Serum Tumor Necrosis Factor and Interleukin 1 in Leprosy and during Lepra Reactions

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INTRODUCTION

Leprosy is a chronic inflammatory disease caused by Mycobacterium leprae. The disease spectrum at one pole has tuberculoid leprosy (TT) having pronounced cell-mediated immunity (CMI) to M. leprae, with skin lesions containing well-organized granulomas of activated macrophages and lymphocytes. At the other end, lepromatous leprosy (LL) patients exhibit deficient CMI with diffuse infiltration of skin and nerves, and undeveloped granulomas containing bacilli-laden macrophages (1). Borderline groups consisting of borderline lepromatous (BL), borderline borderline (BB), and borderline tuberculoid (BT) present dimorphous characters with varying degrees of both polar forms. Pathology is most conspicuous in the peripheral nerves and skin (2).

Tissue damage manifesting as deformities progress with the severity of the disease. The mechanism(s) underlying host tissue damage in leprosy is not well understood. Since M. leprae itself is nontoxic, the tissue damage is thought to be mediated by two types of immunological reactions (lepra reactions): reversal reaction (RR/type 1 reaction) and erythema nodosum leprosum (ENL/type 2 reaction) which frequently occur in leprosy (3). ENL is characterized by the deposition of extravascular immune complexes, high antibody levels, a diminished antigen load (4) and enhanced T helper cell activity (5). The RR usually observed in borderline groups signifies the transient rejuvenation of a competent CMI following chemotherapy (6).

Tumor necrosis factor-α (TNF), the secretory product of monocytes/macrophages (7), large granular lymphocytes (8), and activated peripheral blood lymphocytes (9), is suspected to be one of the inflammatory and pathological mediators in septic shock (10, 11), cerebral malaria (12), and several infectious conditions (13). It has been shown in a murine model that TNF plays a major role in the development of BCG-induced granulomas (14). A recent study has shown elevated TNF levels in parasitic infections ascribing the role of inflammatory cytokines in host defence (15).

This study was initiated to find out the relationship between serum TNF and IL-1 levels in the clinical forms of leprosy and lepra reactions.

MATERIALS AND METHODS

Subjects. Patients attending leprosy clinics in the two urban leprosy centres in New Delhi were the subjects of this study. Most of these patients were newly detected active leprosy patients without any previous history of treatment. Patients were examined clini-
cally and their bacteriological, histopathological, and immunological statuses were determined by slit-skin smear, skin biopsy, and lepromin-A testing, respectively. The patients were classified according to the Ridley–Jopling scale (17) and were subsequently treated with a standard multidrug regimen (rifampicin, clofazimine, and dapsone for lepromatous patients for a minimum of 24 months and rifampicin and dapsone for tuberculoid patients for 6 months). The patients were also routinely screened to exclude other chronic and general illness such as tuberculosis, diabetes, hypertension, allergy, etc.

Ninety patients consisting of 69 males and 21 females in the age range of 7–65 years (31 ± 1.3) consisting of 30 LL, 12 BL, 8 BB, 14 BT-TT, and 26 (14 type 1 and 12 type 2) fresh reaction patients were inducted into the study. Three patients (two in type 1 and one in type 2 reaction) belonged to earlier groups who subsequently developed the reaction following the commencement of therapy. Written consent of each patient was obtained before induction into the study.

Sera collection. Peripheral venous blood was collected and allowed to clot by incubating at 37°C for 1 hr. The samples were kept overnight at 4°C, spun at 2000 rpm for 20 min, sera separated, aliquoted into 0.5-ml volumes, and stored at −70°C.

Assays for TNF and IL-1. Concentrations of TNF and IL-1 were determined by an immunoradiometric assay (IRMA) system with a sensitivity level of 15 and 50 pg/ml, respectively (Medgenix, Belgium). Polypropylene tubes were coated with a combination of monoclonal antibodies (an oligoclonal system) which recognize distinct epitopes of the relevant human cytokine. These tubes were incubated overnight at room temperature with a mixture of the 125I-labeled anti-cytokine antibody and the test samples. After decantation, the bound fraction was counted in a gamma counter, and the level of TNF or IL-1 was expressed in picograms per milliliter in relation to a standard binding curve of the relevant recombinant human cytokine.

STATISTICAL METHODS

The skewed distribution of serum concentrations of TNF necessitated the use of nonparametric Mann–Whitney U–Wilcoxon rank sum W test to compare means. All tests, including the χ² test and the correlation coefficient, were performed with the use of statistical package SPSS/PC +.

RESULTS

Figures 1 and 2 present scattered graphs of TNF and IL-1 levels in leprosy patients belonging to LL, BL, BB, BT-TT, and normal healthy controls. LL patients were histopathologically proven LL, lepromin negative sub-

FIG. 1. Serum TNF levels in leprosy patients across the spectrum of leprosy and normal healthy controls (NHC) in log scale. Bar in each category denotes the mean.

FIG. 2. Serum IL-1 levels in leprosy patients across the spectrum of leprosy and normal healthy controls in log scale. Bar in each category denotes the mean.
patients were followed up clinically for a period of 1 year for the incidence of lepra reaction. There was an increased incidence of reactions in patients with high levels of TNF at recruitment into the study. Four out of 8 patients with TNF levels >100 pg/ml went into reaction within 2–6 months (3 out of 4 with >500 pg/ml and 1 out of 4 with 100–500 pg/ml). Figure 3 presents the data. It can be seen that the incidence of reaction was the lowest (5 out of 56) among patients with low levels of TNF ranging from 0 to 100 pg/ml (433 and 1/23 patients having admission TNF level <15 and 15–100 pg/ml, respectively). The incidence of reaction increased significantly in patients with TNF levels >100 pg/ml ($\chi^2 = 9.7, P < 0.01$). Three out of 4 cases that had levels >500 pg/ml went into repeated (three to four) episodes of reaction within 3–6 months. Tables 1 and 2 give clinical data (histopathological diagnosis, lepromin status, bacteriological index, and clinical diagnosis) of patients in type 1 and type 2 reactions. Figure 4 gives the TNF and IL-1 levels of those patients. In the type 1 reaction, 9 out of 16 cases showed high TNF levels of 50–980 pg/ml (186 ± 71 pg/ml), and in 7 cases it was not detectable. In the type 2 reaction, 8 out of 13 cases showed high TNF levels of 15–2100 pg/ml (255 ± 161 pg/ml). This frequency of high levels of TNF is comparable to that observed in LL categories.

Circulating IL-1 levels were assayed in all the sera tested for TNF. The incidence of increasing IL-1 was much less compared to TNF. Of the 106 sera assayed, detectable IL-1 levels ranging from 56 to 5000 pg/ml were observed in 16 cases (7/30 in LL, 1 of 12 BL, and 8 of the 29 reaction cases). Seven cases of LL had high levels of IL-1 ranging from 68 to 5000 pg/ml (328 ± 184), which is significant as compared to normal controls ($P < 0.01$). In BL patients, 1 out of 12 had IL-1 levels of 60 pg/ml (20 ± 5). In the 8 BB cases (22 ± 4), 14 BT-TT cases (12 ± 3), and 14 normal healthy controls (9 ± 3) studied, none had a IL-1 level >50 pg/ml.

In type 1 reaction patients, 6 out of 15 had IL-1 levels of 56 to 295 pg/ml (83 ± 27) and in type 2 reaction cases, 2 out of 13 showed levels of 1900 and 2550 pg/ml (333 ± 233). It is interesting to note that these sera also have high levels of TNF and that their coefficient of correlation is statistically significant in LL and reaction cases ($r = 0.96, P < 0.001$). All 4 patients having admission IL-1 levels >300 pg/ml went into reactions in subsequent months in contrast to 5 out of 60 patients with IL-1 levels <300 pg/ml. However, considering the small number of this group, this finding is being verified on a larger study group.

**DISCUSSION**

Serum levels of TNF and IL-1 were measured in all categories of leprosy patients as well as in patients undergoing type 1 and type 2 reactions. The patients were classified using the Ridley–Jopling scale. Their clinical, histopathological, bacteriological, and immunological statuses were carefully recorded. High TNF levels were detected in the LL type where there is spe-

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### TABLE 1

<table>
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<tr>
<th>No.</th>
<th>Patient’s initials</th>
<th>H/P diagnosis</th>
<th>Bl score</th>
<th>Lepromin status</th>
<th>TNF level (pg/ml)</th>
<th>IL-1 level (pg/ml)</th>
<th>Reaction stage</th>
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* Downgrading reaction

* Upgrading reaction

* Persistent reaction.

