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Vaccination of Mice with a Soluble Protein Fraction of *Mycobacterium leprae* Provides Consistent and Long-Term Protection against *M. leprae* Infection

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Groups of BALB/c mice were vaccinated intradermally with either Freund's incomplete adjuvant (FIA) alone, 10^7 heat-killed *Mycobacterium leprae* organisms in FIA, or a number of fractions of *M. leprae* containing soluble and/or cell wall components. At 1, 3, 6, 9, and 12 months later, vaccinated mice were challenged in the right hind footpad with 5,000 live *M. leprae* organisms, and vaccine protection was assessed 6 to 8 months later, at the peak of *M. leprae* multiplication in the negative control (FIA alone), by the two-sample rank-sum test. In these studies, a cell wall fraction rich in peptidoglycan was consistently ineffective. Both heat-killed *M. leprae* and a fraction containing cell wall and fixed proteins generally protected when the interval between vaccination and challenge was 1 or 3 months but not subsequently. On the other hand, soluble proteins of *M. leprae* alone or in combination (with cell wall fractions) consistently (14 of 14 instances) afforded highly significant protection ($P \leq 0.01$) at all challenge intervals up to 1 year after vaccination. These results suggest that the soluble protein fraction of *M. leprae* offers promise for a vaccine against leprosy.

Vaccines have proven to be our greatest tool in the prevention of infectious diseases. The mycobacterial diseases leprosy and tuberculosis remain serious public health problems, leprosy afflicting 12 million people worldwide (1) and tuberculosis projected to result in 1 to 2 million deaths per year by the year 2000 (13). The use of vaccines to prevent mycobacterial disease, either tuberculosis or leprosy, has centered on the use of whole attenuated mycobacteria, *Mycobacterium bovis* BCG. Although in some locales BCG vaccination has proved relatively effective (75 to 80%) in preventing both tuberculosis (2–4, 27) and leprosy (23), unfortunately it has not proved generally effective in preventing either tuberculosis (18, 19, 24, 28) or leprosy (9, 17, 22, 26).

Previously it was found that if mice were vaccinated intradermally in the right flank with 10^7 to 10^9 killed *M. leprae* organisms, BCG, or both and challenged 1 month later in the ipsilateral footpad with 5×10^3 live *M. leprae* organisms, growth of *M. leprae* was significantly reduced and usually entirely prevented (5, 20). By using such a scheme, we found that various cell wall preparations derived from 10^5 to 10^9 *M. leprae* organisms were also protective (5). In these studies, it was noteworthy that the more complex of these fractions, the so-called cell wall insoluble fraction (CWIF), afforded protection when the amount of material utilized was as little as that derived from 10^5 or 10^6 *M. leprae* organisms (5). On the other hand, in these same studies, killed *M. leprae* or more refined versions of CWIF, i.e., cell wall core (CWC) and *M. leprae* cell wall protein peptidoglycan complex (CWPPC), each protected but at amounts derived from 10^7 or more *M. leprae* organisms and not at those derived from 10^5 or 10^6 *M. leprae* organisms (5). These results suggested that components of CWIF are superior to killed *M. leprae* and the more refined cell wall-containing preparations. The nature of CWIF, arrived at in subsequent work (7, 14), provided some clues as to the nature of the protective materials. CWIF is essentially the residue remaining after whole *M. leprae* has been thoroughly extracted with chloroform-methanol mixtures (to remove lipids) and aqueous ethanol (to extract lipoarabinomannan and other lipopolysaccharides). It now appears that CWIF contains a sizable proportion of the total somatic proteins of *M. leprae*, particularly the 10-kDa protein, perhaps fixed to the peptidoglycan complex as a result of the extraction process (14). Moreover, because previously it had been demonstrated that the *M. leprae*-specific phenolic glycolipid inhibits T-lymphocyte responses (15) and lipoarabinomannan causes both inhibition of antigen-dependent lymphoproliferation (10) and gamma interferon-induced activation of macrophages (21) and since both of these products had been removed from CWIF, we postulated that the unrefined protein nature and the virtual absence of these immunosuppressive lipids accounted for the impressive vaccine protection afforded by these *M. leprae* preparations, particularly that of CWIF.

