Liposomal delivery of *Mycobacterium leprae* antigen(s) with murabutide and Trat peptide inhibits Fas-mediated apoptosis of peripheral blood mononuclear cells derived from leprosy patients

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Protective immunity against intracellular pathogen *Mycobacterium leprae* is dependent on the activation of T cells. Repeated stimulation of T cells by *M. leprae* antigens MLCwA (*M. leprae* total cell wall antigen) and ManLAM (mannose capped lipoarabinomannan) may lead to apoptosis in leprosy patients. In the present study, inhibition of the Fas-induced apoptosis of peripheral blood mononuclear cells of leprosy patients was investigated using above *M. leprae* antigen(s), in combination with immunomodulators murabutide (MB) and a Trat peptide in particulate form (liposome). Incubation of the cells with particulate mode of antigen presentation led to both decreased percentage of propidium iodide (PI) positive cells and T cells expressing Fas-FasL, as well as decreased caspase-8/-3 activities in the lepromatous patients, thereby inhibiting apoptosis, while converse was true with stimulation with soluble antigen. Concurrently, there was an upregulation of anti-apoptotic protein Bcl-XL in the lepromatous patients, thereby inhibiting apoptosis. Thus, the liposomal formulation of antigen promoted proliferation of anergized T cell by inhibiting apoptosis through decreased expression of death receptors and caspase activities and increased expression of anti-apoptotic protein Bcl-XL in these patients.

**Keywords**: Leprosy, Anergy, Apoptosis, Fas-FasL, Caspase activity, Bcl-xL protein, liposome, Immunomodulator.

Leprosy is caused by *Mycobacterium leprae* and is a chronic disease that affects skin and peripheral nerves. It is characterized by clinical manifestations, largely as a result of the immunological response of the host to the antigens of *M. leprae*. Leprosy exhibits a clear-cut immunological polarity between tuberculoid and lepromatous leprosy showing a CD4⁺ Th1-and Th2-type response, respectively.¹² The Th1-type immune response is maintained by more of IFN-γ and IL-2 production by T cells, which activate macrophages and help in killing intracellular pathogen. Conversely, Th2-type cytokines help in antibody production, leading to unresponsiveness to *M. leprae* and further weakens the cell mediated immunity response³. They are also responsible for inducing or maintaining anergic state in these patients. This is because IL-10 is known to down-regulate co-stimulatory molecules (B-7 family) on antigen presenting cells (APC)⁴,⁵. The levels of Th2 cytokine and certain pro-inflammatory cytokines are increased in reactional states of leprosy patients, making the cells susceptible to undergo apoptosis. Spontaneous apoptosis is also observed in peripheral blood mononuclear cells (PBMC) of leprosy patients without mitogen stimulation, which is inhibited by anti-TNF-α antibodies, as well as ionomycin and zinc, leading to increased IL-2 production and cell proliferation⁶,⁷.

The macrophages derived from lepromatous patients are biochemically and immunologically inert as compared to normal and tuberculoid individuals⁸,¹⁰. The anergy of T cells is reversed using *M. leprae* antigen(s) *in vitro* with two immunomodulators murabutide (MB) and an immunodominant T cell peptide of Trat protein in liposomes¹¹,¹². Proliferation of anergized T cells has been reported with upregulation of Th1-type cytokines, co-stimulatory molecules and IFN-γ releasing CD4⁺ T cells in BL/LL patients¹³,¹⁴. Recently, the involvement of second messengers, kinases, phosphatases and transcription
factors in inducing the IL-2 secretion especially in BL/LL patients has also been studied to understand T cell anergy using the same formulation.\textsuperscript{15}