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### TABLE 2

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<th>No.</th>
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<th>TNF level (pg/ml)</th>
<th>IL-1 level (pg/ml)</th>
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**FIG. 3.** Correlation of serum TNF level and percentage of incidence of lepra reaction subsequent to TNF detection.
The question of specific T cell anergy to *M. leprae* antigens. This finding is similar to the report of Scuderi et al. (18) in visceral leishmaniasis. It appears that T cells of most of these patients do not respond efficiently to intracellular *M. leprae* as measured by lymphocyte transformation tests, IL-2, and IFN-γ production (19–21). Furthermore, high TNF levels in the absence of functional T cell responses indicate that TNF by itself does not confer any resistance in these patients. This is also evidenced by low or undetectable levels in BT-TT categories where the T cell responses are pronounced. Elevated TNF titers, although associated with high antigenic loads (e.g., LL), did not correlate with the bacteriological indices within this group. These observations indicate that TNF in leprosy does not induce septic shock type of toxic response, even with levels as high as 2050–4500 pg/ml. These patients did not manifest symptoms of shock. TNF and IL-1 inhibitors have been recently described (22–25). The possibility of production of such inhibitors during leprosy reactions is under current evaluation. The mechanisms of production and the sources of high levels of TNF in LL patients remain unclear. More recent evidence suggests that although there is no organized granuloma or evidence of activation of macrophages in the lesions, the antigen trapped in lymph nodes could induce local activation of helper T cells as well as antigen-presenting cells within the nodes (26). The other reason could be of impending subclinical or preclinical states of reactions. Many of the patients with high TNF levels at study entry went into repeated episodes of reactions (at least three to four times) over the ensuing months.

The question of *M. leprae* products directly causing release of TNF from the host cells needs to be examined. There is evidence that TNF in *in vitro* tissue culture is able to directly cause destruction of myelin and oligodendrocytes (27, 28). Such an effect is not due to an immune-mediated mechanism, but is due to an alteration in ion channels of oligodendrocytes as well as axons. Similarly, it has a potent effect on bone resorption and collagen synthesis in osteoblast-like cells *in vitro* (29, 30). If these observations are extended to leprosy, then it could perhaps explain nerve damage and deformities in those cases where there is no direct evidence of local granuloma formation. Lack of detectable levels of TNF in the TT and BT categories suggests that TNF release may be mainly local in the granulomatous area and is not high enough to be detected in circulation. In patients with reactions, the high level of TNF could be due to an acute inflammatory response. In ENL reactions, generalized activation of helper T cells has been reported. Similarly, in type 1 reactions, there is histopathological evidence of immunological upgrading associated with the activation of both macrophages and T cells. These events suggest initiation of a competent cell-mediated immune response. Thus the high levels of TNF in these reactional states may be summed up as a result of activated helper T cells as well as macrophages, but the present study does not distinguish the source between these two. Patients having high levels of TNF also have high levels of IL-1, suggesting the synergistic action between the two cytokines in the biological activity and immunopathological process. The role of TNF and other cytokines in leprosy needs to be further elucidated.

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TNF and Mycobacteria

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The clinical manifestations of leprosy and tuberculosis provide an outstanding opportunity to study the relationship between tumor necrosis factor (TNF) and mycobacteria in a biologically relevant system. Both diseases form immunologic spectra, allowing for the correlation of TNF levels with the success of the immune response against infection. Furthermore, the myriad of clinical symptoms present in patients provides a tempting database within which to identify pathologic effects of TNF relevant to human disease.

The disease spectrum of leprosy exhibits two poles: at one pole of the spectrum, patients with tuberculoid leprosy have one or several skin lesions, containing well-organized granulomas in which Mycobacterium leprae can rarely be identified, whereas at the opposite pole, patients with lepromatous leprosy have diffuse infiltration of skin and nerves, and undeveloped granulomas containing bacilli-laden macrophages (1,2). The immunologic state of these patients can be assessed using the lepromin skin test (Mitsuda reaction), in which a granulomatous response to intradermal injection of M. leprae is monitored over 3 to 4 weeks. The test is positive in tuberculoid patients and negative in lepromatous individuals.

Imposed upon this spectrum are the so-called reactional states, i.e., the reversal reaction and erythema nodosum leprosum (ENL). The reversal reaction appears to be a naturally occurring delayed-type hypersensitivity (DTH) response to M. leprae. Clinically, it is characterized by "upgrading" of the clinical picture toward the tubercular pole, with a concomitant reduction in the bacillary load. Immunologically, there is development of strong skin test reactivity to M. leprae antigens as well as lymphocyte responsiveness to M. leprae in vitro (3-7). ENL is characterized by crops of tender erythematous nodules, fever, and arthralgias. The pathogenesis of ENL re-
actions is enigmatic, but they are thought to be due to immune complex deposition in the lesions (8-10) and/or increases in cell-mediated immunity (11,12). Throughout the spectrum of leprosy, nerve damage is a major source of morbidity. Such damage varies from the nerve trunk lesions observed in tuberculoid patients to atrophy of the terminal ramifications of pain fibers. Recently TNF has been shown to cause nerve demyelination in vitro (13).

The clinical outcomes of Mycobacterium tuberculosis infection can also be regarded as a spectrum that reflects the immune response. At one end of the spectrum, most healthy tuberculin reactors have a protective immune response to infection. Patients with pleuritis have mild disease that usually resolves without therapy (14). The disease provides an opportunity to study the immune response at the site of disease activity. Studies of Barnes, et al. (15) have demonstrated that cell phenotypes and cytokine production in pleural fluid and tissue differ significantly from those in blood, and that the evaluation of T cells from pleural lesions yields insights not obtainable through study of peripheral blood mononuclear cells (PBMC) (15,16). Advanced pulmonary tuberculosis is characterized by extensive and life-threatening disease. At the most extreme end of the spectrum, patients with military tuberculosis have a weak immune response to M. tuberculosis infection and a mortality rate approaching 100% without treatment.

Clearly, some of the clinical and histological manifestations of leprosy can be related to TNF production and granuloma formation. Fever, arthralgia, and nerve damage. Similarly, in tuberculosis, granulomas with caseation necrosis, fever, and cachexia may have their basis in TNF release.

MYCOBACTERIA AS A STIMULUS FOR TNF PRODUCTION

While animals are sensitized to respond to bacterial lipopolysaccharide (LPS) by Bacillus Calmette-Guérin (BCG) (17), there is no evidence for a rise of plasma TNF concentration in BCG-treated animals. Valone, et al. (18) have recently demonstrated that BCG and mycobacterial products are capable of directly stimulating both PBMC and alveolar macrophages to produce TNF in vitro. In these studies, four mycobacterial preparations were found to be responsible for this effect: whole BCG, culture filtrate of M. tuberculosis H37Ra, purified protein derivative (PPD), and antigen 5 (a glycoprotein purified from culture filtrate) each stimulated blood monocytes and alveolar macrophages to release TNF, independent of the presence of LPS (18).

Lipoarabinomannan (LAM), another major component of mycobacterial cell walls, is a potent trigger of TNF release from both human and murine macrophages, both in vivo and in vitro (19). Indeed, when LAM is fractionated by electrophoresis on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose, most TNF-induc-
ing capacity coincides with the bulk of the LAM, as estimated by molecular weight and antigenic activity. It has also been shown that LAM is distinct from LPS, although it also binds to polymyxin B. In vivo, mice pretreated with Propionibacterium acnes and then injected with LAM have a TNF response identical to that observed following administration of LPS. The ability of LAM to cause the release of TNF from macrophages activated by a T-cell-mediated pathway may be responsible for some of the characteristics of tuberculosis, such as local necrosis in lesions and skin-test sites, fever, weight loss, and raised plasma concentrations of acute-phase reactants. However, LAM may not be the only inducer of TNF release that is present in mycobacteria.

The fine antigenic moieties of M. tuberculosis responsible for TNF release have been analyzed further. Wallis, et al. (20) have identified mycobacterial proteins that stimulate monocytes to secrete interleukin-1 (IL-1) and TNF. Culture filtrates of M. tuberculosis separated by gel electrophoresis and nitrocellulose blotting display two fractions (46 kD and 20 kD) that are able to induce both monokines. It has been shown that induction of these cytokines was due neither to LAM nor to contaminant LPS. The magnitude of monocyte responses to these proteins was similar to that induced by whole mycobacteria or by LPS. The same fractions also can activate T cells from healthy tuberculin-reactive individuals. These proteins may therefore have potential utility as immune inducers. The 46-kD and 20-kD proteins can also trigger pathologic effects, since Rook (21) postulates that TNF is involved in tissue injury rather than in protection. According to him, the mediators so far elucidated, i.e., interferon-γ, 1,25 dihydroxy-vitamin D₃ (1.25(OH)₂D₃) and TNF, are more likely to lead to immunopathology than to protection.

