

Effective vaccination of mice against *Mycobacterium leprae* with density-gradient subfractions of soluble *M. leprae* proteins: clues to effective protein epitopes

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Summary It had previously been discovered that intradermal mouse vaccination with a protein fraction of *Mycobacterium leprae* (called soluble proteins) in Freund's incomplete adjuvant (FIA) resulted in consistent and long-lived protection against *M. leprae* multiplication from subsequent viable footpad challenges. In this study certain density-gradient subfractions of this soluble protein, but not others, in FIA afforded vaccine protection. The results of this study suggest which *M. leprae* proteins may be involved in protective immunity, particularly 1–3 kD, 10 kD, 65 kD, and those of higher molecular weight.

Introduction

It had previously been discovered that intradermal vaccination of mice with protein subunits of *Mycobacterium leprae* protected mice from multiplication of live *M. leprae*.^{1,2} When the interval between vaccination and infectious challenge was 1 month, protection was observed following vaccination both with various cell-wall fractions of *M. leprae* as well as with a partially purified protein derived from a pellet fraction of *M. leprae*.¹ Also, when the interval between vaccination and infectious challenge was 1 month, it was noted in this study that the most complex of the effective cell-wall vaccines, the so-called cell wall in soluble fraction (CWIF), afforded protection when the amount of material utilized was as little as that derived from 10^5 *M. leprae*, while 10^7 or more killed *M. leprae*, or further refined cell-wall fractions derived from 10^7 or more *M. leprae*, were required to provide protection.¹

In subsequent studies, we found that vaccination with a sodium dodecyl sulphate

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(SDS)-soluble fraction of CWIF, 'soluble proteins', provides both unique and consistently (14 of 14 instances) prolonged mouse protection.² While heat-killed *M. leprae* and progressively more refined cell-wall fractions of *M. leprae* cell wall protein (CWP) and protein peptidoglycan complex (PPC) generally protected when the interval between vaccination and challenge was 1–3 months, only soluble proteins protected when the interval between vaccination and challenge was extended to 6, 9 and 12 months.²

In order to define further the critical protective protein epitopes in these soluble proteins, separation was first performed by SDS PAGE. However, under these conditions migration of individual proteins was not observed. In order to separate these soluble proteins and help define their effective protein components, various superose-12 density-gradient fractions of the *M. leprae* 'soluble proteins' were prepared. Here we report the results of the protective efficacy of vaccination of mice with these fractions and discuss our present state of knowledge concerning the specific proteins responsible for the protection that was observed in this study.

Materials and Methods

Soluble proteins of *M. leprae* were prepared as described previously.² These were passed through a superose-12 column at pH 6.8 and separated into 10 fractions, designated fraction 8, 9, 10, 11, 12, 13, 14, 15, 16 and 22.

Groups of 10 or more mice were vaccinated intradermally in the right-hind flank with 0.01 ml of Freund's incomplete adjuvant (FIA) alone, 10^7 killed *M. leprae*, and the following vaccines emulsified in FIA: 'soluble proteins' derived from 10^7 *M. leprae* and each of the 10 separate subfractions of 'soluble proteins' derived from 10^7 *M. leprae*. Two months after vaccination mice were challenged with 5000 mouse-derived and logarithmically multiplying *M. leprae* in the ipsilateral foodpad. Protection was assessed at the peak of multiplication, when the number of acid-fast bacilli from monthly foodpad harvests of 3 separate mice vaccinated with Freund's incomplete adjuvant alone each reached $\geq 10^5$ *M. leprae* per foodpad (8 months).

Protection was assessed by comparing the number of *M. leprae* organisms in, generally, 10 right-hind foodpads from each vaccine group with the number present in the negative control, FIA alone, by using the Mann-Whitney 2-sample rank-sum test and the Wilcoxon 2-tailed distribution.³

Results

The results of these studies are presented in Figure 1. As was found previously, vaccination with heat-killed *M. leprae* ($P < 0.02$) and soluble proteins ($P < 0.0001$) protected against *M. leprae* multiplication. Fractions 8, 9 and 10 afforded significant protection ($P \leq 0.005$). While fractions 11 and 12 did not protect, fractions 16 and 22 resulted in significant protection, and fractions 13, 14 and 15 resulted in highly significant protection ($P \leq 0.0005$). Moreover, the protection afforded by soluble proteins themselves and certain of the subfractions (8, 13, 14 and 15) was found to be significantly more profound than protection afforded by killed *M. leprae* itself.