Virulent mycobacteria, such as \textit{M. leprae} modulate host cell apoptosis in order to create a protective niche within the cells.\textsuperscript{16-18} They also downregulate protective host cytokines such as TNF-\(\alpha\) and IFN-\(\gamma\)\textsuperscript{19,20} to reduce effector T cell and macrophage functions to the pathogen. However, TNF-\(\alpha\) has a double-edged function, it is essential for protection and is also involved in induction of cell death both \textit{in vivo} and \textit{in vitro}.\textsuperscript{21,22} Moreover, IL-10 has shown to attenuate apoptosis by counteracting TNF-\(\alpha\) function.\textsuperscript{23} T cell and macrophage responses are less affected in paucibacillary leprosy than in multibacillary leprosy, where effector T cell and macrophage responses are severely compromised. Multibacillary leprosy granulomas show increased expression of Fas ligand, which may protect these sites from attack by Fas-expressing cytotoxic T lymphocytes.\textsuperscript{24} Thus, clinical manifestation of infection with mycobacteria is thought to occur from immune evasion of viable bacilli, or from immune effector mechanisms, which are themselves contributing to tissue damage.

Macrophages loaded with \textit{M. leprae} have shown to undergo apoptosis, which is under the control of cytokines via the downregulation of Bcl-2 \textit{in vitro} in mononuclear cells.\textsuperscript{16} As the immune system is overburdened with mycobacterial bacilli in BL/LL patients, it is likely that continuous activation of T cells by circulating \textit{M. leprae} antigens may lead to apoptosis, a key pathogenic mechanisms involved in reduction of peripheral lymphocytes and other immune effector cells in these patients. Regulation of apoptosis occurs via either extrinsic pathway, involving the stimulation of death receptors (Fas/FasL) expressed on cells surface and activation of caspase-8 or the intrinsic pathway involving mitochondrial release of cytochrome \(c\) and activation of caspase-9. Since T cells are persistently stimulated by circulating \textit{M. leprae} antigens and are also categorized as type I cells, it is reasonable to believe that extrinsic pathway could be predominant in causing cell death in leprosy patients. In the present study, we have evaluated, whether reduction of peripheral lymphocytes or in other words, low T cell proliferation in LL patients in response to \textit{M. leprae} antigens MLCwA (\textit{M. leprae} total cell wall antigen), and ManLAM (mannose capped lipoarabinomannan) activation is associated with upregulation of apoptosis of peripheral mononuclear cells. And if so, whether liposomal delivery of \textit{M. leprae} antigen with these two immunomodulators (MLCwA or ManLAM + MB + Trat) could reverse this phenomenon. Cross-linking of Fas with FasL leads to activation of initiator caspase-8 which leads to activation of effector caspase-3 downstream in the apoptotic pathway. Thus, the expression of Fas/FasL and activities of caspase-8/-3 in PBMC of leprosy patients and normal individuals, in response to \textit{M. leprae} antigen presentation \textit{in vitro} in soluble/particulate form (in liposomes) with the immunomodulators have been investigated.

Materials and Methods

Subjects

The study was approved by Human Ethics Committee of All India Institute of Medical Sciences (AIIMS), New Delhi. Blood samples were collected from leprosy patients registered in the Department of Dermatovenereology, AIIMS. The patients were diagnosed according to Ridley and Jopling classification.\textsuperscript{25} These patients were of age group 20-45 years and of both sexes and were divided into two groups BT/TT (borderline tuberculoid and tuberculoid) and BL/LL (borderline lepromatous and polar lepromatous patients). Newly registered cases or patients with less than two months treatment of standard WHO-MDT regimen for BT/TT and less than six months for BL/LL were included in the study. Healthy young individuals, who were neither in close contact nor had any regular closeness with leprosy patients served as controls.

Immunomodulators

Murabutide (MB), an active analog of muramyl dipeptide was kindly provided by Dr. W Haq, Central Drug Research Institute, Lucknow, India and T cell peptide of Trat protein, NH\textsubscript{2}-(GLQGKIADAVKAKG)-COOH was synthesized using solid-phase peptide technique and was >95\% pure.