Other mycobacterial structures are also found to induce cytokine release. Silva and Faccioli (22) have found that cord factor (CF), a toxic glycolipid from mycobacteria, induces cachexia in BALB/c mice. Body weight falls markedly 48 hours after CF injection, and animals display hypertriglyceridemia, hypoglycemia, and high blood levels of TNF. These effects are abolished by administration of anti-TNF antibody.

More recently, Ab, et al. (23) have shown that heat shock protein 65 (HSP 65) from Mycobacterium bovis or M. tuberculosis stimulates the production of both interferon-γ and TNF by fresh PBMC from BCG-responsive individuals. However, TNF production by HSP-65-stimulated PBMC is significantly lower than that induced by PPD. In addition, cytotoxic HSP-65-stimulated effector cells appear to be very efficient in inhibiting the growth of live BCG inside autologous macrophages. HSP 65 also induced major histocompatibility complex (MHC) class II restricted CD4 cytotoxic T lymphocytes (CTL). The fact that HSP-65-educated effector cells display strong nonspecific cytotoxicity towards HSP 65 cells as targets may be important with regard to the possible role of this HSP in autoimmunity, manifested by nonspecific lysis of tissue macrophages and other cells.
Mycobacterial cell wall components that induce TNF release have been characterized. The cell walls *M. leprae* and *M. tuberculosis* are highly immunoreactive and have analogous structural components. Two major components of the mycobacterial cell walls are LAM and the cell wall core. LAM is a lipopolysaccharide that consists of a phosphatidylinositol molecule covalently linked to peptidoglycan, which is in turn tightly associated with cell wall protein (P. Barnes, personal communication). It has been confirmed that mycobacterial LAM is a potent stimulus for TNF release and that this activity is not due to contaminating LPS. To determine the specific component of LAM responsible for TNF release, arabinose residues and also most of the mannose groups in LAM have been chemically removed. Removal of all mannose groups (phosphatidylinositol) or all acyl functions (deacylated LAM) abrogated TNF release. Thus, acylated polysaccharides appear to be the minimal biochemical structures necessary to stimulate TNF release.

The protein–peptidoglycan complex of *M. tuberculosis*, but not its arabinogalactan, stimulates TNF release from mononuclear cells. Peptidoglycan, but not the cell wall protein, is a potent stimulus for TNF release. Peptidoglycan is composed of polymerized N-glycolylmuramic acid and N-acetylglucosamine, intercalated with a tetrapeptide. To further investigate the minimal biochemical constituents of peptidoglycan that are necessary for TNF release, the capacity of glucosamine derivatives to elicit TNF release has been determined. Poly-N-acetylglucosamine stimulates TNF release, but monomeric N-acetylglucosamine and glucosamine do not, suggesting that, as is the case for LAM, polymeric acylated sugars are capable of inducing TNF release (P. Barnes, personal communication).

**DETECTION OF TNF IN PATIENTS WITH MYCOBACTERIAL INFECTION**

The production of TNF and prostaglandin E$_2$ (PGE$_2$) by PBMC from tuberculosis patients and healthy controls has been investigated (24). PBMC from tuberculosis patients constitutively generate more TNF than do control PBMC. This constitutive production is significantly higher among patients with high-grade fever and cachexia than among patients without these manifestations. The increase of TNF production by PBMC from tuberculosis patients is associated with a comparatively smaller elevation of PGE$_2$ synthesis, which does not parallel fever or weight loss. Treatment of control PBMC with tuberculin PPD *in vitro* promotes increased TNF production similar to that of untreated PBMC from tuberculosis patients. Thus, the increased TNF production in tuberculosis could be explained by the exposure of PBMC to mycobacterial antigens *in vivo*. In contrast, the concentration of PGE$_2$ is lower in the medium of untreated PBMC from tuberculosis pa-
patients than in the medium of PPD-treated control PBMC, suggesting that in tuberculosis PGE₂ synthesis by PBMC is limited by unidentified factors. *M. tuberculosis* appears to be a strong inducer of TNF release, and the incidence of mycobacterial infections is particularly high in human immunodeficiency virus (HIV)-infected individuals. This fact might have important consequences in malaria-endemic areas. Indeed, it is conceivable that excessive mycobacterium-induced TNF release could aggravate the pathology witnessed in patients with malaria, especially cerebral malaria (25-27).

To investigate the roles of TNF, as well as interferon-γ *in vivo* at the site of disease activity in human tuberculosis, local cytokine production has been evaluated in patients with tuberculous pleuritis (15). Both TNF and interferon-γ are selectively concentrated 5- to 30-fold in pleural fluid, as compared with blood from the same patients. Messenger RNAs for both cytokines are detected in pleural tissue by *in situ* hybridization, suggesting that selective cytokine concentration is due to local cytokine production. Two *M. tuberculosis* cell wall components, the protein–peptidoglycan complex and LAM, cause dose-dependent release of TNF by pleural fluid mononuclear cells and may constitute the stimuli for TNF production in the pleural space. In contrast to results obtained for TNF release, the protein–peptidoglycan complex, but not LAM, stimulates interferon-γ release by pleural fluid mononuclear cells. The clinical manifestations of tuberculous pleuritis, such as fever, exudative pleural effusion, and tissue necrosis, may thus be due to the effects of elevated local TNF concentrations, occurring in response to mycobacterial cell wall components. The differential effects of mycobacterial antigens in stimulating cytokine release appear to regulate the immune response at the site of infection.

Circulating concentrations of cytokines in patients with mycobacterial infections have been investigated by several groups. Sera from patients with the multiparasitic/bacillary type of disease (visceral or diffuse leishmaniasis and lepromatous leprosy), known to be associated with absent or low specific T-cell response, contain significantly higher TNF titers than do those of patients with pauciparasitic/bacillary disease (localized cutaneous leishmaniasis and nonlepromatous leprosy) (28). High titers of TNF, therefore, do not seem to confer resistance in the absence of a functioning T-cell response.

In contrast to these findings in leprosy patients, there was no elevation of TNF concentration in serum of patients with tuberculosis (29). Rather, bioassays have revealed markedly elevated levels of TNF inhibitory activity in serum from patients with tuberculosis as well as sarcoidosis.

The possible role of cytokines in leprosy reactions has been investigated by analyzing the concentration of TNF and IL-1β in serum samples from 39 leprosy patients. 22 of them presenting either type 1 (upgrading) or type 2 (ENL) reactions (30). Fifty percent of the patients shows elevated concentrations of TNF and IL-1 in at least one of the serum samples tested. This
was the case in all four patients undergoing type I reversal reaction and in
nine (50%) of the ENL patients studied. Concentrations of TNF above 1,000
pg/ml have been found in four patients with ENL. Development of erythema
multiforme in these ENL patients represents an aggravating factor. and all
four patients suffering from this type of lesion demonstrated increased
serum TNF levels. All BT patients tested presented elevated IL-1 levels,
while only half of them presented elevated levels of TNF. No correlation has
been found between any particular systemic symptoms and the levels of
TNF and IL-1. These results suggest that TNF and IL-1 may be implicated
in leprosy reactions, either acting directly or in synergism with other
cytokines.

In another study, serum TNF levels were examined in 64 patients spanning
the spectrum of leprosy (LL = 30, BL = 12, BB = 8, and BT-TT = 14) and
in 29 cases during lepra reactions (type 1 = 16 and type 2 = 13), and
followed up for a period of 1 year (Parida, et al., ms. submitted). TNF was
detected in dramatically high concentrations (15-4,500 pg/ml; mean. 399 ±
189 pg/ml) in lepromatous leprosy cases and in low levels (15-160 pg/ml) in
tuberculoid leprosy. Patients also showed high TNF levels during lepra re-
actions (15-2,100 pg/ml; 186 ± 71 pg/ml in type 1 and 255 ± 161 pg/ml in
type 2 disease). IL-1 levels were also studied in these cases and found to be
high in LL cases in comparison with the normal controls. The coefficient of
correlation between TNF and IL-1 levels is statistically significant in LL
and in reaction cases (r = 0.96, p < 0.001). It was further observed that
50% of the patients with TNF levels exceeding 100 pg/ml within 2 to 6
months of study entry developed lepra reaction (either type 1 or type 2) in
the course of their treatment. In contrast, only 9% of the patients having
TNF levels less than 100 pg/ml developed this complication in a mild form
(χ² = 9.7, p < 0.01). Similarly, all of the patients (100%) with IL-1 levels
exceeding 300 pg/ml developed this reaction, in contrast to 8% of patients
with IL-1 levels beneath 300 pg/ml. These data strongly suggest that cyto-
kine determination in leprosy (more specifically, the measurement of TNF
and IL-1) might have prognostic value.