The present study was initiated to test that hypothesis, further define which components of CWIF previously afforded such profound vaccine protection, and determine whether increasing the interval between vaccination and challenge could distinguish between epitopes found protective when this interval was confined to 1 month. In this study, we found that vaccination with a fraction of CWIF, which we have termed the soluble proteins of *M. leprae*, provided consistent and prolonged immunity with challenge intervals of up to 1 year later, and that while killed *M. leprae*, CWC, and CWPPC generally protected when the interval between vaccination and challenge was 1 to 3 months, they did not protect when viable *M. leprae* challenge occurred 6, 9, and 12 months after vaccination. These results demonstrate that by increasing the interval between vaccination and *M. leprae* challenge distinctions can be made between the protective efficacies of vaccines previously all found effective when evaluated with only a single *M. leprae* challenge interval, 1 month after vaccination, and

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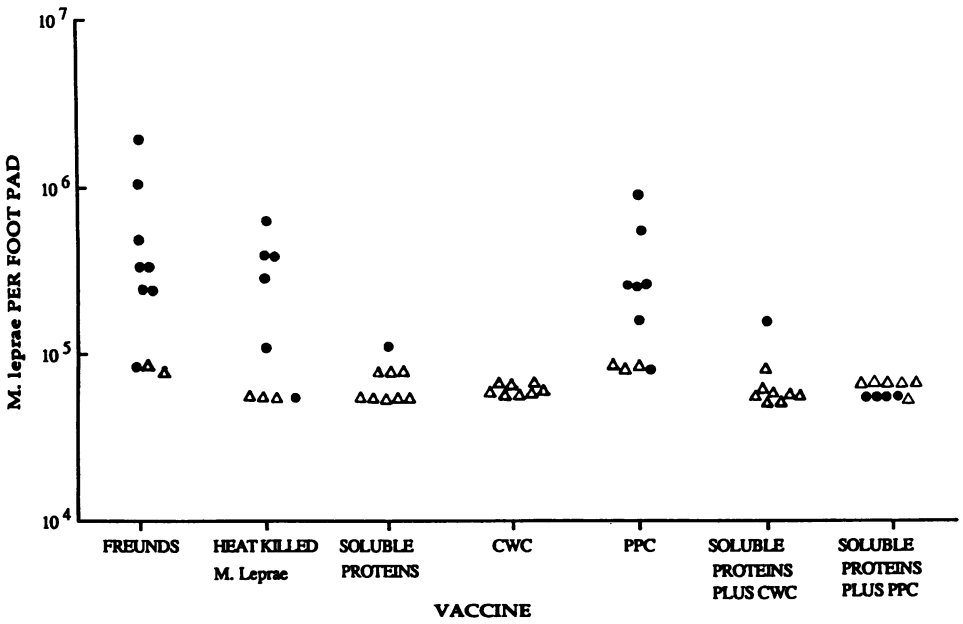

FIG. 1. One-month interval between vaccination and live *M. leprae* challenge. Symbols: ●, number of *M. leprae* organisms in one right hind footpad; △, no *M. leprae* found, plotted at a level equal to or less than the number of acid-fast bacilli present in one right hind foot pad.

that the soluble protein fraction of *M. leprae* affords both consistent and long-lasting protection.

MATERIALS AND METHODS

In these studies, groups of BALB/c mice were vaccinated in the right flank intradermally with 0.01 to 0.02 ml containing 10^7 killed *M. leprae* organisms or various *M. leprae* preparations derived from 10^7 *M. leprae* organisms, each diluted in Freund's incomplete adjuvant. The *M. leprae* preparations utilized in these studies were obtained from the same infected armadillo liver as was the killed *M. leprae*. Vaccination with 0.01 ml of Freund's incomplete adjuvant itself served as the negative control for these studies. Vaccine moieties that were tested included soluble proteins, CWC, CWPPC, soluble proteins plus CWC, and soluble proteins plus CWPPC. Most of the moieties used for vaccination, their properties, and their preparation have been described elsewhere (5); they were provided by P. J. Brennan and S. W. Hunter through National Institute of Allergy and Infectious Diseases contract ND1 A105074. A preparation utilized in this study which was not studied previously for mouse protection, the soluble proteins, is the sodium dodecyl sulfate-soluble fraction of the previously described CWIF.

