which components are potent inducers of TNF. We therefore evaluated the capacity of purified LAM derived from *M. leprae* to elicit TNF production across the spectrum of leprosy. TNF release paralleled the response to sonicated *M. leprae*, being highest in patients with active ENL and next highest in individuals with tuberculoid leprosy (Fig. 3). The levels of TNF release induced by LAM were similar to those induced by sonicated *M. leprae* but did not achieve statistical significance.

Recent research on the elucidation of the fundamental structure of the cell wall core or skeleton of *Mycobacterium* species has resulted in the availability of this core, or parts of it, in quantities such that their biological properties can be examined. The cell wall core or skeleton may be given the chemical name mAGP, since it consists of a covalently linked complex of mycolic acids, arabinogalactan, and peptidoglycan (12, 21). In addition, several subfractions of the cell wall core, notably the PPC, have been generated. Because some of these fractions are potent stimuli for T lymphocyte proliferation (4, 22) and because the arabinan components of both mAGP and LAM are structurally very similar (11), we wished to evaluate their capacity to induce TNF release. The mAGP complex and the PPC were as potent as sonicated *M. leprae* in eliciting TNF production (Fig. 4). In addition, the muramyl dipeptide of mycobacterial cell walls induced TNF secretion, although to a lesser extent than the other constituents of *M. leprae*. These results indicate that components of the core of mycobacterial cell walls are also capable of inducing TNF release.

**DISCUSSION**

The immunologic role of TNF in leprosy is not clearly defined, although TNF is thought to be a major factor in the pathogenesis of mycobacterial infection. TNF may augment the immune response towards the elimination of the pathogen and/or mediate the pathologic manifestations of the disease. In the present study, we correlated TNF release by *M. leprae* with the clinical manifestations of leprosy. We found evidence supporting a role for TNF in the resistant immune response to infection, since higher TNF release was evident in patients resistant to *M. leprae* (patients with the paucibacillary tuberculoid form of the disease) than in those susceptible to *M. leprae* (patients with the multibacillary lepromatous form).

Several previous studies evaluated TNF production in patients with leprosy, specifically the tuberculoid and lepromatous forms, to gain insight into the role of this cytokine in the pathogenesis of disease. Serum TNF levels, as measured by a bioassay, were reported to be higher in patients with the tuberculoid form than in those with the lepromatous form (36). However, serum TNF concentrations, as measured by an ELISA, were higher in individuals with the lepromatous form (25). This discrepancy has led to speculation that TNF inhibitors in the serum of patients with the lepromatous form interfere with the bioassay for TNF, decreasing the measured amount and perhaps the biologic effect in these individuals (33). Inhibitors of TNF bioactivity have been described in the serum of patients with tuberculosis and sarcoidosis (16).

Investigations of TNF release by PBMC and in the lesions of patients have clearly demonstrated higher cytokine pro-

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**FIG. 2.** TNF release induced by sonicated *M. leprae* in patients with ENL. PBMC were obtained from six patients with active ENL (■) and from the same patients 5 to 46 days later, when the episode of ENL had subsided (□).

**FIG. 3.** TNF release induced by *M. leprae* LAM. PBMC were obtained from patients with lepromatous leprosy (LL; $n = 11$), reversal reactions (RR; $n = 6$), inactive ENL [ENL(in); $n = 14$], active ENL [ENL(ac); $n = 12$], and borderline tuberculoid leprosy (BT; $n = 20$).

**FIG. 4.** TNF release induced by *M. leprae* cell wall components. PBMC were obtained from five patients in whom the TNF level in response to sonicated *M. leprae* was higher than 400 pg/ml. Sonicated *M. leprae* (MLS), the mAGP complex, the PPC, and muramyl dipeptide (MDP) were studied.
duction in patients with the tuberculoid form than in those with the lepromatous form. Stimulation of PBMC with LPS (36) and with *M. leprae* (present study) induced significantly higher release of TNF in the former patients than in the latter. Immunohistochemical studies of skin biopsies with anti-TNF monoclonal antibodies revealed higher numbers of TNF-containing cells in tuberculoid than in lepromatous leprosy tissue lesions (2). Similarly, higher concentrations of TNF mRNA in tissue were found in tuberculoid leprosy lesions, as detected by in situ hybridization (38) and by polymerase chain reaction amplification (44). These data suggest that the more marked *M. leprae*-induced production of TNF by mononuclear cells in patients with tuberculoid leprosy results in elevated levels of TNF in tissue lesions. However, because the total number of skin lesions and the total number of mononuclear cells exposed to *M. leprae* are far higher in lepromatous leprosy patients, the total TNF secretary capacity of lepromatous leprosy patients may result in higher serum TNF levels, although the biologic effect may be diminished by circulating inhibitors. The sum of these findings could be interpreted to indicate that the net biologic effect of TNF correlates with resistance to mycobacterial disease.