These observations indicate that in leprosy, TNF does not induce a toxic
response of the type witnessed in septic shock. even when concentrations
are as high as 2,050-4,500 pg/ml. TNF and IL-1 inhibitors have recently been
described (31-34). The possibility that such inhibitors may be produced dur-
ing leprosy reactions is currently under evaluation. The mechanisms re s pon-
sible for production of high levels of TNF in LL patients remain unclear.
Recent evidence suggests that although there is no organized granuloma or
evidence of macrophage activation in the lepromatous lesions, mycobacte-
rial antigens trapped in lymph nodes can induce local activation of helper T
cells as well as antigen-presenting cells.

BT, borderline tuberculoid; 11, lepromatous; BL, borderline tuberculoid; BB, border-
line, BT-TT, borderline tuberculoid or tuberculoid.
It has also been found that release of TNF into the peripheral blood of leprosy patients is significantly higher in ENL and BT patients than in LL, reversal reaction, and inactive ENL patients. Interestingly, this TNF release in ENL cases decreases after thalidomide treatment. Furthermore, M. leprae LAM induces TNF release from PBMC in parallel to whole M. leprae.

In immunohistochemical studies, granulomas visualized in biopsies of untreated patients with tuberculoid leprosy have shown large numbers of cells that stain positive for IL-1, TNF, interferon-γ, and CD 1 antigen (which is indicative of subepidermal Langerhans cells), whereas no positive signals can be detected in untreated patients with lepromatous leprosy (35). However, in those biopsies obtained from lepromatous leprosy patients undergoing chemotherapy, positive staining for cytokines, as well as subepidermal Langerhans cells, was observed. Remarkably, in tuberculoid leprosy patients, the number of IL-1-positive cells does not vary under therapy, while the number of TNF and interferon-γ reactive cells decreases. These results suggest that immunohistochemical determination of cytokine production, in combination with the detection of subepidermal Langerhans cells, may be used as a parameter for assessment of the patient’s cell-mediated immune status in the course of chemotherapeutic treatment.

More recently, the degree of TNF gene expression has been analyzed in leprosy lesions. The relative intensity of TNF mRNA-positive cells, in terms of grain density, is constant throughout the leprosy spectrum, allowing for quantitation according to the percentage of positive cells. Greater than 0.5% of the cells in Mitsuda reactions (n = 8), reversal reactions (n = 7), and tuberculoid lesions (n = 9) are found to express TNF, while this cytokine gene is found only in about 0.2% of the cells in lepromatous lesions (n = 9) and ENL lesions (n = 14). Although levels of TNF are relatively low in the latter two conditions, they appear sufficient, in ENL, to synergize with the available interferon-γ, thereby to inducing intracellular adhesion molecule-1 (ICAM-1) expression by keratinocytes (Sullivan, et al., ms. submitted).

Yamamura, et al. (ms. in preparation) have extracted total RNA from skin biopsies of lepromatous skin lesions, synthesized cDNA, and amplified it by polymerase chain reaction (PCR) using cytokine-specific primers. Intensity of PCR bands on ethidium-bromide-stained agarose gels reveals that TNF mRNA is distributed in a manner similar to that determined by in situ hybridization.

EFFECTS OF TNF ON MYCOBACTERIAL FUNCTIONS:
PATHOLOGY VERSUS PROTECTION

It is clear that mycobacterial antigens can induce TNF release and that TNF can be detected in the blood and lesions of patients with mycobacterial disease. Given the myriad inflammatory effects of TNF, a central issue is
the relationship of TNF to the outcome of mycobacterial infection. Specifically, does TNF release contribute to protective immunity, or does TNF mediate the pathologic manifestations of mycobacterial disease?

A role for TNF in protective immunity is suggested by data implicating this cytokine in the killing of mycobacteria. TNF, like other cytokines (such as interferon-\(\gamma\) and IL-4) is able to activate tuberculostatic macrophage functions (36). TNF, alone or in combination with IL-2, can activate human and murine macrophages in \textit{vitro} to kill \textit{M. avium} strains isolated from disseminated infections (37,38). In \textit{vivo}, the combination of IL-2 and TNF caused only a trend toward an absolute decrease in the number of bacteria in blood, suggesting the possible usefulness of such cytokines, alone or in combination with one another, in the therapy of disseminated mycobacterial infection.

The ability of TNF to cause killing of mycobacteria by macrophages may be influenced by a number of factors. For example, insulin, epinephrine, and somatostatin decrease the ability of macrophages to kill \textit{M. avium}, and cause a concomitant decrease in macrophage expression of TNF receptors (39). Vitamin \(D_3\) may activate macrophages to kill intracellular mycobacteria or inhibit their growth via induction of TNF and granulocyte-macrophage colony-stimulating factor (GM-CSF) release (40). Vitamin \(D_3\)-induced macrophage activation is significantly reduced in the presence of anti-TNF or anti-GM-CSF. Both interferon-\(\gamma\) and vitamin \(D_3\) increase LPS-triggered release of TNF (41). Live virulent \textit{M. tuberculosis} organisms can substitute for LPS, and are markedly more effective as triggers than several strains of BCG prepared in an identical manner. It is noteworthy that concentrations of vitamin \(D_3\) are increased at the site of disease activity in tuberculous pleuritis, a condition in which bacterial growth is relatively contained (42).

The granuloma is an organized collection of macrophages and lymphocytes that contains the foreign invader, facilitating the disposal of pathogens. Granuloma formation in the liver of mice with BCG coincides with local TNF synthesis (43). Injection of rabbit anti-TNF antibody, 1 to 2 weeks after initiation of infection dramatically interferes with the development of granulomas and subsequent mycobacterial elimination. Furthermore, fully developed BCG granulomas, formed after 3 weeks of infection, rapidly regress after anti-TNF antibody treatment. Antibody treatment also prevents or suppresses accumulation of TNF mRNA and protein, which resumes after disappearance of the antibody. TNF released from macrophages into the microenvironment of developing granulomas thus appears to be involved in a process of autoamplification: acting in an autocrine or paracrine fashion, it enhances its own synthesis and release, favoring further macrophage accumulation and differentiation, and leading to bacterial elimination.

A protective role for TNF in the immune response to mycobacterial infection is further supported by its production at the site of disease activity in patients resistant to mycobacterial infections. TNF mRNA is relatively more
abundant in resistant tuberculoid lesions (containing organized granulomas) compared with susceptible lepromatous lesions (containing poorly developed granulomas), as determined by in situ hybridization and by PCR-based detection of tissue RNA. TNF protein, as detected by immunohistochemical analysis, is also more abundant in tuberculoid lesions. In tuberculous pleuritis, there is a transpleural gradient of TNF, and abundant TNF mRNA in pleural tissue.

Other aspects of TNF distribution and function in patients with mycobacterial disease suggest that TNF may mediate pathologic manifestations of mycobacterial disease. Serum TNF levels and M. leprae-induced TNF release from PBMC are greatest in patients with ENL, a reactional state of lepromatous leprosy. These patients have fever, a manifestation that may be due to TNF release. The caseation necrosis characteristic of tuberculous granulomas may be due to local TNF production, a hypothesis supported by the finding of TNF mRNA in such granulomas. In addition, TNF release may provoke the cachexia frequently seen in patients with tuberculosis.

There is evidence that in vitro, TNF is able to cause destruction of myelin and oligodendrocytes directly; (13). This effect is not due to immune-mediated mechanisms, but results from alteration of ion channels in oligodendrocytes and neuronal axons. Similarly, TNF has a potent effect on bone resorption and collagen synthesis in osteoblast-like cells in vitro (see Mundy, this volume). TNF may also contribute to nerve damage by acting in conjunction with interferon-γ to induce class II MHC determinants on Schwann cells (44). Such Schwann cells may then serve as targets for sensitized CD4 cytotoxic cells. It is conceivable that local TNF production in leprosy lesions could, through these effects, result in nerve damage and deformities.

