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Cell-Mediated Immune Responses of Healthy Laboratory Volunteers to Sonicate Antigens Prepared from the Most Prevalent Strains of *Mycobacterium tuberculosis* from South India Harboring a Single Copy of IS*6110*

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Our restriction fragment length polymorphism (RFLP) studies have shown that the most prevalent (40%) strains of *Mycobacterium tuberculosis* from South India contain a single copy of the IS*6110* insertion sequence and are of importance in studying virulence and immunity. Sonicate antigens from seven such strains were used to study in vitro T-cell proliferation and gamma interferon (IFN-γ) and interleukin-12 (IL-12) secretion as markers of protective immunity in 25 healthy subjects positive for purified protein derivative (PPD). The standard PPD and heat-killed H37Rv antigens induced the maximum levels of T-cell proliferation and IFN-γ secretion but low levels of IL-12. All sonicate antigens induced T-cell proliferation and IFN-γ secretion with strong positive correlation. Our results suggest that sonicate antigens from the most prevalent and recent strains of *M. tuberculosis* from clinical isolates have the potential to induce T-cell activation and may allow newer and specific antigens to be further characterized for diagnosis and vaccine development.

Tuberculosis remains a major international health problem which is likely to become even more critical in coming years because of the high incidence of human immunodeficiency virus disease in regions where infection with the intracellular pathogen *Mycobacterium tuberculosis* is endemic. Although BCG (live attenuated *Mycobacterium bovis*) vaccine used against tuberculosis is available, its protective efficacy ranged from 0 to 80% in different clinical trials (9, 16). Therefore, more effective antituberculosis vaccines are required to combat the tuberculosis epidemic. The criterion for developing such vaccines is to select the mycobacterial antigens from the current and most prevalent *M. tuberculosis* strains from clinical isolates in the community, which may induce a greater immune response than the standard laboratory strains do. In many reports, extracellular antigens or the secretory proteins released by live *M. tuberculosis* in the culture medium have been tested for their vaccine potential and demonstrated to induce substantial levels of protection in animal models (2, 13, 18) and also in in vitro studies (8, 21). However, the culture filtrate proteins used in these studies were conditionally secreted and showed varied protein profiles. In recent studies, whole-cell lysate or sonicate antigens of *M. tuberculosis* inclusive of cytosolic proteins and membrane proteins have also been used for diagnostic purposes (11, 19) and to induce protective immunity (23).

Our restriction fragment length polymorphism studies have shown that about 40% of *M. tuberculosis* strains from South India contain a single copy of the IS*6110* insertion sequence in the genomic DNA and are widely spread in the community (6). The insertion of this mobile genetic element occurs at different sites in the genome and may influence the phenotype of the strains, which is of importance in studying their virulence and role in immunity (20). In this study, we prepared sonicate antigens from the most prevalent strains of *M. tuberculosis* obtained from the BCG trial area of Tiruvallur District, South India, and characterized them epidemiologically by restriction fragment length polymorphism studies using an IS*6110* probe (15). We assessed their ability to induce protective immunity in healthy subjects by studying the lymphoproliferative response and cytokine response to these antigens as markers of T-cell activation and compared them with standard purified protein derivative (PPD)- and *M. tuberculosis* H37Rv-induced responses.

In total 14 clinical strains with a single copy of IS*6110* were selected based on different insertion sites of this mobile genetic element in the genome and designated S1 to S14. Out of 14, seven sonicate antigens (S1, S2, S6, S7, S8, S10, and S12) showed differential expression of proteins in a low-molecular-mass region (12 to 30 kDa) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (J. Madhumathi et al., personal communication) and hence were selected for further studies of T-cell activation markers. The sonicate antigens were prepared by sonicating the bacilli in Soniprep 150. The supernatants after centrifugation were filtered through a 0.45-μm-pore-size membrane and stored in aliquots at −70°C. The protein content was determined by Lowry's method (Bangalore Genei).

