Immune Response to Recombinant Mycobacterial Proteins in Patients with Tuberculosis Infection and Disease

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The capacity of four Mycobacterium tuberculosis recombinant antigens to elicit proliferation and cytokine production by human T cells was evaluated. Proliferative responses of peripheral blood mononuclear cells (PBMC) to all antigens were greater in healthy tuberculin reactors than in pulmonary tuberculosis patients, and proliferative responses of pleural fluid cells were greater than those of PBMC from patients with tuberculous pleuritis. The proliferative responses to the four recombinant antigens were similar in all patient groups, and there was no selective unresponsiveness to any antigen in pulmonary tuberculosis patients. The 38-kDa antigen induced less interferon-γ than did the 10-, 30-, and 65-kDa antigens, and all four antigens induced similar amounts of interleukin-10. These results suggest that none of the four recombinant antigens are immunodominant, and that the 10-, 30-, and 65-kDa antigens are similar in their capacity to induce a potentially protective Th1-like response.

Protective immunity against Mycobacterium tuberculosis is thought to be mediated by T cells that recognize mycobacterial antigens and secrete the Th1 cytokine interferon-γ [1]. Identification of mycobacterial antigens that stimulate a human Th1 response is critical to development of an effective antituberculosis vaccine. Conversely, antigens that primarily elicit production of the Th2 cytokines interleukin (IL)-4 and -10 may suppress cellular immunity and should probably be excluded from a vaccine. One approach to identify mycobacterial antigens that induce protective immunity is to characterize antigens recognized by T cells from healthy tuberculin reactors with protective immunity but not by T cells from patients with advanced pulmonary tuberculosis and ineffective immunity. A second approach is to identify mycobacterial antigens selectively recognized by T cells at the site of disease from patients with a resistant immune response, such as those with tuberculous pleuritis, in whom disease often resolves without therapy [2].

Although many studies have evaluated the capacity of individual mycobacterial antigens to elicit immune responses in animals and lymphocyte proliferation in humans, few reports have compared human lymphocyte proliferative responses and cytokine production elicited by different mycobacterial antigens [3–5], and none have compared these responses to a series of recombinant antigens. Four antigens (10-, 30-, 38-, and 65-kDa) are of particular interest because they elicit strong proliferative responses by human T cells [3, 6–9]. The recent availability of these antigens in recombinant form provided the opportunity to evaluate the proliferative and cytokine responses to these proteins in the absence of proteins that contaminate purified antigen preparations. We therefore evaluated the capacity of these four recombinant antigens to elicit proliferation and cytokine production by peripheral blood mononuclear cells (PBMC) from healthy tuberculin reactors and tuberculosis patients and from pleural fluid mononuclear cells from patients with tuberculous pleuritis.

Methods

Patient population. Blood was obtained from 15 healthy tuberculin reactors, 5 healthy tuberculin-negative donors, and 15 patients with a first episode of culture-proven pulmonary tuberculosis and positive acid-fast sputum stains. Pleural fluid and blood were obtained from 8 patients with pleural tuberculosis. Six of these 8 patients had culture-proven tuberculosis; the other 2 had exudative pleural effusions with a lymphocytic predominance, positive tuberculin skin tests, histologic evidence of granulomatous pleuritis, and a clinical response to antituberculosis therapy. All patients had negative ELISAs for human immunodeficiency virus antibody and had received antituberculosis therapy <1 week before blood and pleural fluid samples were obtained.

Recombinant antigens. The 38-, 65-, and 70-kDa recombinant mycobacterial proteins were provided by J. van Embden (UN Developmental Program/World Bank/WHO Special Joint Program for Research in Tropical Diseases, Bilthoven, Netherlands). Using the pMAL expression system, recombinant 10-kDa antigen was...
produced by a method used to produce the *Mycobacterium leprae* 10-kDa homologue [10]. Recombinant 30-kDa α antigen was expressed and purified using the QIA expression system (Qiagen, Chatsworth, CA) according to the manufacturer’s instructions. Briefly, the gene encoding the 30-kDa mature protein, without the signal sequence, was cloned into a pQE vector at BamHI-SphI sites downstream of a 6× histidine affinity tag. The cells expressing the fusion protein were grown to log phase, and the recombinant protein was induced by isopropyl-β-D-thiogalactopyranoside at 37°C for 3 h. It was then purified from the bacterial lysate by the affinity of 6× histidine for Ni, using a Ni-nitrilotriacetic acid resin column. Recombinant 30-kDa antigen was eluted with a 10–250 mM imidazole gradient and separated from the contaminating proteins by fast-performance liquid chromatography using a mono Q anion exchange column with 20 mM–1 M salt gradients. Fractions containing the 30-kDa protein were pooled and dialyzed against PBS. All recombinant proteins were freed of endotoxin by passage over Detoxigel affinity columns (Pierce Chemical, Rockford, IL), after which endotoxin contamination was 0.002–0.008% for the 10-, 30-, 38-, and 65-kDa antigens, and >0.1% for the 70-kDa antigen (limulus amebocyte assay; BioWhittaker, Walkersville, MD). Because of significant endotoxin contamination, the 70-kDa antigen was not used in further experiments.