Groups of 10 or more mice were vaccinated with each of these preparations and challenged 1, 3, 6, 9, and 12 months subsequently with 5,000 mouse-derived and logarithmically multiplying *M. leprae* organisms in the ipsilateral footpad. For each of these challenge intervals, protection was evaluated at the peak of multiplication (6 to 8 months postchallenge), when the number of acid-fast bacilli from each of three separate mice vaccinated with Freund's incomplete adjuvant alone reached $\geq 5 \times 10^5$ *M. leprae* organisms per footpad. Protection was assessed by comparing the number of *M. leprae* organisms in, generally, 10 right hind footpads from each vaccine group with the number present in the negative control, Freund's incomplete adjuvant alone, by using the Mann-Whitney two-sample rank-sum test and the Wilcoxon two-tailed distribution (12).

RESULTS

The results of these studies are presented in Fig. 1 to 5. Only vaccination with soluble proteins alone or combined with CWC or CWPPC consistently (14 of 14 times) protected ($P \leq 0.01$) mice from growth of *M. leprae* inoculated 1, 3, 6, 9, or 12 months after vaccination. When live *M. leprae* challenge occurred 1 month following vaccination (Fig. 1), most moieties (soluble proteins, CWC, soluble proteins plus CWC, and soluble proteins plus CWPPC) afforded significant protection ($P < 0.02$). At that challenge interval, only CWPPC and heat-killed *M. leprae* were ineffective. At a postvaccination challenge interval of 3 months (Fig. 2), only CWPPC was not protective while all of the other moieties, including heat-killed *M. leprae*, resulted in very highly significant protection ($P < 0.001$). When challenge occurred 6 months after vaccination (Fig. 3), heat-killed *M. leprae*, CWPPC, and for the first time CWC were not protective. On the other hand, soluble proteins, soluble proteins plus CWC, and soluble proteins plus CWPPC maintained very highly significant vaccine efficacy ($P < 0.001$). Similarly, when the intervals between vaccination and challenge were both 9 (Fig. 4) and 12 (Fig. 5) months, the only moieties that afforded protection were soluble proteins alone or combined with CWC or CWPPC.

DISCUSSION

We found in these studies that a soluble protein fraction of *M. leprae* afforded consistent and prolonged protection of mice from subsequent *M. leprae* infection. The finding of the importance of soluble *M. leprae* proteins in protective immunity against *M. leprae* was anticipated by our previous work (5), wherein vaccination with CWIF (containing soluble proteins) derived from 10^5 and 10^6 *M. leprae* organisms