Many unexplained findings still obfuscate the role of TNF in mycobacterial disease. To complicate the issue of protection versus pathology, certain studies indicate that TNF may not directly mediate killing of M. tuberculosis (21). The role of TNF in mycobacterial killing could be mediated by its ability to induce interferon-γ release from natural killer (NK) cells (45). Furthermore, despite the fact that TNF release occurs frequently in leprosy, the occurrence of tissue necrosis is uncommon. This may be related to the presence of TNF inhibitors in leprosy patients (29). TNF is plentiful in the serum of ENL patients, and although TNF mRNA is weakly expressed in lesions, the level of tissue TNF appears sufficient to act with interferon-γ to induce ICAM-1 expression by keratinocytes. Yet, the concentration of TNF in the tissues is apparently not sufficient to effect removal of intracellular bacilli.

CONCLUSIONS

We have summarized the evidence supporting a role for TNF in protective immunity to mycobacteria, and the evidence that TNF may contribute to
the pathologic manifestations of mycobacterial disease. It may be overly simplistic to envision the protective and pathologic roles of TNF as mutually exclusive. Instead, TNF may cause both protection and pathology, with pathology seen as the consequence of the protective mechanisms. Identification of regulatory effects of other cytokines on TNF function, as well as the definition of TNF inhibitors, is likely to elucidate further the role of TNF in mycobacterial diseases.

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Immunotherapeutic trials with a candidate anti-leprosy vaccine based on Mycobacterium w


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Mycobacterium w was selected from amongst 15 coded Mycobacteria screened for their nearness to M. leprae in cell mediated immune reactions (antigen driven blast transformation and leucocyte migration inhibition) with peripheral blood leukocytes of a panel of tuberculoid leprosy (TT) patients (Mustafa and Talwar, 1978). Another requirement was the ability of the bacillus to evoke response with cells of lepromatous leprosy (LL) patients, who are anergic of response with M. leprae (Talwar, 1978; Mustafa and Talwar, 1978). Five of the first stage selected mycobacteria were investigated for their immunogenecity in mice and their ability to elicit delayed hypersensitivity reaction with homologous and M. leprae antigens in guinea pigs. Finally lepromin like preparations from the selected mycobacteria were evaluated for Dharmendra & Mitsuda reactions in TT and LL patients in six renowned leprosy control centres. Mycobacterium w was the best mycobacterium fulfilling the requirement of M. leprae like reactivity with TT cells and capability of evoking responsiveness in LL patients.

These “traits” of Mycobacterium w have recently been further supported by observations on B and T cell epitopes. CD4+ clones from subjects immunized with BCG and M. leprae were found to react with Mycobacterium w (Mustafa, 1988). Similarly the reactivity pattern of T cell lines developed in response to M. leprae recombinant antigens demonstrated reaction of M. w with 18 KDa and 65 KDa M. leprae antigens (Mustafa, 1988). Monoclonal antibodies hitherto considered specific to M. leprae or to M. tuberculosis (Engers et al., 1986), gave reactions with Mycobacterium w antigen in Western blots (Ganju et al., 1990).

Mycobacterium w is a fast grower resembling bacilli in Runyon’s group IV on basis of its growth and metabolic properties. It is, however, not identical to any currently listed mycobacteria in this group (Saxena et al., 1978; Katoch, 1981). M. w is a nonpathogen. Following extensive toxicology studies, Phase I clinical trials were conducted in 32 BL and LL patients. The vaccine was well tolerated. A single injection converted 62% of patients to lepromin positivity status (Chaudhuri et al., 1983).

Phase II/III Immunotherapeutic trials with M. w started in December 1986 in two hospitals of Delhi. Subjects enrolled are multibacillary cases belonging to BB, BL or LL spectrum of leprosy. All of them were lepromin negative to start with. Patients are given standard multi-drug therapy (MDT). However, the experimental group receives also the M. w vaccine given intradermally at 3 months interval. The control group receives MDT + injection of micronised starch as placebo. Group I consisting of 103 patients, on whom results are available, is a single blind study, where the head of the clinic was aware of the code, but not the attending physician. In this group, 96 patients are availing regular treatment and have completed 20-24 months of follow up. Group II is a double blind study in progress on 180 multibacillary leprosy patients.

Patients receiving immunotherapy with M. w vaccine, in addition to drugs, have shown distinctly better clinical improvement than those receiving multi drug therapy only. A twelve month report has been published (Talwar et al., 1990). Bacterial clearance was accelerated by the vaccine. 5 BL and 2 LL patients became bacillary negative (BI = 0) within 6 to 12 months. An LL patient with initial BI of 6 had a BI of 0.16 at 12 months after receiving 4 doses of the vaccine. By 15 months, this patient became bacteriologically negative as determined by bacillary count in slit skin smears taken from 6 sites in the body.

In the control LL group (patients treated with MDT + placebo injection), the fall in BI ranged between 0.9 & 1.2 units over 12 months. By 24 months, 11 patients in this group were bacillary negative, most of whom were BB or BL type with initial BI of 1.29 ± 0.20. The protocol envisaged treatment of patients for 24 months. The number of patients released from treatment at this time period are given in Table 1. It is evident that vaccine had beneficial effects. 100% of the subjects of BB or BL type were ready for release as also 12 out of 22 LL patients receiving the vaccine. None of the LL patient receiving MDT only were ready for release at 24 months, and only 40% in the BB or BL group.

Upgrading of immune status to M. leprae was evident from Mitsuda lepromin reaction and histopathology. All BB and BL patients, and 75% of LL patients became lepromin positive on progressive treatment with drugs and the M. w vaccine. None of the LL and BL category of patients converted in a stable manner to lepromin positivity on treatment with drugs only. Drugs, though effective in clearing bacteria,
failed to improve immunoreactivity to \textit{M. leprae} antigens. Thus immunotherapy with the vaccine may prevent relapses in the patients showing “conversion”. This property would be particularly useful in leprosy control programme.

Histopathologically, 75% of patients given the vaccine have shown upgrading. A notable feature is the absence of granuloma in the lesions, with nonspecific infiltration of cells and complete clearance of bacilli. With drugs only, even in paucibacillary cases, granuloma is observed to persist for long periods (A. Mukherjee, unpublished data). \textit{M. w} vaccine may thus be conferring the ability to clear the antigens, besides killing of the bacteria.

\textbf{Mycobacterium \textit{w} protective against Tuberculosis in animals}

Previous studies have shown the inability of BCG (Bacillus Calmette Guerin) to confer protective immunity against \textit{M. tuberculosis} in certain genetic strains of mice such as C3H and CBA, A/J (Pelletier et al., 1982). In some countries like, Burma, and India, BCG gave a fairly low degree of protection in carefully conducted clinical trials (Stanford and Rook, 1983 and Tripathi, 1983). Some other genetic strains of mice such as BALB/C and C57BL/6 do get immunized with BCG (Pelletier et al., 1982). Mycobacterium \textit{w} immunizes both the BCG “responder” and the BCG “nonresponder” strains of mice against challenge with live \textit{M. tuberculosis} H\textsubscript{37} RV (Guleria Singh et al. to be published). \textit{M. w} has also conferred protection in guinea pigs against \textit{M. tuberculosis}.

\textbf{Table 1} Number of patients completing 24 months and no. released from treatment

<table>
<thead>
<tr>
<th>Type of leprosy</th>
<th>Number at 24 months</th>
<th>Number released from treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V</td>
<td>P</td>
</tr>
<tr>
<td>BB</td>
<td>12</td>
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</tr>
<tr>
<td>BL</td>
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<td>8</td>
</tr>
<tr>
<td>LL</td>
<td>22</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>44</td>
<td>26</td>
</tr>
</tbody>
</table>

V – Vaccine group  
P – Placebo group

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Immunotherapeutic effects of a vaccine based on a saprophytic cultivable mycobacterium, *Mycobacterium w* in multibacillary leprosy patients


Immunotherapy with a vaccine consisting of autoclaved *Mycobacterium w* was given in addition to chemotherapy in 54 multibacillary, lepromin negative patients belonging to BB, BL and LL types of leprosy. Thirty-seven patients with similar types of diseases received chemotherapy and placebo injections. The 'vaccine' was repeated every 3 months. Bacterial clearance was more rapid in the vaccinated patients. Two lepromatous leprosy patients with initial bacterial index (BI) of 1.8 and 2.8 became bacteriologically negative in 1 year. One LL patient with BI of 6.0 had a BI fall to 0.16 after four doses of the vaccine. None of the LL patients belonging to placebo group during the same time period became bacteriologically negative. Rapid bacterial clearance was accompanied by distinct signs of clinical improvement. One hundred percent of BB, 85.7% of BL patients and 61.5% of LL patients converted to lepromin positivity after four doses of the vaccine. A significant number of vaccinated patients demonstrated an upgrading in skin lesions histopathologically.