The peripheral blood mononuclear cells (PBMC) were prepared from 25 healthy, PPD-positive laboratory volunteers by Ficoll-Hypaque (Amersham) density gradient centrifugation. In total 0.1×10^6 cells/well were cultured in round-bottomed 96-well plates (Nunclon; Nunc, Roskilde, Denmark) in triplicate with PPD (10 μg/ml) (Ministry of Fisheries, Weybridge, Surrey, United Kingdom), heat-killed H37Rv (10 μg/ml; whole bacilli killed at 80°C for 30 min), and sonicate antigens (10 μg/ml). Proliferation was assessed by measuring [^{3}H]thymidine

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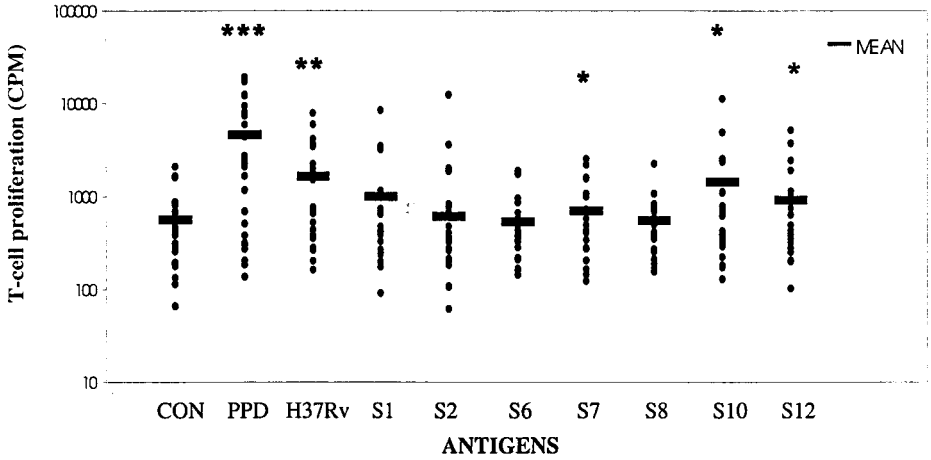

FIG. 1. Lymphoproliferative responses of 25 healthy subjects to sonicate antigens from clinical isolates (S1, S2, S6, S7, S8, S10, and S12) of *M. tuberculosis* and standard antigens, PPD and H37Rv. Results are individual counts per minute and means. Statistical significance: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. CON, control.

incorporation (0.5 μCi/well) by liquid scintillation counting (Wallac 1409 liquid scintillation counter).

For the cytokine assay, 0.5×10^6 cells/ml were cultured in flat-bottomed 48-well plates (Costar, Cambridge, Mass.) with the above antigens and incubated at 37°C in a 5% CO_2 incubator for 5 days. The culture supernatants were harvested and stored immediately at −70°C until the enzyme-linked immunosorbent assay was done. Gamma interferon (IFN-γ) and interleukin-12 (IL-12) levels were estimated by using a sandwich enzyme-linked immunosorbent assay kit (R & D Systems) according to the manufacturer's instructions. Arithmetic means and standard errors of the means were calculated. The significance of differences was estimated by Student's paired *t* test.

Tuberculosis is endemic in South India, and the population there is already sensitized to mycobacterial antigens and/or most of it is infected with *M. tuberculosis*. These individuals would have developed protective immunity to *M. tuberculosis*, and studying their immune response to these newer sonicate antigens would be more relevant to correlating effective protective immunity. Hence, we studied the in vitro response of PPD-positive healthy laboratory volunteers to the above antigens. Lymphoproliferation and IFN-γ secretion are the parameters of T-cell activation and establish the induction of immune response (1, 10). PBMC are rich in T cells that actively proliferate and secrete IFN-γ in vitro. Our results for T-cell proliferation and IFN-γ levels are summarized in Fig. 1 and 2. As expected, the standard antigens, PPD and H37Rv, showed the maximum proliferative response and IFN-γ production ($P < 0.01$) compared to the control cells. In total 80 to 92% of the