**Proliferative responses.** Pleural fluid mononuclear cells or PBMC were isolated by standard techniques [6] and plated in 200-μL flat-bottomed wells at 10^5 cells/well in RPMI (GIBCO, Grand Island, NY) containing penicillin/streptomycin (GIBCO) and 10% heat-inactivated human serum. PBMC were cultured in triplicate in the presence of medium alone, heat-killed *M. tuberculosis* Erdman (10 μg/mL), or the recombinant antigens. Preliminary experiments revealed that the maximal proliferative responses were elicited by the 30-, 38-, and 65-kDa antigens at 10 μg/mL. Different individuals showed maximal proliferation to the 10-kDa antigen at either 5 or 20 μg/mL so we used both concentrations. Proliferative responses were determined by measurement of [3H]thymidine incorporation, as previously described [6], and were highest after 6 days of culture for all antigens. Responses were expressed as changes in counts per minute (Δ cpm), which is the difference between cpm in the presence of antigen and that in medium alone.

**Measurement of cytokine concentrations by ELISA.** Cell cultures were established as outlined above except that 2 × 10^5 cells/well were used. Cytokine concentrations were measured by ELISA using antibodies provided by Genentech (South San Francisco; interferon-γ) and M. Gately (Hoffmann-La Roche, Nutley, NJ; p70, heterodimeric IL-12) or purchased from Monoclonal Antibody Technology (Stockholm; IL-4) and Pharmingen (San Diego; IL-10). Detection limits for interferon-γ and IL-10 were 25 and 20 pg/mL, respectively. The detection limit for IL-4 and IL-12 was 10 pg/mL. Cytokine concentrations are shown as the concentration in supernatants of antigen-stimulated cells. Cytokines were not detectable in supernatants of cells cultured in medium alone. Preliminary experiments established the time points for maximal cytokine concentrations; results are reported for those time points (24, 48, and 72 h for IL-12, -4, and -10, respectively, and 96 h for interferon-γ).

**Results**

**Proliferative responses.** Proliferative responses of PBMC to *M. tuberculosis* and to the recombinant antigens were greater in 11 healthy tuberculin reactors than in 13 patients with pulmonary tuberculosis (*P < .05* for all antigens, Student’s *t* test; figure 1A). There were no significant differences in proliferative responses to any recombinant antigens among healthy tuberculin reactors or pulmonary tuberculosis patients. PBMC from healthy tuberculin-negative donors proliferated only in response to the 65-kDa protein (mean Δ cpm, 8050 ± 1256). This protein is highly conserved among many bacteria to which people are exposed, and lymphocyte responses in tuberculin-negative persons probably reflect recognition of conserved epitopes.

Proliferative responses to the recombinant antigens were all markedly enhanced in pleural fluid cells, compared with PBMC, of 8 patients with tuberculous pleuritis (*P < .05* for all antigens, paired *t* test; figure 1B). These findings suggest there is no selective proliferative response to any of the four recombinant antigens by T cells from persons with protective immunity to tuberculosis nor by T cells from the site of disease in patients with a resistant immune response. Furthermore, no selective unresponsiveness to any recombinant protein was observed in patients with pulmonary tuberculosis.

**Cytokine production.** When PBMC from 12 healthy tuberculin reactors were cocultured with the recombinant antigens, there was wide variation in interferon-γ production, but mean concentrations were similar for the 10-, 30-, and 65-kDa antigens (figure 2A). Interferon-γ production in response to the 38-kDa antigen was significantly reduced compared with responses to the 30- and 65-kDa antigens (*P < .02*, paired *t* test). Antigen-stimulated PBMC from 13 tuberculosis patients produced very low levels of interferon-γ in response to all antigens. PBMC from healthy tuberculin-negative donors produced small amounts of interferon-γ only in response to the 65-kDa protein (mean, 37 ± 25 pg/mL).