Keywords: Leprosy; combined chemotherapy and immunotherapy; bacterial clearance; clinical score; lepromin conversion; lepromin reaction; histopathological upgrading

Introduction

Leprosy is a chronic infectious and debilitating disease caused by an acid fast bacillus, *Mycobacterium leprae*. It affects an estimated 10-15 million people in the world. The disease manifests itself as a spectrum which ranges from the paucibacillary tuberculoid type (TT) at one pole to the lepromatous type (LL) at the other, where the bacilli are abundant and *M. leprae* specific immune response is maximally depressed. BT, BB, and BL denote the intermediate states based on the classification proposed by Ridley and Jopling.1

Several attempts have been made to develop vaccines against the disease and currently three vaccines are in various stages of clinical trials. A vaccine consisting of armadillo grown killed *M. leprae* together with live BCG has been employed by Convit et al. in Venezuela for immunoprophylaxis and immunotherapy in leprosy.2,3 Two vaccines based on cultivable mycobacteria are in clinical trials in India, the ICRC vaccine4,5 and the *Mycobacterium w* (*M. w*) vaccine.6,7

Reported in this communication are the results of an immunotherapeutic trial with *M. w* vaccine in borderline borderline (BB), borderline lepromatous (BL) and lepromatous (LL) leprosy groups of patients. The choice of conducting trials in these categories of patients was based on the consideration that they represent cases of pronounced immunological deficiency. Thus the ability of a vaccine to enhance immunity can be critically assessed. Moreover, multibacillary LL cases require several years of treatment with drugs and in case a vaccine can expedite cure, it will be of benefit to the patient.

* M. w was amongst the 16 mycobacteria screened for their antigenic similarity to *M. leprae*, on the basis of antigen driven blast transformation and leukocyte migration inhibition with peripheral blood cells of tuberculoid leprosy patients.8 It induced cross-reactive skin responses with *M. leprae* in guinea-pigs, gave strong delayed type hypersensitivity (DTH) reactions and caused an enlargement of draining lymph nodes in mice.11 It produced Dharmendra and Mitsuda reactions similar to *M. leprae* lepromin in tuberculoid leprosy patients, but unlike *M. leprae* lepromin, it evoked positive responses in lepromatous leprosy patients.12-17

*Mycobacterium w* is a non-pathogenic, rapidly growing atypical mycobacterium classifiable in Runyon’s group IV, but differs in one respect or the other from bacilli currently listed in this group.18

Phase I clinical trials with this vaccine showed that it was well tolerated. After a single vaccine dose (5 x 10⁷ killed bacilli) 20 out of 32 treated BL and LL leprosy cases converted to lepromin positivity.6 The effect was
of a stable nature and lepromin conversion and some other in vitro CMI functions were manifest after 8-11 months of vaccination.

After obtaining necessary approval from the Drug Controller of India and clearance from the Institutional Ethics Committees of the respective hospitals, Phase II immunotherapeutic trials with this vaccine began in two urban leprosy centres of New Delhi. This report pertains to results obtained at the end of one year in 91 patients belonging to BB, BL and LL types of leprosy enrolled in the trial. A part of these findings was discussed at the workshop for vaccines in leprosy at the 13th International Leprosy Congress.

Materials

Vaccine preparation

From master seed stock M. w. was initially grown in Lowenstien-Jens (L-J) medium. After thorough characterization of growth and metabolic properties, seed lots are taken for preparation of the vaccine. This was prepared in Middlebrook medium (Difco Laboratories, Detroit, Michigan) with bovine albumin, dextrose and casein (ADC) enrichment. The bacteria were harvested during the log phase on 8-9th day of culture. The pellet was centrifuged and washed thrice with normal saline (0.085% NaCl). The purity of the preparation was assessed in nutrient agar to confirm the absence of other bacilli. The bacilli were suspended in saline at a concentration of 10^8/ml-1 and autoclaved for 15 min at 15lb inch-1 pressure. Completion of inactivation was tested by loopfuls on L-J medium on which no live mycobacteria was detected after two weeks of culture.

The stability of the vaccine preparation was tested in thioglycolate and soya bean casein digest medium to exclude presence of aerobic and anaerobic contaminants. One percent thiomersal was added to a final concentration of 0.01% as a preservative.

Placebo preparation

One gram of micronized starch (Sarabhai Chemicals, Baroda, India) was dissolved in 100 ml distilled water. This was autoclaved at 15lb inch-1 pressure for 15 min and dispensed in sterile vials.

Lepromin preparation

Armadillo lepromin containing 40 x 10^6 killed bacilli ml-1 was kindly made available by WHO.

Methods

Vaccine dosage, administration, regimen

The first dose of the vaccine was 1 x 10^6 autoclaved bacilli dispensed in 0.1 ml of physiological saline (0.85% NaCl). Subsequent doses contained 5 x 10^6 killed bacilli. The vaccine was administered in the deltoid region, intra-dermally (i.d.) distributed over three sites.

Multidrug therapy (MDT)

Consisted of 2 weeks of intensive therapy with 600 mg Rifampicin, 50 mg Clofazimine and 100 mg Dapson (DDS) daily. Subsequently patients received for two years the WHO regimen of MDT of 600 mg Rifampicin and 300 mg Clofazimine once a month supervised, continued with 50 mg Clofazimine and 100 mg DDS daily. Adherence to regular drug intake was counter-checked by randomly conducting spot test for DDS in urine.

Subjects and design

Patients reporting to the leprosy clinics at the two major hospitals were screened on clinical, bacteriological, histopathological and immunological criteria. Only those falling in the category of BB, BL and LL types of leprosy on the Ridley-Jopling scale, with evidence of bacilli in slit skin smears and who were negative to Mitsuda lepromin were taken for the study. The patients were divided in the vaccine/placebo groups. All received MDT. The vaccine group in addition received M. w vaccine every 3 months, whereas the placebo group received placebo injection of micronized starch. The allotment of patients was done in a randomized manner according to a code supplied to the attending physician. The trials were single blind, in which the Head of the Dermatology, Venereology and Leprology unit of the hospital knew of the group to which the patient belonged, whereas the investigators carrying out the histopathology, BI, clinical scores and other investigations did not know whether the patient was receiving the vaccine or the placebo.

Clinical scores

An entire body chart of the lesions was made at the start of the trial and after 1 year. Clinical scoring was, however, done every 6 months according to Ramu. In this scoring system, the body is divided into seven regions, i.e. the face, head and neck, right and left upper limb, chest and abdomen, back and buttock, and right and left lower limbs. Each region is independently scored. A score of one is given to predominantly macular lesions, two to diffusely infiltrated, three to few papules or plaques and four to predominantly papulonodular lesions. Thus a score from one to four is given to each of the seven regions. The maximum score for a patient on this scale would be 7 x 4 = 28, and the score at a given time would vary with the type of lesion and the number of body regions involved.

Bacteriological status

The bacteriological status of the patient was established by measurement of bacterial index (BI). For this, skin smears were taken from six sites. These included the right and left ear lobes, the right and left eyebrows and two representative skin patches. The smears were stained by the Ziehl-Neelsen method and average bacterial density calculated according to the Ridley's logarithmic scale. The bacterial index was determined every 3 months.

Lepromin testing

Standard armadillo lepromin (0.1 ml) was injected intradermally (i.d.) with a 1 ml tuberculin syringe and 30G needle on the flexor aspect of the left forearm. Readings for the late Mitsuda reaction were taken 3-4 weeks after the injection. An induration of 3 mm and above (with or without erythema), was taken as positive. Lepromin testing was repeated every 3 months.

Histopathological examination

Skin biopsies were taken from typical lesions and sent for histopathological examination at the start of the trial.
Figure 1  Clinical changes in patients after administration of M.w vaccine. Upper panel illustrates clinical improvement on the back of an LL patient. (a) Before and (b) after four doses of vaccine. Middle panel represents the back of a patient with BL type leprosy. (c) Before vaccination and (d) after three doses of vaccine. Lower panel presents the thighs and upper leg of a patient with BB leprosy. The hypopigmented lesion (e) cleared after two doses of vaccine (f).
Effects of mycobacterium-based vaccine on leprosy patients: G.P. Talwar et al.