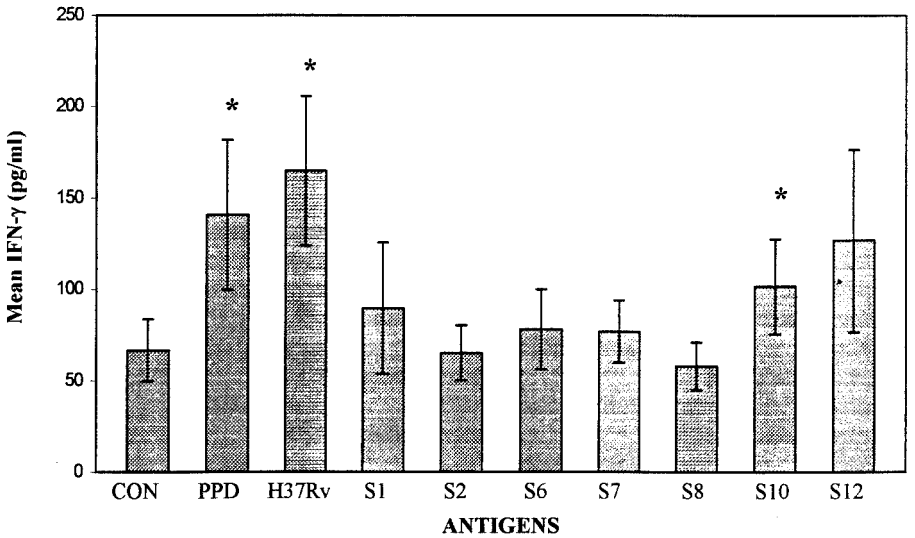

FIG. 2. IFN-γ levels in healthy laboratory volunteers induced by *M. tuberculosis* sonicate antigens from clinical isolates (S1, S2, S6, S7, S8, S10, and S12) and standard antigens, PPD and H37Rv. Results are means ± standard errors of the means. Statistical significance: *, $P < 0.05$. CON, control.

healthy subjects responded to PPD and H37Rv, while only 48 to 76% of subjects showed a lymphoproliferative response to sonicate antigens. Among them, the antigens S7, S10, and S12 induced statistically significant T-cell proliferation ($P < 0.05$) compared to the control. All sonicate antigen preparations (except S2 and S8) were potent inducers of IFN-γ, but only S10 induced statistically significant IFN-γ levels ($P < 0.05$). The sonicate antigen preparations also induced IL-12 secretion but not to a statistically significant extent (data not shown). In our earlier study, we reported similar types of responses induced by PPD and heat-killed H37Rv in PPD-positive subjects (7). In other in vitro studies using PPD and heat-killed mycobacteria as antigens, the authors reported increases in IFN-γ levels and the Th1 type of immune response, thus supporting our observations (4, 17, 22).

Though PPD and heat-killed H37Rv generated a stronger immune response, it is highly cross-reactive and nonspecific in the case of PPD, and many purified antigens of H37Rv, though specific, are not enough for developing an ideal diagnostic test or vaccine (14). Hence, there are a need and scope for adding newer antigens from the most recent and prevalent strains of *M. tuberculosis* to induce more specific and stronger immune responses. In one of the recent reports, Siddiqui et al. have studied the efficacy of culture filtrate proteins from clinical isolates of *M. tuberculosis*; they reported increased T-cell activation by these antigens compared to that for the standard laboratory strains and asserted that these culture filtrate proteins are a better source of potential candidates for a tuberculosis vaccine (21). Similarly, we planned this study with a view to identifying the best sonicate antigen from the highly prevalent strains from South India that induce protective immunity. We studied two parameters of protection for these antigens and found that most antigens induced both T-cell proliferation and IFN-γ secretion with a strong positive correlation ($r = 0.782$) (Fig. 3). Among them S10 and S12 showed comparatively higher levels of T-cell activation; however, S10 showed the best correlation of these two parameters (Fig. 3). Hence, these antigens look like promising immunogens, which could be exploited to study their role in mycobacterial immunity and virulence. The major contribution to IFN-γ production was from the activated-sensitized T cells rather than from NK cells, as the NK cell population in total PBMC is very low.

IL-12 is a crucial cytokine in controlling *M. tuberculosis* infection. It is secreted by macrophages after stimulation with mycobacterial antigens and induces IFN-γ secretion by T cells, driving the development of the Th1 response (5, 12). All sonicate antigens induced uniform secretion of IL-12, but no significant results were observed. However, the antigens PPD, H37Rv, S10, and S12, which induced significant levels of IFN-γ, induced lower levels of IL-12 than did the control and other antigens (data not shown). This may be due to IL-12 utilization by appropriate T-cell receptor engagement for stimulation of T-cell proliferation and IFN-γ secretion (3).