Antigen-induced IL-10 production by PBMC from healthy tuberculin reactors and tuberculosis patients was highly variable, and there were no significant differences in IL-10 concentrations elicited by any of the four proteins (figure 2B). Although mean IL-10 production in response to some antigens was higher in tuberculosis patients than in healthy tuberculin reactors or in tuberculosis-negative persons, these differences were not statistically significant. IL-12 and -4 were not detected in any supernatants.

**Discussion**

Our results demonstrate that 10-, 30, 38-, and 65-kDa recombinant *M. tuberculosis* antigens are similar in their capacity to induce proliferation by lymphocytes from persons with protective immunity and by lymphocytes from the site of disease, suggesting that none of the four antigens are immunodominant. Proliferative responses to all antigens were reduced in PBMC from tuberculosis patients with ineffective immunity. The 38-kDa antigen induced less interferon-γ production by PBMC from healthy tuberculin reactors than did the other three anti-
Figure 1. Proliferative responses to heat-killed *Mycobacterium tuberculosis* Erdman (TBE) and recombinant *M. tuberculosis* proteins. A, Responses of peripheral blood mononuclear cells (PBMC) from healthy tuberculin reactors (PPD+) and pulmonary tuberculosis patients (TB). B, Responses of pleural fluid mononuclear cells and PBMC from patients with tuberculous pleuritis. Data are mean ± SE. Delta cpm is difference between cpm in presence of heat-killed *M. tuberculosis* and cpm in presence of medium alone. Mean cpm (±SE) with medium alone were 951 ± 320 for healthy tuberculin reactors and 1203 ± 462 for pulmonary tuberculosis patients. In patients with tuberculous pleuritis, mean cpm with medium alone for PBMC and pleural fluid cells were 1243 ± 421 and 5346 ± 1235, respectively.

gens, but all four antigens were similar in their capacity to elicit IL-10 production, and IL-4 was not detectable in any supernatants. When PBMC are cocultured with mycobacterial antigens, T cells are the major source of interferon-γ [11], whereas monocytes are the major source of IL-10 [12]. Thus, the 10-, 30-, and 65-kDa antigens are similar in their capacity to induce a Th1-like response dominated by T cell production of interferon-γ but not IL-4. The reduced interferon-γ production induced by the 38-kDa antigen suggests that this protein does not elicit a strong Th1 response and is therefore less likely to induce protective immunity.

Havlíř et al. [3] used the T cell Western blot technique and demonstrated lower proliferative responses in tuberculosis patients to a broad variety of antigens of 30–90 kDa [3]. They found selectively reduced reactivity to the 30-kDa antigen in tuberculosis patients, which was not confirmed in the current study. This difference may be due to differential responses to the native and recombinant 30-kDa antigen. The native antigen

Figure 2. Concentrations of interferon (IFN)-γ (A) and interleukin (IL)-10 (B) elicited by coculture of recombinant *Mycobacterium tuberculosis* proteins with peripheral blood mononuclear cells from healthy tuberculin reactors (PPD+), pulmonary tuberculosis patients (TB), or healthy tuberculin-negative persons (PPD−).
may be contaminated with small amounts of other proteins or glycolipids, which may suppress proliferative responses in tuberculosis patients. Alternatively, there may be selective lack of reactivity in tuberculosis patients to conformational or glycosylated epitopes on the native protein that are not present on the recombinant protein.

Our results confirm and extend those of Boesen et al. [4], who recently showed that lymphocytes from healthy tuberculosis reactors proliferate and produce interferon-\(\gamma\) in response to a wide variety of culture filtrate proteins separated by SDS-PAGE, whereas lymphocytes from patients with advanced tuberculosis do not reduce proliferative responses. However, they noted minimal proliferative responses to a variety of purified recombinant proteins, including the 10-, 30-, and 38-kDa antigens. The higher proliferative response we observed is probably due to the systematic removal of immunosuppressive lipopolysaccharide from the preparations used in our study and provides a more accurate reflection of the relative capacity of these antigens to induce lymphocyte proliferation and cytokine production.

Recent advances in mycobacterial genetics have laid the groundwork for development of an antituberculosis vaccine by insertion of mycobacterial genes encoding "protective antigens" into bacterial vaccine vectors [13]. Development of such a vaccine would be simplified if a single immunodominant mycobacterial antigen induced protective immunity in the vast majority of the population. However, the current and previous reports indicate that human T cells respond to a broad diversity of mycobacterial antigens and that none of the available recombinant mycobacterial antigens are clearly immunodominant in eliciting lymphocyte proliferation or producing Th1 cytokines. Vaccination with multiple antigens, an approach that can protect against tuberculosis in guinea pig and murine models [14, 15], is likely to be needed to provide effective immunity against tuberculosis.

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References