Table 1 Number of patients enrolled and the number having completed 12 months of treatment

<table>
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<th>Type of leprosy</th>
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<th>Placebo group</th>
<th>Total</th>
<th>Number completing 12 months</th>
<th>Vaccine</th>
<th>Placebo</th>
<th>Total</th>
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</thead>
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<tr>
<td>BB</td>
<td>15</td>
<td>13</td>
<td>28</td>
<td>14</td>
<td>9</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>BL</td>
<td>18</td>
<td>13</td>
<td>31</td>
<td>14</td>
<td>13</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>LL</td>
<td>32</td>
<td>20</td>
<td>52</td>
<td>26</td>
<td>15</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>65</td>
<td>46</td>
<td>111</td>
<td>54</td>
<td>37</td>
<td>91</td>
<td></td>
</tr>
</tbody>
</table>

Table 2 Clinical scores before and after treatment with MDT + vaccine or placebo

<table>
<thead>
<tr>
<th>Type of leprosy</th>
<th>Group</th>
<th>Number of patients</th>
<th>Initial score</th>
<th>Score after 6 months</th>
<th>Score after 12 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>LL</td>
<td>Vaccine</td>
<td>26</td>
<td>13.87 (± 1.51)</td>
<td>10.65 (± 1.70)</td>
<td>6.73 (± 1.78)</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>15</td>
<td>12.97 (± 1.49)</td>
<td>10.75 (± 1.63)</td>
<td>9.70 (± 1.46)</td>
</tr>
<tr>
<td>BL</td>
<td>Vaccine</td>
<td>14</td>
<td>8.98 (± 1.67)</td>
<td>6.25 (± 1.51)</td>
<td>4.07 (± 1.48)</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>13</td>
<td>9.99 (± 1.51)</td>
<td>8.19 (± 1.59)</td>
<td>6.54 (± 1.60)</td>
</tr>
<tr>
<td>BB</td>
<td>Vaccine</td>
<td>14</td>
<td>6.40 (± 1.46)</td>
<td>4.19 (± 1.63)</td>
<td>1.92 (± 1.90)</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>9</td>
<td>8.03 (± 1.34)</td>
<td>7.12 (± 1.52)</td>
<td>5.53 (± 1.29)</td>
</tr>
</tbody>
</table>

Initial score and score at 12 months were statistically analysed using two way analysis of variance. In the BB and BL types given vaccine, the decrease in clinical scores was statistically significant as compared to the group given placebo (p < 0.01).

Figure 2 Clinical changes in patients after administration of placebo. Upper panel illustrates the thighs of a patient with LL leprosy, (a) Initially and (b) after four doses of placebo. Lower panel represents the back of a patient with BB Hansen’s, (c) before and (d) after four doses of placebo.
and at 6 months intervals thereafter. The repeat biopsies were taken from the same lesion as far as possible. In cases where the originally biopsied lesions had disappeared, other lesions were biopsied. Tissues were routinely fixed in 10% buffered formalin and processed for paraffin sections. Five micron sections were stained with Haematoxylin and Eosin and Fite–Faraco stains. Lesions were classified according to Ridley–Jopling grading system.20

Results

Table 1 gives the total number of patients belonging to different categories of leprosy enrolled in the study and those completing 12 months of treatment and observation. Twenty patients (11 in vaccine group and nine in placebo group) were lost to follow up.

Local reactions to M.w

Local reactions after vaccination consisted of erythema and induration appearing usually 5–7 days after vaccination in all patients. In the majority of cases, by 2 weeks there was formation of a well defined ulcer which healed spontaneously with scar formation in 3–4 weeks.

Clinical improvement

By 6 months, clinical improvement was more marked in individuals in the vaccinated group than those in the control, with a rapid clearing of papulonodular lesions (score 4) and marked decrease in infiltration. The relative improvement in the two groups is reflected by changes in clinical scores (Table 2). Figures 1 and 2 are representative photographs of changes in the lesions of the patients in the two groups.

Bacterial index (BI)

Changes in the bacterial index (BI) were documented at intervals of 3 months. The data given in Figures 3a, b and c describe the BI changes in these patients. Each patient bears a number. Patients from Dr Ram Manohar Lohia Hospital bear the suffix R and those from Safdarjung Hospital bear the suffix S. It is thus possible from these figures to follow the change in BI taking place in individual patients. The lines drawn give the arithmetic means. Table 3 gives the details of BI changes in the two groups. In the patients with BB and BL types of leprosy and LL leprosy (BI 4–6), the vaccine caused a more rapid fall in BI as compared with that induced by placebo. This difference was statistically significant (p < 0.01).

Lepromin reaction

At the start of the trial all patients were lepromin negative. It was observed in all groups that with progressive immunization there was a gradual increase in patients showing lepromin conversion, which is in a way an index of the delayed type hypersensitivity (DTH) of the patient to M. leprae antigens. Figures 4a, b and c give the number of patients becoming lepromin positive after receiving one to four doses of the vaccine or the placebo. In the BB category of patients 100% lepromin conversion was observed in the vaccine group, whereas in the placebo group only 44.4% of the patients attained positivity on reduction of the bacterial load by chemotherapy. The difference was more marked in the BL and LL leprosy patients. Only one out of 13 manifested a lepromin conversion in the placebo group of patients with BL type leprosy, whereas in the vaccine group 12 out of 14 (~86%) were lepromin positive at the end of 12 months. Similarly, in the LL group of patients, 16 out of 26 (61.5%) became positive to lepromin after 1 year of immunotherapy. Only one out of 15 reached this status in the placebo group.

Histopathological trends

Table 4a gives the analysis of histopathological changes seen after four doses of the vaccine. In the 1 year period, eight out of 24 LL patients upgraded to sub polar LL (LLs), and BL while three patients showed features of non-specific infiltration (i.e. perivascular lymphocytic infiltration) or subsided disease. In the placebo group (Table 4b), three out of 11 LL patients upgraded to LLs. In the BB type leprosy, 10 out of 13 in the vaccine and three out of 11 in the placebo group showed histopathological upgrading after twelve months of therapy. One patient of BL (placebo group) downgraded to LL. Of the 14 patients clinically diagnosed as Borderline-borderline (BB), and given vaccine, six had a definite histopathology of BB, one had histological features of BB/BB, five of BT and two showed non-specific infiltration in dermis. After twelve months, 10 patients out of 14 showed varying grades of upgrading ranging from BT/BB, BT, non-specific infiltrates to subsided disease. One BB patient however, downgraded to BL after four doses of the vaccine. This patient was pregnant during the course of the trial. In the placebo group, of the nine patients clinically diagnosed as BB, six had a histopathological picture of BB, two of BT and one of Indeterminate disease. Eight of the nine patients showed features of upgrading at the end of twelve months. Figure 5 shows representative histopathological pictures of a BL patient before and after vaccination with M. w showing histopathological upgrading to BT type leprosy.

Table 3: Bacterial Index (BI) of patients given vaccine or placebo

<table>
<thead>
<tr>
<th>Type of leprosy</th>
<th>Group</th>
<th>Number of patients</th>
<th>Initial BI</th>
<th>BI at 3 months</th>
<th>Variation (%)</th>
<th>BI at 6 months</th>
<th>Variation (%)</th>
<th>BI at 9 months</th>
<th>Variation (%)</th>
<th>BI at 12 months</th>
<th>Variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BB and BL</td>
<td>Vaccine</td>
<td>20</td>
<td>1.6±0.24</td>
<td>0.8±0.16</td>
<td>44.54</td>
<td>0.4±0.12</td>
<td>48.60</td>
<td>0.39±0.10</td>
<td>15.31</td>
<td>0.13±0.05</td>
<td>65.90</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>22</td>
<td>1.24±0.21</td>
<td>1.26±0.22</td>
<td>-1.17</td>
<td>-1.09±0.21</td>
<td>12.79</td>
<td>0.88±0.18</td>
<td>19.81</td>
<td>0.86±0.16</td>
<td>22.48</td>
</tr>
<tr>
<td>LL</td>
<td>Vaccine</td>
<td>13</td>
<td>2.92±0.17</td>
<td>2.20±0.22</td>
<td>24.74</td>
<td>1.85±0.22</td>
<td>15.73</td>
<td>1.62±0.20</td>
<td>12.61</td>
<td>1.16±0.21</td>
<td>28.49</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>6</td>
<td>2.56±0.28</td>
<td>2.30±0.29</td>
<td>9.92</td>
<td>1.86±0.31</td>
<td>18.29</td>
<td>1.74±0.27</td>
<td>7.70</td>
<td>1.53±0.25</td>
<td>12.23</td>
</tr>
<tr>
<td>LL (BI up to 3.6)</td>
<td>Vaccine</td>
<td>13</td>
<td>5.02±0.23</td>
<td>2.42±0.25</td>
<td>15.80</td>
<td>3.58±0.24</td>
<td>15.12</td>
<td>3.09±0.24</td>
<td>13.09</td>
<td>2.84±0.27</td>
<td>8.11</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>7</td>
<td>4.26±0.10</td>
<td>4.13±0.23</td>
<td>3.02</td>
<td>3.62±0.30</td>
<td>12.35</td>
<td>3.51±0.25</td>
<td>8.16</td>
<td>3.30±0.27</td>
<td>6.10</td>
</tr>
</tbody>
</table>