Antigens

MLCwA (\textit{M. leprae} total cell wall antigen), ManLAM (mannose capped lipoarabinomannan) and PGL-I (phenolic glycolipid-I) were generously provided by Colorado State University and the NIH, NIAID contract NOI AI-75320 entitled “Tuberculosis Research Materials and Vaccine Testing”,
respectively. Endotoxins content in antigens was tested using Limulus amebocyte Assay and was found to be 0.06 ng/mg of antigen.

Antigen formulations and liposome preparation
Both antigens were presented to the cells in optimal amounts in two formulations viz. Ag (5 µg/well) in RPMI medium, and Ag + MB + Trat peptide (1:2:2 µg/well) in liposomes (particulate form). Cells were also stimulated with murabutide or Trat peptide individually or combination of both in RPMI medium or liposome or combination of both with the antigen in medium under identical experimental conditions. Liposomes were prepared by freeze and thaw method as per the reported protocol. After entrapment of antigen with immunomodulators, the percentage entrapment of the antigen was assayed by BCA method and was found to be 47-52%.

Isolation and culture of peripheral blood mononuclear cells (PBMCs)
PBMCs were isolated by density gradient centrifugation on histopaque (Sigma, USA). Viability of the cells was tested by trypan blue exclusion dye. Cells were plated at a density of 1 × 10⁶ cells/well in duplicate in the presence of *M. leprae* antigens in medium or in liposomes entrapped with MB and Trat peptide for 5 days at 37°C in 5% CO₂-95% air.

Propidium iodide (PI) staining
Freshly isolated PBMCs were stimulated with MLCWA/ManLAM in soluble or particulate form with the immunomodulators as described above, fixed with 70% ethanol and then stained with PI (250 µg/ml, Sigma, USA) for 30 min at 4°C. Apoptosis was quantified by flow cytometric analysis of proportion of cells with hypodiploid DNA.

Flow cytometric analysis
Expression of Fas (CD95), FasL (CD95L) and lineage markers CD3 was analyzed using specific FITC/PE conjugated monoclonal antibodies (mAb) by flow cytometry. Briefly, aliquots of cultured cells (1 × 10⁶/well) were stained for 30 min with specific mAb (1 µg/well). Cells were then washed and fixed with paraformaldehyde (2%) and analyzed by two-color flow cytometry using a Coulter Epics XL flow cytometer and WinMDI software. Suitable isotype controls were used. Results were expressed as percentage of cells expressing the marker out of a gated population. The data generated were analyzed from the total number of 5,000 events for each labeled cell population.

Caspase activity
Freshly isolated PBMCs (1 × 10⁶ cells/well) treated with Ag/Ag + MB + Trat for 5 days were lysed in lysis buffer (10 mM Tris HCl, Na₂HPO₄, NaH₂PO₄, 130 mM NaCl, 1% Triton X-100; pH 7.5) and the cell lysate (50 µl) was then evaluated for caspase-8/-3 activities using 30 ng of synthetic fluorogenic substrates Ac-IETD-AMC (N-acetyl-Ileu-Glu-Th-Asp-7-amino-4-methylcoumarin) or Ac-DEVD-AFC (N-acetyl-Ileu-Glu-Th-Asp-7-amino-4-trifluoromethylcoumarin) (Sigma, USA) respectively as per the reported protocol. The caspase activity was expressed as mean arbitrary fluorescence units (AFU) per mg of cell lysate. Caspase-8 (Ac-IETD-CHO) and caspase-3 inhibitors (Ac-DEVD-CHO) were used to determine the specificity of the assay. Protein content in the cell lysate was determined using the BCA method.

Immunoblotting
After incubation of PBMC with both the formulations for 60 min, cells were washed twice with PBS and lysed in phospholipase Cγ lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, and 1 mM EGTA, pH 7) on ice for 1 h. The lysates were resolved by SDS-PAGE using 10% polyacrylamide gels, electrotransferred on to PVDF membranes (Millipore, Bedford, MA), and probed with rabbit polyclonal anti-human Bcl-xL-specific Ab (1/200 dilution, Santa Cruz Biotechnology, USA). The blot was washed, exposed to HRP-conjugated goat anti-rabbit IgG for 2h and finally detected by 3, 3 diaminobenzidine (10 mg/ml) in sodium acetate (pH 5.0) containing 4 µl H₂O₂. Densitometric analysis was performed to compare the intensity of bands (Quantity One Software). β-Actin (Sigma, USA) was used as a loading control.