Silver-stained SDS PAGE profiles of the density-gradient fractions are presented in

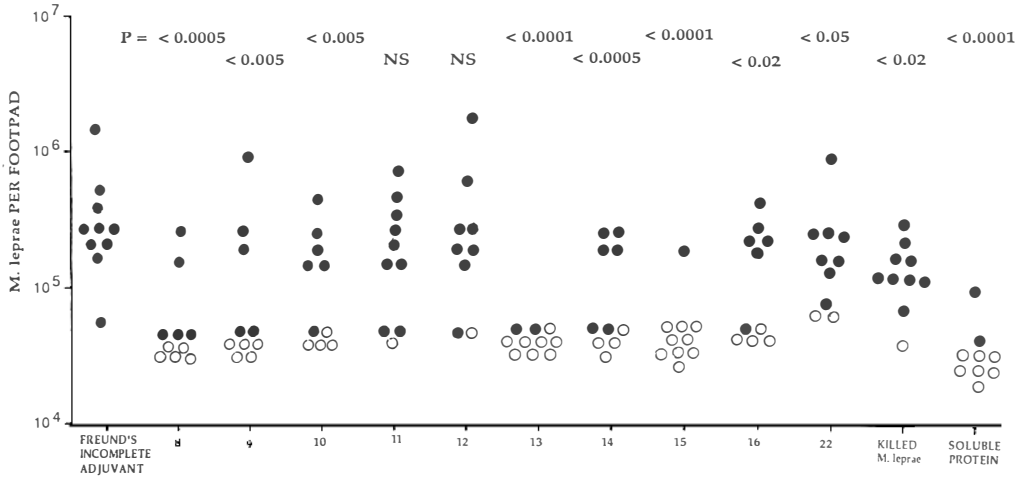


Figure 1. Top: Protection of mice from *M. leprae* multiplication by vaccination. Bottom: ● Actual number of *M. leprae*/footpad; ○ no AFB found, plotted at \leq the number of AFB this represents.

Figure 2. While fractions 8–10 contain only minimal amounts of various visible proteins, they mainly contain proteins of high molecular weight that do not migrate on the gels. Fractions 11 and 12 contain moderate amounts of protein but no substantial amounts of 1–3 kD or 10 kD proteins. Fractions 13–15 contain copious amounts of the major *M. leprae* 10 kD protein, a substantial amount of the 1–3 kD proteins, and also a minimal amount of what appears to be the 65 kD *M. leprae* protein. Fraction 16 contains mostly 1–3 kD proteins. Fraction 22 appears to contain a moderate amount of a single *M. leprae* protein, the 65 kD protein.

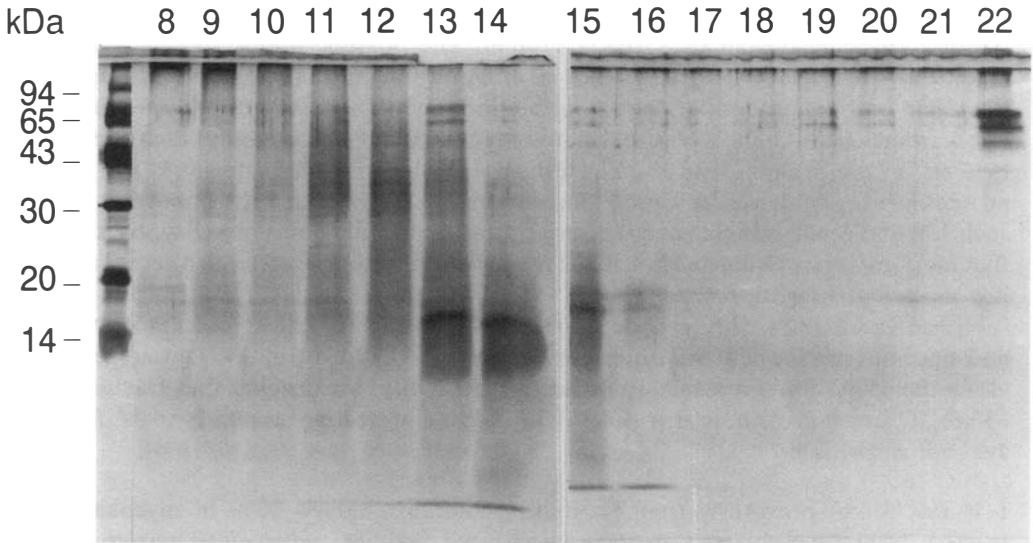


Figure 2. Silver stain of SDS polyacrylamide gel of the density-gradient subfraction vaccines utilized.

Discussion

Since the amount of protein contained in several of the protective density-gradient subfractions (particularly 8–10) was derived from less than 10^6 *M. leprae*, these findings confirm our previous results that smaller amounts of components of *M. leprae*-soluble proteins are more protective than whole killed *M. leprae* and more refined *M. leprae* cell walls.² More importantly, since we found in this study that while vaccination with certain density-gradient subfractions of *M. leprae* 'soluble proteins' prevented footpad multiplication of *M. leprae* following a subsequent challenge and others did not, this study provides some clues as to which *M. leprae* proteins are important for protective immunity. This study suggests that *M. leprae* protein epitopes of 1–3 kD, 10 kD, 65 kD and high molecular weight provide protective immunity.