So, in our search for protective antigens, we identified two South Indian clinical isolates, S10 and S12, which are potent inducers of T-cell proliferation and also IFN-γ secretion in PPD-positive laboratory volunteers. The specific epitopes of these antigens involved in T-cell activation have to be identified to obtain a greater protective immune response. These

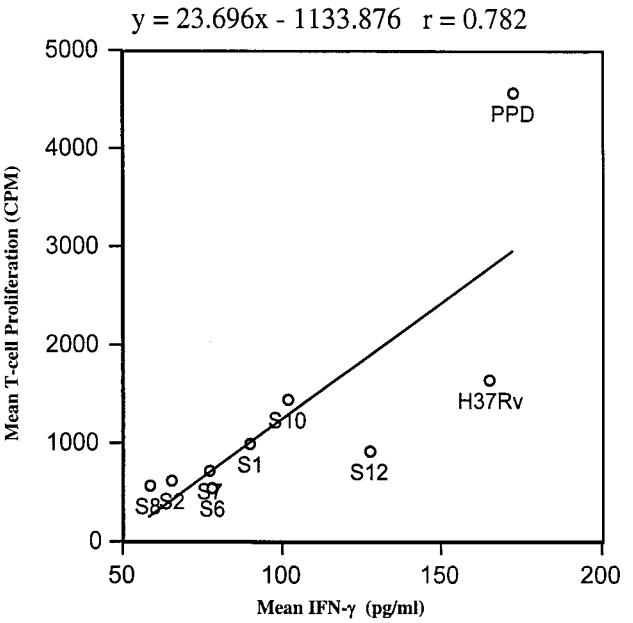

FIG. 3. Correlation of T-cell proliferation response and IFN-γ secretion induced by various sonicate antigens from clinical isolates of *M. tuberculosis* and standard antigens, PPD and H37Rv.

protective epitopes can then be utilized either for vaccine development or for diagnostic tests.

It is known that the occurrence of insertion sequence IS*6110* at different sites in the genome may influence phenotypes of the strains, but at this stage we are not able to directly correlate the phenotypic differences observed in the protein profiles of these strains with the immunological parameters studied here. We are currently looking at the infectivity and apoptosis induced by these strains in relation to immune response. Further molecular studies are required to establish the influence of mobile genetic elements in immune response.

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REFERENCES

1. **Almeida, M. G. B., S. Chitale, and I. Boutsikakis.** 1998. Induction of *in vitro* human macrophage anti-*Mycobacterium tuberculosis* activity: requirement of IFN-γ and primed lymphocytes. J. Exp. Med. **160:**4490–4499.
2. **Anderson, P.** 1994. Effective vaccination of mice against *Mycobacterium tuberculosis* infection with a soluble mixture of secreted mycobacterial proteins. Infect. Immun. **62:**2536–2544.
3. **Bertagnoli, M. M., B.-Y. Lin, D. Young, and S. H. Herrmann.** 1992. IL-12 augments antigen-dependent proliferation of activated T lymphocytes. J. Immunol. **149:**3778–3783.
4. **Cher, D. J., and T. R. Mosmann.** 1987. Two types of murine helper T cell clones. II. Delayed type hypersensitivity is mediated by Th1 clones. J. Immunol. **138:**3688–3694.
5. **Cooper, A. M., J. Margram, J. Ferrante, and I. M. Orme.** 1997. IL-12 is crucial to the development of protective immunity in mice intravenously infected with *Mycobacterium tuberculosis*. J. Exp. Med. **186:**39–45.
6. **Das, S., C. N. Paramasivan, D. B. Lowrie, R. Prabhakar, and P. R. Narayanan.** 1995. IS*6110* restriction fragment length polymorphism typing of clinical isolates of *Mycobacterium tuberculosis* from patients with pulmonary tuberculosis in Madras, south India. Tuber. Lung Dis. **76:**550–554.
7. **Das, S. D., P. R. Narayanan, C. Kolapan, and M. J. Colston.** 1998. The cytokine response to bacille Calmette Guerin vaccination in South India. Int. J. Tuberc. Lung Dis. **2:**836–843.
8. **Demissie, A., P. Ravn, J. Olobo, T. M. Doherty, T. Eguale, M. Geletu, W. Hailu, P. Andersen, and S. Britton.** 1999. T-cell recognition of *Mycobacte-*