Statistical analysis
Data were represented as mean ± standard deviation (S.D). Comparison between multiple groups was performed with one-way ANOVA, followed by Bonferroni ‘t’ test. *P<0.05 was considered significant.

Results
Measurement of apoptosis with PI staining
Freshly isolated PBMC from normal and leprosy patients showed negligible percentage of PI positive cells. But when PI staining was done for PBMC of all
the three groups, after 5 days of culture without any stimulation, BL/LL cells showed spontaneous apoptosis compared with the cells from normal and BT/TT groups. In BL/LL patients, the degree of apoptosis after 5 days of incubation with mycobacterial antigens (MLCwA/ManLAM) was significantly \( (P<0.005) \) higher than BT/TT and normal groups (Table 1). When PBMC of the same patients were cultured with Ag + MB + Trat formulation, the degree of apoptosis was significantly lower in cells treated with Ag + MB + Trat formulation, especially in particulate form in BL/LL patients, this formulation inhibited apoptosis by approximately 40% only, irrespective of the nature of the mycobacterial antigen used. The percentage of hypodiploid cells decreased significantly \( (P<0.005) \), when cells were treated with Ag + MB + Trat formulation in liposomes, especially in BL/LL patients (Table 1) as compared to BT/TT and normals. It was also observed that in BL/LL patients the degree of apoptosis was significantly \( (P<0.005) \) lower in cells treated with Ag + MB + Trat formulation, compared to cells treated with MLCwA or ManLAM.

**Effect of antigen formulation on Fas and FasL expression**

The initial experiments were carried out to standardize the time required for optimal expression of Fas and FasL. The time required for maximal expression of Fas and FasL in the normal and leprosy groups was optimized by stimulating the PBMC at different time intervals (24, 48, 72, 96 and 120 h) using the stimulant Ag + MB + Trat in liposome or Ag alone in medium. The optimal time interval for the maximal expression of these molecules was 120 h (5 days) (data not shown). The basal levels of expression of these molecules were studied immediately after the isolation of PBMC (i.e., 0 day) without any stimulant. The percentage of T cells expressing Fas in constitutive state was marginally higher in BT/TT group than BL/LL, followed by normal group on day 0 (Table 2). When the same cultures were kept unstimulated for 5 days, minimal increase in expression of these markers was observed, which was statistically insignificant in normal and BT/TT patients. However, expression of

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**Table 1**—Percent apoptosis in PBMC of leprosy patients in the presence of mycobacterial antigens in soluble and particulate form

<table>
<thead>
<tr>
<th>Patients (n = 10)</th>
<th>0 Day</th>
<th>5th Day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unstimulated cells</td>
<td>Unstimulated cells</td>
</tr>
<tr>
<td>Controls</td>
<td>1.40 ± 0.25</td>
<td>9.18 ± 3.4( ^{i} )</td>
</tr>
<tr>
<td>BT/TT</td>
<td>2.52 ± 0.85</td>
<td>15.3 ± 5</td>
</tr>
<tr>
<td>BL/LL</td>
<td>2.84 ± 0.72</td>
<td>25.0 ± 9.2( ^{b} )</td>
</tr>
</tbody>
</table>

\( ^{a} \) vs \( ^{b} \); \( ^{c} \) vs \( ^{d} \); \( ^{e} \) vs \( ^{f} \); \( ^{g} \) vs \( ^{h} \); \( ^{i} \) vs \( ^{j} \); \( ^{k} \) vs \( ^{l} \), \( P<0.005 \).