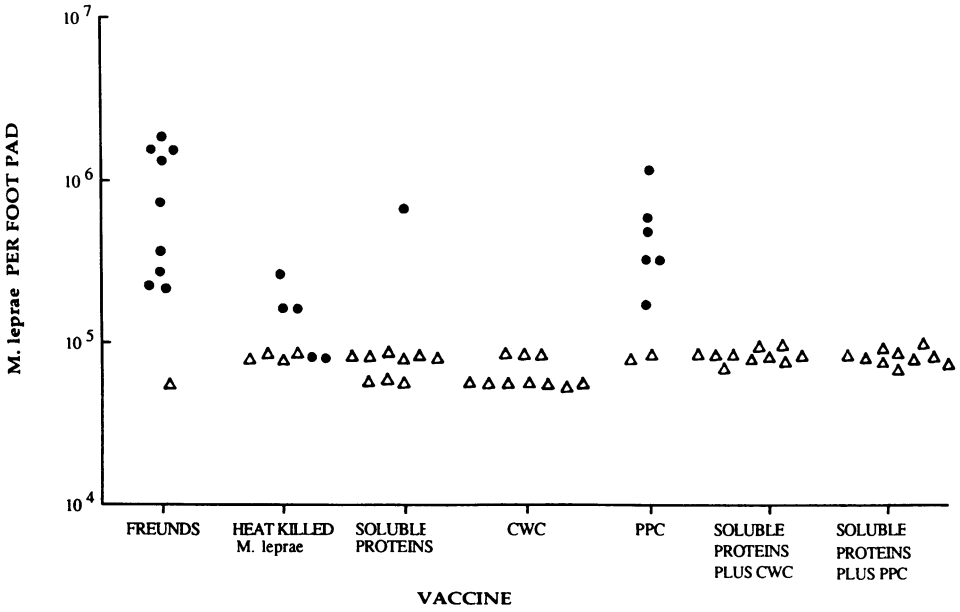

FIG. 2. Three-month interval between vaccination and live *M. leprae* challenge. The symbols are as in Fig. 1.

was found protective, while 10^7 or more heat-killed *M. leprae* organisms or CWC or CWPPC derived from 10^7 or more *M. leprae* organisms, but not those obtained from 10^5 and 10^6 *M. leprae* organisms, were required for protection.

In previous studies (5, 20) of *M. leprae* vaccine protection in mice, *M. leprae* challenge was limited to a single interval, 1 month, after vaccination. This did not allow distinctions to be made between degrees of protection resulting from vaccination with (i) heat-killed *M. leprae*, BCG, or both (20) and (ii) killed *M. leprae*, various *M. leprae* cell wall preparations, and a partially purified *M. leprae* 35-kDa protein (5). Although we previously postulated that the lack of consistent protection of BCG vaccination against leprosy infection in countries where leprosy is endemic was a consequence of the demonstrated immunosuppressive activity of complex fatty acids and carbohydrates of mycobacteria on T-lymphocyte (10, 15) and macrophage (21), functions, this study provides the first experimental evidence that a protein fraction of *M. leprae*, largely devoid of lipids, affords more consistent and prolonged protective immunity against *M. leprae* than do whole mycobacteria.

In these studies, vaccination with the soluble proteins of *M. leprae* also resulted in protection superior to that obtained with more purified *M. leprae* cell walls (CWC and

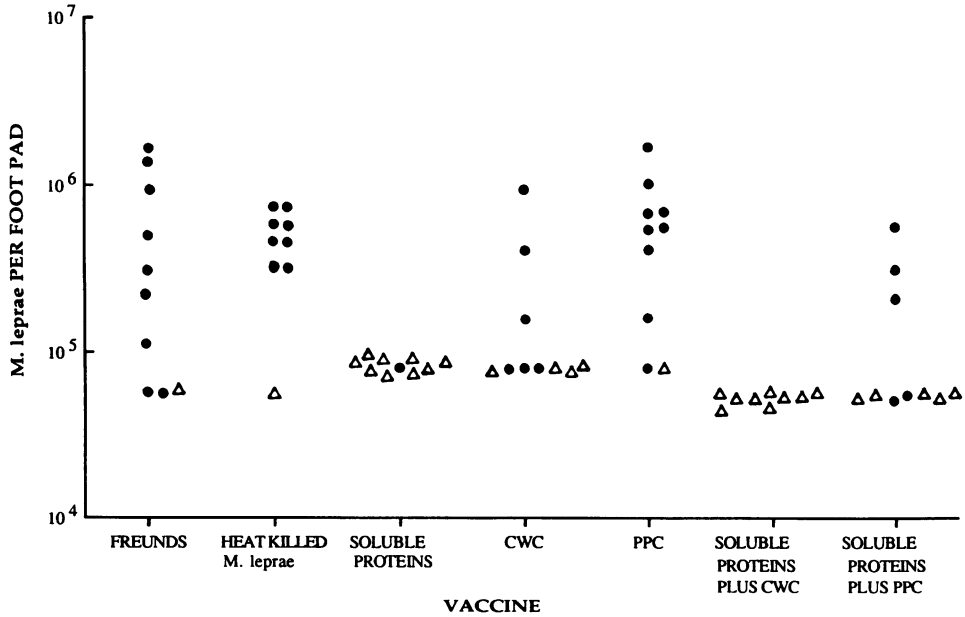

FIG. 3. Six-month interval between vaccination and live *M. leprae* challenge. The symbols are as in Fig. 1.