rium tuberculosis culture filtrate fractions in tuberculosis patients and their house contacts. Infect. Immun. **67:**5967–5971.
9. **Fine, P. E. M.** 1989. The BCG story: lessons from the past and implications for the future. Rev. Infect. Dis. **12:**353–359.
10. **Flynn, J. A., J. Chan, K. J. Triebold, D. K. Dalton, T. A. Stewart, and B. R. Bloom.** 1993. An essential role for IFN-γ in resistance to *Mycobacterium tuberculosis*. J. Exp. Med. **178:**2249–2251.
11. **Katial, R. K., J. Hershey, T. Purohit-Seth, J. T. Belisle, P. J. Brennan, J. S. Spencer, and R. Engler.** 2001. Cell-mediated immune response to tuberculosis antigens: comparison of skin testing and measurement of in vitro gamma interferon production in whole-blood culture. Clin. Diagn. Lab. Immunol. **8:**339–345.
12. **Ladel, C. H., G. Szalay, D. Reidel, and S. H. E. Kaufmann.** 1997. Interleukin-12 secretion by *Mycobacterium tuberculosis*-infected macrophages. Infect. Immun. **65:**1936–1938.
13. **Lindblad, E. B., M. J. Ellay, R. Silva, R. Appelberg, and P. Andersen.** 1997. Adjuvant modulation of responses to tuberculosis subunit vaccines. Infect. Immun. **65:**623–629.
14. **Mustafa, A. S.** 2002. Development of new vaccines and diagnostic reagents against tuberculosis. Mol. Immunol. **39:**113–119.
15. **Narayanan, S., S. Das, R. Garg, L. Hari, V. B. Rao, T. R. Frieden, and P. R. Narayanan.** 2002. Molecular epidemiology of tuberculosis in a rural area of high prevalence in South India: implications for disease control and prevention. J. Clin. Microbiol. **40:**4785–4788.
16. **Orme, I. M.** 1999. Beyond BCG: the potential for a more effective B vaccine. Mol. Med. Today **5:**487–492.
17. **Orme, I. M.** 1988. Induction of nonspecific acquired resistance and delayed-type hypersensitivity, but not specific acquired resistance, in mice inoculated with killed mycobacterial vaccines. Infect. Immun. **56:**3310–3312.
18. **Pal, P. G., and M. A. Horwitz.** 1992. Immunization with extracellular proteins of *Mycobacterium tuberculosis* induces cell-mediated immune responses and substantial protective immunity in a guinea pig model of pulmonary tuberculosis. Infect. Immun. **60:**4781–4792.
19. **Raja, A., K. R. Uma Devi, B. Ramalingam, and P. J. Brennan.** 2002. Immunoglobulin G, A, and M responses in serum and circulating immune complexes elicited by the 16-kilodalton antigen of *Mycobacterium tuberculosis*. Clin. Diagn. Lab. Immunol. **9:**308–312.
20. **Sampson, S. L., R. M. Warren, M. Richardson, G. D. Van der Spuy, and P. D. Van Helden.** 1999. Disruption of coding regions by IS*6110* insertion in *Mycobacterium tuberculosis*. Tuber. Lung Dis. **79:**349–359.
21. **Siddiqui, S. M., I. M. Orme, and R. K. Saxena.** 2000. Efficacy of culture filtrate protein preparations from Indian isolates of *Mycobacterium tuberculosis* to activate T cells derived from healthy donors. Int. J. Tuberc. Lung Dis. **4:**980–987.
22. **Wisher, M. L., C. Hagan, R. Prestidge, A. U. Wells, and G. Murison.** 1999. Human *in vitro* immune responses to *Mycobacterium tuberculosis*. Tuber. Lung Dis. **79:**371–377.
23. **Worku, S., and D. F. Hoft.** 2000. *In vitro* measurement of protective mycobacterial immunity: antigen-specific expansion of T cells capable of inhibiting intracellular growth of Bacille Calmette-Guerin. Clin. Infect. Dis. **30**(Suppl. 3)**:**8257–8261.