**Table 2**—Expression of Fas/FasL on T cells in the absence and presence of M. leprae antigens with or without immunomodulators

<table>
<thead>
<tr>
<th>Groups Stimulant</th>
<th></th>
<th>Normal</th>
<th>BT/TT</th>
<th>BL/LL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fas</td>
<td>FasL</td>
<td>Fas</td>
<td>FasL</td>
</tr>
<tr>
<td>Unstimulated cells, day 0</td>
<td>28.4 ± 9.05</td>
<td>1.7 ± 1.8( ^{j} )</td>
<td>36.4 ± 10.2</td>
<td>4.1 ± 1.2( ^{f} )</td>
</tr>
<tr>
<td>Unstimulated cells, day 5</td>
<td>36 ± 8.1</td>
<td>2.8 ± 1.5</td>
<td>42 ± 6.2</td>
<td>5.6 ± 1.3</td>
</tr>
<tr>
<td>MLCwA</td>
<td>42.2 ± 8.5</td>
<td>5.4 ± 2.1</td>
<td>48.1 ± 12.5</td>
<td>7.0 ± 2.7</td>
</tr>
<tr>
<td>MLCwA+MB+Trat</td>
<td>33.2 ± 9.7</td>
<td>4.4 ± 1.5</td>
<td>35.3 ± 12.1</td>
<td>5.4 ± 2.4</td>
</tr>
<tr>
<td>ManLAM</td>
<td>45.4 ± 11.4</td>
<td>6.1 ± 2.6</td>
<td>50.6 ± 7.3</td>
<td>6.1 ± 3.0</td>
</tr>
<tr>
<td>ManLAM+MB+Trat</td>
<td>36.3 ± 12.4</td>
<td>5.2 ± 3.2</td>
<td>37.5 ± 10.5</td>
<td>4.7 ± 2.5</td>
</tr>
</tbody>
</table>

\( ^{a} \) vs \( ^{b} \); \( ^{c} \) vs \( ^{d} \); \( ^{e} \) vs \( ^{f} \); \( ^{g} \) vs \( ^{h} \); \( ^{i} \) vs \( ^{j} \); \( ^{k} \) vs \( ^{l} \), \( P<0.005 \).
FasL was significantly \((P<0.005)\) higher on unstimulated T cells of BL/LL patients, as compared to normal and BT/TT patients on 5\(^{th}\) day. Interestingly, BL/LL patients showed two-fold increased expression of FasL than normals or BT/TT patients (Table 2).

Upon activation by MLCwA or ManLAM in soluble form, all the groups showed increased expression of Fas/FasL on T cells, which was highly significant \((P<0.005)\) in BL/LL group. The percentage of T cells expressing FasL was increased by three-fold and Fas by 1.5-fold in BL/LL patients with MLCwA or ManLAM from its constitutive level (0 day/5\(^{th}\) day). When the same group of cells were presented with MLCwA or ManLAM with MB and Trat in particulate form, decreased expression of Fas/FasL on T cells was observed in all the groups, with more pronounced effect seen in BL/LL group \((P<0.005)\) as compared to soluble mode of antigen presentation.

Effect of antigen formulation on caspase-8 and -3 activities

The constitutive level of caspase-8 and -3 activities was comparatively higher in BL/LL patients, followed by BT/TT and normal healthy controls (0 day/5 day) (Table 3). In BL/LL group, 46% and 47.48% increase in caspase-8 activity and 38.47% and 39% increase in caspase-3 activity was observed upon stimulation with MLCwA and ManLAM respectively, as compared to constitutive level (5 day). Upon activation by MLCwA or ManLAM, caspase-8 and -3 activities increased in all the groups, which was highly significant \((P<0.005)\) in BL/LL patients, as compared to normal and BT/TT groups. MLCwA or ManLAM with MB and Trat in particulate form significantly \((P<0.005)\) decreased caspase-8 and -3 activities (Table 3) in BL/LL patients, as compared to normal and BT/TT patients. Particulate delivery of antigen decreased these activities in all groups, as compared to soluble form, but was significant in BL/LL group. Overall, in BL/LL group, 26.9% and 22.3% decrease in caspase-8 activity and 26.4% and 24.6% decrease in caspase-3 activity respectively was observed with particulate delivery of MLCwA and ManLAM combined with immunomodulators, respectively as compared to constitutive level (5\(^{th}\) day). The caspase inhibitors showed >50% inhibition of caspase activity on 5\(^{th}\) day (inducible) in leprosy patients and healthy controls.