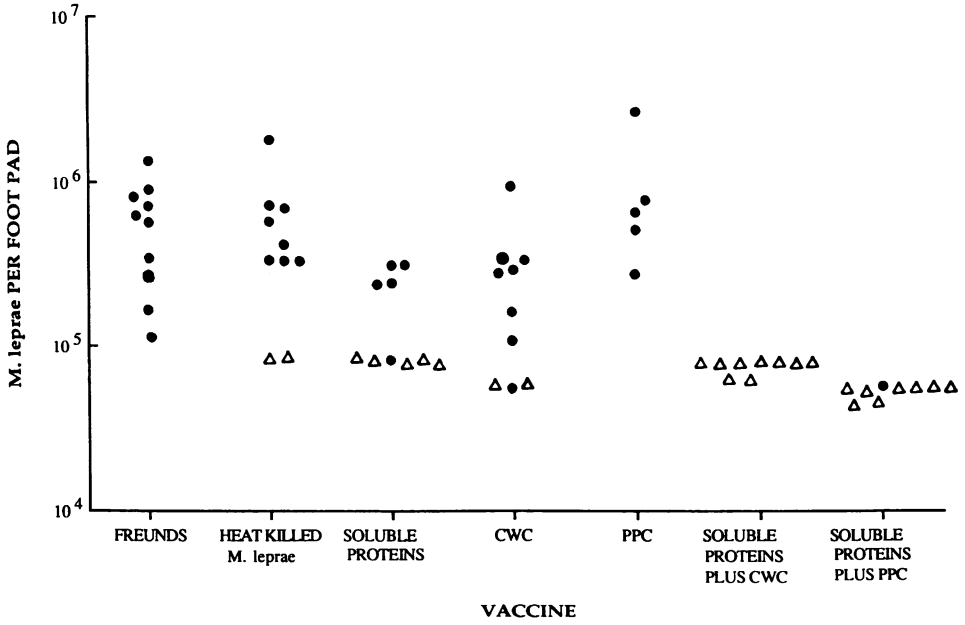

FIG. 4. Nine-month interval between vaccination and live *M. leprae* challenge. The symbols are as in Fig. 1.

CWPPC). Previously it was demonstrated that the array of cell wall proteins of *M. leprae* as they appear in CWC and CWPPC are large and insoluble, perhaps once soluble and then fixed by the extraction process (7). On the other hand, the soluble native protein fraction of *M. leprae* contains a vast number of individual proteins, including the major 10-kDa cytosolic protein, the cell membrane 35-kDa protein, and other cytosolic and membrane proteins (8). It is unclear whether the impressive protection found in this study by the *M. leprae* soluble protein fraction is a function of its simply having a larger number of protein epitopes which participate in protective immunity or amounts of one or more proteins critical to the initiation of salutary immunity. With the recent availability of purified *M. leprae* somatic proteins (8), recombinant synthesized *M. leprae* proteins and polypeptides (6, 11, 16, 25), and further subfractionation of the *M. leprae* soluble proteins, we are attempting to dissect further the role of specific *M. leprae* proteins in mouse protection against *M. leprae* infection. However, whatever the molecular basis for the prolonged and consistent protection by *M. leprae* soluble proteins found, the finding herein that such an *M. leprae* protein fraction provides protection superior to that of whole bacilli lends hope that a more effective human vaccine for leprosy and perhaps other mycobacterial diseases is possible.