Values of BI are given as mean ± s.e.m. Initial BI and BI at 12 months were statistically analysed using two way analysis of variance. In the BB and BL and LL (BI 4–6) types of leprosy given vaccine, the fall of BI was statistically significant as compared to the group given placebo (p < 0.01).
Effects of mycobacterium-based vaccine on leprosy patients: G.P. Talwar et al.

Figure 3 Changes in BI after administration of vaccine or placebo. The arithmetic means of BI in patients in vaccine group are given as (●—●) and the placebo group as (○—○). Each patient bears a number. Each patient also bears the suffix S (Safdarjung Hospital) or R (Ram Manohar Lohia Hospital). (a) LL patients with initial BI between 4 and 6. (b) LL patients with BI up to 3.9. (c) BB and BL leprosy patients. Vaccinated patients (●); placebo patients (○)
Reational states and neuritis

Type 1 reaction in leprosy is delayed hypersensitivity associated with a rapidly changing cell mediated immunity (CMI), whereas Type 2 reaction (Erythema nodosum leprosum) has an immune complex component. Four out of 14 BB patients in the vaccine group and three out of nine in the placebo showed Type 1 reactions. In the BL type of leprosy, six out of 14 after vaccination and only one out of 13 after placebo had Type 1 reaction. Eight out of 26 and four out of 15 LL patients showed Type 2 (ENL) reaction after vaccine and placebo administration respectively (Table 5). Two patients, who upgraded to subpoliar lepromatous (LS) showed Type 1 upgrading reaction to BL after vaccination. Neuritis and motor disturbances were seen with equal frequency in both groups, ulnar nerve involvement being the most common. Facial palsy was seen in 1 patient (vaccine group) from which he subsequently recovered. One patient of BB type after three doses of vaccine complained of paraesthesia. Type 2 reactions appeared less frequent and less severe with subsequent vaccine doses, possibly due to rapid bacterial clearance in these patients.

Discussion

Vaccines have traditionally been employed for immunoprophylaxis. Recently, however, immunotherapy with vaccine as an adjunct to chemotherapy has been employed in many chronic infectious diseases. In leprosy, the incubation period as well as the clinical course of the disease is very long. Treatment with Dapsone lasts for 15–20 years. However, use of multidrug regimen has significantly reduced this period of therapy to two to five years. At the end of the two years of MDT, most of the lepromatous patients are still loaded with killed bacilli indicating poor ability to clear the bacilli. It is in this context, that immunotherapeutic trials were initially undertaken. Trials were conducted in patients sifted by nature to be highly immunodeficient with respect to M. leprae, i.e., LL, BL and BB types of leprosy patients with high bacillary load, who were unable to mount DTH skin reaction to M. leprae antigens. Ethical considerations demanded treatment of all patients with the recommended drug therapy. The patients in this trial were however divided into two groups, one received the ‘vaccine’ every three months and the other a placebo injection. The distribution of patients into two groups was not equal and a larger number of patients were taken in the ‘vaccine’ group, owing to the encouraging results observed by vaccination in those initially enrolled. The trials were single blind where the investigators reporting BL,

![Image](image-url)
Effects of mycobacterium-based vaccine on leprosy patients: G.P. Talwar et al.

Table 4a: Histopathological findings after administration of two and four doses of vaccine at 6 and 12 months

<table>
<thead>
<tr>
<th>Clinical diagnosis (number)</th>
<th>Initial histopathology (number)</th>
<th>6 months (2 doses)</th>
<th>12 months (4 doses)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LL (26)</td>
<td>LL (24)</td>
<td>LLs (2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16</td>
<td>1</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
</tr>
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<td>BL (13)</td>
<td>NSI (1)</td>
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<td>BB (6)</td>
<td>BB/BL (1)</td>
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</tr>
</tbody>
</table>

Table 4b: Histopathological findings after administration of two and four doses of placebo at 6 and 12 months

<table>
<thead>
<tr>
<th>Clinical diagnosis (number)</th>
<th>Initial histopathology (number)</th>
<th>6 months (2 doses)</th>
<th>12 months (4 doses)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LL (15)</td>
<td>LL (11)</td>
<td>LLs (3)</td>
</tr>
<tr>
<td></td>
<td></td>
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<tr>
<td>BL (13)</td>
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<td>BL (11)</td>
<td>BB (1)</td>
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<tr>
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<td>BB (6)</td>
<td>BT (2)</td>
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</tr>
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<tr>
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<td></td>
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<td>1</td>
</tr>
</tbody>
</table>

*NSI: Non-specific infiltration; *SD: Subsided disease; *Indel: Indeterminate; *LLs: Sub Polar LL

Table 5: Incidence of Type I and Type II (ENL) reactions and neuritis during the course of immuno and chemotherapy

<table>
<thead>
<tr>
<th>Group</th>
<th>Type of leprosy (no. of patients)</th>
<th>No. of patients showing Type I reactions</th>
<th>No. of patients showing Type II reactions</th>
<th>No. of patients with previous history of reactions</th>
<th>No. of patients with neuritis and nerve(s) involvement and resultant deformity if any</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccine</td>
<td>BB (14)</td>
<td>4</td>
<td>-</td>
<td>1</td>
<td>1—Right radial and ulnar nerve</td>
</tr>
<tr>
<td></td>
<td>BL (14)</td>
<td>6</td>
<td>1</td>
<td>3</td>
<td>1—Parasthesia</td>
</tr>
<tr>
<td></td>
<td>LL (26)</td>
<td>2</td>
<td>8</td>
<td>6</td>
<td>1—Left ulnar nerve with clawing</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1—Left ulnar nerve with clawing</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1—Left facial nerve and left ulnar nerve with clawing</td>
</tr>
<tr>
<td>Placebo</td>
<td>BB (9)</td>
<td>3</td>
<td>-</td>
<td>1</td>
<td>1—Right ulnar nerve</td>
</tr>
<tr>
<td></td>
<td>BL (13)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1—Right ulnar nerve with clawing</td>
</tr>
<tr>
<td></td>
<td>LL (15)</td>
<td>4</td>
<td>-</td>
<td>1</td>
<td>1—Right radial nerve</td>
</tr>
</tbody>
</table>

Vaccination and chemotherapy alone are not rendered bacteriologically negative even after two years of MDT22-24. The average BI decline with drugs is stated to be between 0.6 and 1 unit per year. Most patients receiving immunotherapy have demonstrated faster clearance of bacilli and two LL patients were rendered bacteriologically negative in one year. The significant finding was the ability of the vaccine to induce rapid bacterial clearance even in LL patients with high BI.

Type I reactions were more common in patients...
receiving the vaccine, whereas type 2 reactions were seen with equal frequency in both the groups. Most patients showing type 2 (ENL) reactions had previous history of such reactions before vaccination. On repeat vaccinations, there was a reduction in frequency and severity of such reactions. Neuritis and associated motor disturbances were not aggravated by the immunotherapy. No systemic side-effects were observed in the vaccinated individuals. The present study suggests that M. w can be beneficially used for immunotherapy of leprosy as an adjunct to chemotherapy.

References
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14 Mustafa, A.S. and Talwar, G.P. Early and late reactions in tuberculoid and lepromatous leprosy patients with lepromins from Mycobacterium leprae and five selected cultivable mycobacteria. Lep. India 1978, 50, 566
16 Girdhar, B.K. and Desikan, K.V. Results of skin tests with five different mycobacteria. Lep. India 1978, 50, 550
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