The major finding of this study is that fractions 13–16 were all protective, fractions 13–15 being significantly more protective than killed *M. leprae*, and that the major protein constituent of all 3 of these fractions was a 10 kD protein. The likely importance of a 10 kD antigen to T-cell-mediated protective immunity found herein is consistent with that of previous studies, where it had been found that the *M. leprae* 10 kD antigen was, in general, a strong stimulus of T-cell responses:

- 1 It elicited PBMC responses of similar magnitude to those of *M. leprae* itself, which in patients, parallel their status in the clinical and immunologic disease spectrum.
- 2 It provoked PBMC responses greater than for other purified and recombinant *M. leprae* antigens with limiting-dilution analyses demonstrating that 1/3 of *M. leprae*-reactive T-cell precursors respond to its 10 kD antigen.
- 3 T-cells derived from lepromin skin-test-positive sites respond *in vitro* to the 10 kD antigen.
- 4 Like whole *M. leprae*, the *M. leprae* 10 kD protein elicited delayed-type hypersensitivity responses in *M. leprae*-sensitized guinea-pigs.⁴

Each of these protective fractions 13–16 also contains large amounts of 1–3 kD proteins, fraction 16 being devoid of a 10 kD protein. The possible importance of such low-molecular-weight proteins for protective immunity against *M. leprae* was previously suggested by the finding that peripheral blood mononuclear cells of *M. leprae*-reactive donors were found to incorporate tritiated thymidine most profoundly when stimulated by 2 *M. leprae* proteins, the 1–3 kD protein complex and the major 10 kD *M. leprae* protein.⁴ Also, Andersen & Heron⁵ have recently demonstrated the important role of such low-molecular-weight mycobacterial proteins in infection immunity, by showing that in *M. tuberculosis*-infected mice the majority of memory T-cells are directed at these low-molecular-weight proteins.

Since vaccination with fraction 22 resulted in lesser but still significant protection, and since the vaccine contains almost exclusively a 65 kD protein, this also suggests that alone the 65 kD protein results in protective immunity. The finding that fraction 22, a 65 kD *M. leprae* protein, is also effective in eliciting protective immunity to *M. leprae* is also not surprising:

- 1 It was found previously that by limiting-dilution analysis 20% of mycobacterial-reactive CD4⁺ lymphocytes in mice immunized with *M. tuberculosis* recognize that single protein.⁶

2 We have previously found that various, largely proteinaceous, *M. leprae* cell-wall fractions provide vaccine protection in mice and that the *M. leprae* 65 kD protein is known to be cell-wall associated.⁷

Vaccination with fractions 8–10, and especially 8, each of which contains very minimal amounts of proteins and almost exclusively those of high molecular weight, all resulted in highly significant protection. In fact, mouse vaccination with fraction 8 was found to be significantly more protective than heat-killed *M. leprae* in previous studies. Such largely insoluble high-molecular-weight proteins associated with the cell wall⁷ have been demonstrated to specifically elicit delayed-type hypersensitivity reactions and stimulate lymphoproliferative responses both from leprosy patients who limit their infection as well as from the contacts of leprosy patients.⁸

It is finally noteworthy in this study that fractions 11 and 12, though containing considerable *M. leprae* protein, afford no significant mouse protection and are devoid of large amounts of the 4 proteins implicated here to provide protective immunity.

In unpublished studies we have found that a purified and a recombinant *M. leprae* 10 kD GroEL homologue and a recombinant 65 kD *M. leprae* protein, each diluted in FIA, result in protective immunity in mice when the interval between vaccination and live *M. leprae* challenge was 1 month. Since these 2 heat-shock proteins are widely conserved across both bacterial and mammalian species, their ability to result in general immunity, species-specific immunity, and indeed autoimmunity remains both intriguing and perplexing. In order to further elucidate the issue of species-specific immunity, studies are in progress with vaccination and live *M. leprae* challenge, such as was carried out in the present study, comparing vaccine protection with: (1) a recombinant 10 kD *M. leprae* protein and the analogous recombinant 10 kD *M. tuberculosis* protein, (2) the recombinant *M. leprae* 65 kD protein and the homologous recombinant 65 kD BCG protein, and (3) wild BCG and BCG containing and expressing the 10 kD *M. leprae* protein. As in previous studies^{1,2} discrimination of relative protective efficacy is being done by utilizing the strategy of assessing results both of different amounts of vaccine and by varying the interval from vaccination to live *M. leprae* challenge from 1 month up to 1 year subsequently.

Acknowledgments

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