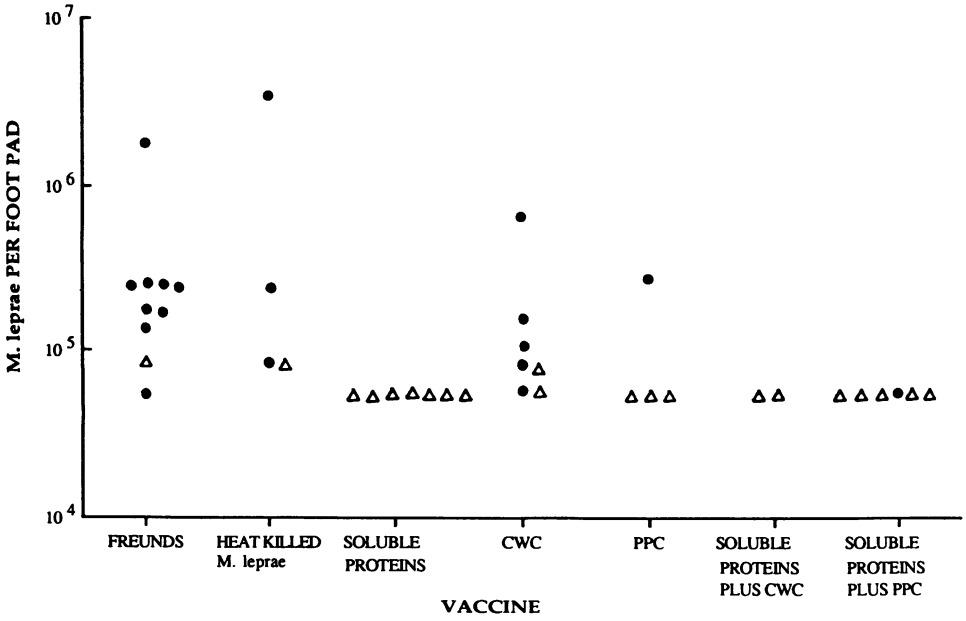

FIG. 5. Twelve-month interval between vaccination and live *M. leprae* challenge. The symbols are as in Fig. 1.