A role for TNF in protective immunity is further suggested by its capacity to inhibit mycobacterial growth in murine and human macrophages (6). TNF is necessary for granuloma formation and mycobacterial elimination in mice infected with *M. bovis* BCG (20). Furthermore, TNF mRNA is abundant in tissues of patients with resistant immune responses to other mycobacterial infections, such as tuberculous pleuritis (3). Monocytes from patients with chronic refractory tuberculosis released significantly lower amounts of TNF than did those from patients with newly diagnosed tuberculosis (39). TNF may contribute to protective immunity against mycobacteria and other intracellular organisms through the production of reactive nitrogen intermediates. TNF enhances the production of nitric oxide by murine macrophages (13). Nitric oxide has been shown to inhibit the growth of and/or to kill leishmaniae (18), *M. leprae* (1), and *M. tuberculosis* (10).

In addition to its potential contribution to antimycobacterial defense mechanisms, TNF may mediate the pathologic manifestations of mycobacterial disease. TNF administered to experimental animals results in fever, weight loss, and cachexia (7) and may cause these characteristic clinical features of mycobacterial disease. The administration of TNF to animals primed with *M. tuberculosis* results in necrotic reactions (31), raising the possibility that this cytokine contributes to the caseation characteristic of tuberculosis. TNF production within leprosy lesions may result in nerve damage and deformities (34, 40). A surprising and striking finding of the present study was the extremely high levels of TNF produced by sonicated *M. leprae* in PBMC from patients with active ENL. This TNF release decreased dramatically with the concomitant clinical response to thalidomide. These findings extend the recent observation that the addition of thalidomide to mononuclear cells in vitro suppresses TNF production induced by LPS and mycobacterial antigens (32). Serum TNF levels are also markedly higher in patients with ENL than in those with tuberculoid or lepromatous leprosy (33). Although relatively low levels of TNF mRNA are present in the skin lesions of these patients (38), careful longitudinal studies of patients may show augmentation of local TNF production. Because ENL reactions are characterized by fever, weight loss, and tissue injury but not the elimination of bacilli, elevated TNF levels in these patients support the hypothesis that TNF production may mediate the immunopathologic manifestations of the reactions.

To define the constituents of *M. leprae* which elicit TNF secretion, we examined two major entities of the cell wall of *M. leprae*. We used LAM, which is now recognized as the major ‘‘LPS’’ of mycobacteria. LAM from *M. tuberculosis* has been shown to induce TNF release by human and murine macrophages (25). In addition, we used the mAGP complex (mAGP is the chemical name for the entire insoluble substructure of cell walls) and the PPC (a subsegment of mAGP with some associated proteins). The combined results indicate that several entities within the cell walls of mycobacteria, including LAM, the mAGP complex, the PPC, and muramyl dipeptide, are capable of eliciting TNF. LAM is a well-defined molecule, marked by a phosphatidylinositol at one end (the reducing end) and linear and branched arabinans at the opposite (nonreducing) end (Fig. 5A). Recent studies have clarified the structure of the mAGP complex (Fig. 5B) (12, 21). The fact that both share similar arabinan motifs would seem to implicate arabinan in TNF evocation. However, we have shown (2a) that an intact phosphatidyl unit in LAM is required for TNF release. Likewise, the present results indicate that much of the potency of the mAGP complex lies in the peptidoglycan unit. Alternatively, since mycobacterial proteins are inducers of TNF (41), the potency of the PPC may be related to the associated proteins. Thus, it appears that the ability of mycobacterial cell walls to evoke TNF is multifaceted.  

In defining the role of TNF in the immunopathogenesis of leprosy, it may be overly simplistic to envision TNF as having exclusively protective or pathologic effects. Instead,
the pathologic effects of TNF may be the consequence of overexuberant immunoprotective mechanisms. Identification of the constituent molecules of mycobacteria which stimulate TNF release, as well as identification and characterization of TNF inhibitors in leprosy patients, will allow a more complete definition of the role of TNF in mycobacterial infection.

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