**Effect of antigen formulation on expression of Bcl-xL**

To evaluate whether soluble antigen or particulate antigen formulation had any effect on the expression of anti-apoptotic protein Bcl-xL, cells were lysed and lysate was used for immunoblot. Immunoblot analysis of Bcl-xL showed that unstimulated cells of normal and leprosy patients (Fig. 1A, lane 1) showed a faint band at ~30 kDa. In the presence of MLCwA, stimulation resulted in an increased level of Bcl-xL in BT/TT patients, which was significant \((P<0.05)\) as compared to unstimulated cells (Fig. 1A, lane 2), while BL/LL patients had no effect on Bcl-xL expression. Upon activation with MLCwA + MB + Trat in liposome, there was increase of Bcl-xL level in

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**Table 3**—Caspase-8 and -3 activities in the absence and presence of *M. leprae* antigens with or without immunomodulators

<table>
<thead>
<tr>
<th>Groups</th>
<th>Stimulant</th>
<th>Mean arbitrary fluorescence unit per mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Caspase-8</td>
<td>Caspase-3</td>
</tr>
<tr>
<td>Unstimulated cells</td>
<td>401.5 ± 47.2</td>
<td>1840.1 ± 331.72</td>
</tr>
<tr>
<td>day 0</td>
<td>521 ± 44.8</td>
<td>2120 ± 64</td>
</tr>
<tr>
<td>MLCwA</td>
<td>627.8 ± 88.7</td>
<td>2386.4 ± 201</td>
</tr>
<tr>
<td>ManLAM</td>
<td>438 ± 53.3</td>
<td>1953.2 ± 483.6</td>
</tr>
<tr>
<td>MLCwA + MB + Trat</td>
<td>639.1 ± 92.3</td>
<td>2440 ± 456.2</td>
</tr>
<tr>
<td>ManLAM + MB + Trat</td>
<td>472.7 ± 46.4</td>
<td>2184 ± 285.5</td>
</tr>
</tbody>
</table>

*indicates a vs. b; b vs. c; d vs. e; b vs. f; b vs. g; h vs. i; i vs. j; k vs. l; i vs. m, i vs. n; s vs. d; t vs. k; t vs. l; t vs. k; P<0.005*
BL/LL patients (Fig. 1A, lane 3), which was significant ($P<0.005$) as compared to MLCwA-stimulated cells (Fig. 1A, lane 2) as well as unstimulated cells (Fig. 1A, lane 1, Fig. 1B). Normal and BT/TT patients showed marginal increase in Bcl-x$_L$ levels in the presence of same formulation (Fig. 1B). Together, these results suggested that particulate mode of antigen presentation induced increased expression of Bcl-x$_L$ levels in BL/LL patients.