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REFERENCES

1. **Bloom, B. R., and T. Godal.** 1983. Selective primary health care: strategies for control of disease in the developing world. V. Lepr. Rev. Infect. Dis. **5**:765–780.
2. **Comstock, G. W., and C. E. Palmer.** 1966. Long-term results of BCG vaccination in the southern United States. Am. Rev. Respir. Dis. **93**:171–183.
3. **Comstock, G. W., and R. G. Webster.** 1969. A twenty-year evaluation of BCG vaccination in a school population. Am. Rev. Respir. Dis. **100**:839–845.
4. **Frimodt-Moller, J., G. Acharyulu, and K. Pillai.** 1973. Observations on the protective effect of BCG vaccination in a South Indian rural population: fourth report. Bull. W.H.O. **48**:40–52.
5. **Gelber, R. H., P. J. Brennan, S. W. Hunter, M. W. Munn, J. M. Monson, L. P. Murray, P. Siu, M. Tsang, E. G. Engleman, and N. Mohagheghpour.** 1990. Effective vaccination of mice against leprosy bacilli with subunits of *Mycobacterium leprae*. Infect. Immun. **58**:711–718.
6. **Harris, D. P., B. T. Backstrom, R. J. Booth, S. G. Love, D. R. Harding, and J. D. Watson.** 1989. The mapping of epitopes of the 18 kDa protein of *Mycobacterium leprae* recognized by murine T cells in a proliferation assay. J. Immunol. **143**:2006–2011.
7. **Hunter, S. W., M. McNeil, R. L. Modlin, V. Mehra, B. R. Bloom, and P. J. Brennan.** 1989. Isolation and characterization of the highly immunogenic cell wall-associated protein of *Mycobacterium leprae*. J. Immunol. **142**:2864–2872.
8. **Hunter, S. W., B. Revoire, V. Mehra, B. R. Bloom, and P. Brennan.** 1990. The major native proteins of the leprosy bacillus. J. Biol. Chem. **265**:1–3.
9. **Irwin, K. T., T. Sundaresan, M. M. Gyi, et al.** 1985. BCG vaccination of children against leprosy: fourteen-year findings of the trial in Burma. Bull. W.H.O. **63**:1069–1078.
10. **Kaplan, G., R. R. Gandhi, D. E. Weinstein, W. R. Levis, M. E. Patarroyo, P. J. Brennan, and Z. A. Cohn.** 1987. *Mycobacterium leprae* antigen-induced suppression of T cell proliferation in vitro. J. Immunol. **138**:3028–3034.
11. **Lamb, F. I., A. E. Kingston, I. Estrada-G, and M. J. Colston.** 1988. Heterologous expression of the 65-kilodalton antigen of *Mycobacterium leprae* and murine T-cell responses to the gene product. Infect. Immun. **56**:1237–1241.
12. **Lehmann, E. L.** 1975. Nonparametric statistical methods based on ranks, p. 5–13. Holden-Day, Inc., San Francisco.
13. **Lowell, A. M.** 1976. Tuberculosis in the world. Health Education Welfare publication no. 76-8317. Centers for Disease Control, Atlanta.
14. **Mehra, V., B. R. Bloom, A. C. Bajardi, C. L. Grisso, P. A. Sieling, et al.** 1992. A major T cell antigen of *Mycobacterium leprae* is a 10-kD heat-shock cognate protein. J. Exp. Med. **117**:275–284.
15. **Mehra, V., P. J. Brennan, E. Rada, J. Convit, and B. R. Bloom.** 1984. Lymphocyte suppression in leprae induced by unique *Mycobacterium leprae* glycolipid. Nature (London) **308**:194–196.
16. **Nomaguchi, H., M. Matsuoka, K. Kohsaka, A. Nakata, and T. Ito.** 1989. Overproduction, affinity purification and characterization of 65-kDa protein of *Mycobacterium leprae* in *Escherichia coli*. Int. J. Lepr. **57**:817–824.
17. **Noordeen, S. K.** 1985. Vaccination against leprosy: recent advances and practical applications. Lepr. Rev. **56**:703–710.
18. **Research Committee of the British Thoracic Association.** 1980. Effectiveness of BCG vaccination in Great Britain in 1978. Br. J. Dis. Chest **74**:215–227.
19. **Rosenthal, S., E. Loewinsohn, M. Graham, D. Liveright, et al.** 1961. BCG vaccination against tuberculosis in Chicago: a twenty-year study statistically analyzed. Pediatrics **28**:622–641.
20. **Shepard, C. C., R. M. Van Landinghkam, L. L. Walker, and S. Z. Ye.** 1983. Comparison of the immunogenicity of vaccines prepared from viable *Mycobacterium bovis* BCG, heat killed *Mycobacterium leprae*, and a mixture of the two for normal and *Mycobacterium leprae*-tolerant mice. Infect. Immun. **40**:1096–1103.
21. **Sibley, L. D., S. W. Hunter, P. J. Brennan, and J. L. Krahenbuhl.** 1983. Mycobacterial lipoarabinomannan inhibits gamma interferon-mediated activation of macrophages. Infect. Immun. **56**:1232–1236.
22. **Stanford, J. L.** 1983. The BCG trials. Practitioner **227**:10–13.
23. **Stanley, S. J., C. Howland, M. M. Stone, and I. Sutherland.** 1981. BCG vaccination of children against leprosy in Uganda: final results. J. Hyg. **87**:233–248.
24. **Stein, S., and J. Aronson.** 1953. The occurrence of pulmonary lesions in BCG-vaccinated and unvaccinated persons. Am. Rev. Tuberc. Pulm. Dis. **68**:695–712.
25. **Thangaraj, H. S., F. I. Lamb, E. O. Davis, P. J. Jenner, L. H. Jeyakumar, and M. J. Colston.** 1990. Identification, sequencing, and expression of *Mycobacterium leprae* superoxide dismutase, a major antigen. Infect. Immun. **58**:1937–1942.
26. **Tripathy, S. P.** 1983. The case for BCG. Ann. Natl. Acad. Med. Sci. (India) **19**:11–21.
27. **Tuberculosis Prevention Trial, Madras.** 1979. Trial of BCG vaccines in South India for tuberculosis prevention: first report. Bull. W.H.O. **57**:819–827.
28. **Tuberculosis Vaccine Clinical Trials Committee.** 1972. BCG and vole bacillus vaccines in the prevention of tuberculosis in adolescence and early adult life. Fourth report to Medical Research Council. Bull. W.H.O. **46**:371–385.