Discussion

Mycobacteria are known to manipulate host cell to persist within the immune cells. Apoptosis or programmed cell death is essential for the homeostatic regulation of cells, restriction of intracellular pathogen and also for stimulation of host adaptive immune response. Earlier correlation was observed between pro-inflammatory cytokines and induction of apoptosis in some of the immune cells$^{29}$. Similarly, we also observed inhibition of T cell proliferation with elevated levels of Th2 cytokines, especially in lepromatous patients after stimulation of mononuclear cells with mycobacterial antigens$^{13}$. This study prompted us to delineate the actual mechanism(s) underlying the activation-induced cell death (AICD) in vitro of these cells derived from leprosy patients. It was found that PBMC of leprosy patients, in particular lepromatous cases showed a higher degree of apoptosis upon treatment with mycobacterial antigens (MLCwA or ManLAM), as compared to normal healthy controls (Table 1). High expression of Fas-FasL on T cells of BL/LL patients (Table 2) suggested that Fas-triggered apoptosis of PBMC could be a possible mechanism in these patients. Secondly, down-regulation of co-stimulatory molecules in these patients could result in T cell inactivation thereby leading to cell death, which could be the cause of T cell anergy$^{14}$. Lepromatous patients showed constitutively (day 5) higher expressions of Fas/FasL compared to other groups, indicating that some percentage of cells were undergoing spontaneous apoptosis. The increased expression of Fas/FasL in BL/LL patients in presence of MLCwA or ManLAM could lead to cell death as well as making the cells unresponsive to $M$. leprae antigens (Table 2). This was in accordance with the observations by others, who showed increased FasL expression on macrophages infected with mycobacterium leading to cell death$^{30}$. This observation was further confirmed by cell viability using trypan blue, which showed nearly 40% of the cells undergoing cell death after 5 days of incubation with the $M$. leprae antigens.

As caspases lie downstream in the apoptotic cascade, high activities of caspase-8 and -3 along with high expression of apoptotic markers (Fas/FasL) were observed in BL/LL patient in unstimulated as well as in stimulated cells, which may account for cell death in these patients (Table 3). Since activation of caspase-8 initiates both mitochondria independent and dependent pathways, high caspase-8 activity in leprosy patients provided evidence that caspase-8 was activated by Fas-AICD pathway. This was further supported by PI staining, which showed a sharp hypodiploid peak in the presence of mycobacterial antigen in BL/LL patients.

When the cells were cultured with Ag + MB + Trat, both the apoptotic markers (Fas/FasL) and caspase-8 and -3 activities were reverted nearly to their constitutive levels, especially in BL/LL patients, indicating the anergized cells were slowly returning to proliferating state (Table 3). This observation was in accordance with our previous reports, wherein unresponsive PBMC were made to proliferate in vitro.
with the same formulation, especially in BL/LL patients. When PBMC of all the three groups were presented with MB or Trat or a combination of both in liposome or a combination of both with antigen in medium, these cells did not show much change in the expression of either of the apoptotic markers or caspase activities. Their values remained slightly higher than the constitutive levels. Results of the above study indicated that Ag + MB + Trat combination in particulate mode of presentation to the mycobacterial sensitized PBMC appeared to be a requisite for down-regulating the Fas-AICD pathway, leading to cell survival and proliferation.

Since we observed spontaneous apoptosis of mononuclear cells in unstimulated conditions, which was reversed using the same formulation, we studied the expression of anti-apoptotic protein Bcl-xL in leprosy patients. The increased expression of Bcl-xL in BL/LL patients in presence of liposomal formulation could prevent cells from undergoing death (Fig. 1). This was in accordance with observations by others, who showed Bcl-xL is known to protect cells against apoptotic death.

Earlier, we demonstrated that co-stimulatory molecules such as CD28, B7.1 and B7.2 present on APC and T cells were downregulated upon presentation with mycobacterial antigens. The downregulation of these co-stimulatory molecules could lead to apoptosis, which was evident from PI study. This could be one of the main causes of T cell anergy. When PBMC were stimulated with Ag+MB+Trat formulation in liposomes, an upregulation of CD28, CD80 and CD86 was observed especially in BL/LL patients, followed by IL-2 release from these cells. This could explain the relieving of T cell anergy in leprosy patients. In the present study, an upregulation of Bcl-xL protein was observed in Western blot analysis, thus it could be inferred that IL-2 positively modulated Bcl-xL and Bcl-2, there by inhibiting apoptosis. Moreover, this formulation also upregulated Th1 cytokines and downregulated Th2 cytokines (viz., IL-4 and IL-10), thereby reversing T cell anergy in these patients. The present study clearly demonstrated reversal of T cell anergy by decreasing the apoptosis of T cells using potential immunomodulators viz., murabutide and Trat in particulate form.

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References