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# Effect of Vaccination with Refined Components of the Organism on Infection of Mice with *Mycobacterium leprae*

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**Only native products of *Mycobacterium leprae*, whether cell wall, cytosol, or membrane derived, can confer protective immunity against challenge in the mouse footpad. Previously, recombinant proteins were shown to be ineffective. The cell wall skeleton—the mycolyl-arabinogalactan-peptidoglycan complex—devoid of proteins is not protective.**

Largely as the result of the implementation of effective multiple-drug chemotherapy, the number of cases of leprosy worldwide has plunged from approximately 10 million in 1985 to fewer than 700,000 in 1999 (27, 28). However, there are large areas throughout the world in which heightened surveillance and an apparent ineffectiveness of multiple-drug chemotherapy to fully sterilize all sources of infection have resulted in a sizable increase of the estimated number of new cases (29, 30). As a result, the earlier, optimistic goal of reducing the worldwide prevalence of the disease to less than one case per 10,000 population by the year 2000 was not achieved (29). Thus, the prospects of a leprosy vaccine are being seriously addressed for the first time since the early 1990s. The mouse footpad model of leprosy, in use in only a few laboratories today, is still the only laboratory means for testing vaccines.

The results of earlier efforts to produce a leprosy vaccine have been mixed. An immunoprophylaxis trial in south India, launched in 1991 with 171,400 volunteers, compared four vaccines: (i) a combination of *Mycobacterium bovis* BCG and heat-killed *Mycobacterium leprae* (HKML); (ii) a cultivable mycobacterium, called ICRC, originally isolated in Mumbai, India, from a lepromatous nodule; (iii) another cultivable mycobacterium, isolated in New Delhi, India, and also from a lepromatous nodule, called *Mycobacterium w*; and (iv) BCG. Normal saline was employed as a placebo. BCG-HKML conferred 64% protection and ICRC conferred 65.5% protection; the other vaccines were ineffective (9). However, earlier trials of HKML in Venezuela (5) and Malawi (10) showed no protection, perhaps because of poor quality control of the vaccines or because the absence of a placebo obscured any protective effects.

The first mouse study employing a subunit vaccine was that of Shepard and Ribi (21), who demonstrated protection of mice against *M. leprae* challenge in a hind footpad by administration of BCG cell walls emulsified in 7-*n*-hexyloctadecane. Subsequently, Gelber et al. (7, 8) showed that immunization of mice with crude cell wall fractions of *M. leprae*, as well as with proteins derived from a pelleted fraction of sonicated *M. leprae*, conferred significant protection against subsequent challenge with *M. leprae* in the footpad. Continuing with this approach, we have demonstrated that three fractions of *M. leprae* all conferred protection against *M. leprae* infection when the materials were administered emulsified in Freund's incomplete adjuvant (FIA) (14, 15). These three fractions were the following: the soluble fraction of *M. leprae* from which much of the soluble carbohydrates and lipids, especially lipoarabinomannan (LAM), had been removed by extraction with Triton X-114 [*M. leprae* soluble antigen minus LAM (MLSA-LAM)]; the remaining insoluble pellet of *M. leprae* (MLCwA); and the insoluble pellet that had been extracted with Triton X-114 (MLCwA-LAM). However, pools of the recombinant versions of some of the major protein antigens of *M. leprae* and *M. tuberculosis*, emulsified in either FIA or monophosphoryl lipid A, were all found to be ineffective in experiments in which MLSA-LAM emulsified in monophosphoryl lipid A was again shown to be protective (15). Reasoning that the failure of the recombinant bacterial proteins to confer protection resulted from the absence of suitable posttranslational changes, such as glycosylation or esterification (6), a further study was undertaken to examine the effects of more refined, well-defined fractions of *M. leprae* on immunocompetent mice challenged in the hind footpad with *M. leprae*.

Female BALB/c$^{+/+}$ mice, purchased from CLEA JAPAN, Inc., Meguro-ku, Japan, and housed at the National Institute of Health, Nonthaburi, Thailand, under specific-pathogen-free conditions, were divided among various groups, as shown in Tables 1 and 2. HKML and fractions of *M. leprae* were prepared at Colorado State University (13). The various materials, suspended in phosphate-buffered saline (PBS) or emulsified in FIA, were injected intradermally into each flank to groups of 12 to 15 mice in a dosage of 20 μg per mouse on three occasions 3 weeks apart. An additional group of 15 mice was administered FIA. Twenty-eight days after the third injection, the antigen-treated mice were inoculated with $5 \times 10^3$ *M. leprae* each in the right hind footpad, and the adjuvant-treated and

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TABLE 1. Results of harvests of *M. leprae* from mice inoculated with the first inoculum

| Material[a] | No. of AFB per footpad ($10^5$) | | $P^b$ |
|---|---|---|---|
| | Individual harvests | Median | |
| Control | 33.6, 20.2, 18.9, 15.1, 11.5, 8.70, 6.39, 6.04, 5.06, 4.70, 4.62, 3.90, 3.37, 3.36, 1.69 | 6.04 | |
| FIA | 8.17, 6.12, 3.55, 3.28, 2.31, 2.22, 1.60, 1.07, 0.89, 0.44, 0.18, 0.089, <0.089 | 1.60 | |
| CW skeleton | 73.3, 13.5, 9.14, 8.61, 7.72, 7.46, 5.24, 1.51, 1.42 (2)[c] | 7.59 | 0.782 |
| CW skeleton in FIA | 2.13, 2.04, 0.98, 0.80, 0.53 (2), 0.44, 0.27, 0.18, 0.089 (2), <0.089 | 0.48 | 0.0602 |
| ML cytosol/MLSA | 3.37, 2.75, 2.13, 1.78, 1.60, 1.24 (2), 1.15, 0.62, 0.44, 0.27, 0.18 | 1.24 | 0.00004 |
| ML cytosol/MLSA in FIA | 0.53, 0.36 (2), 0.27, 0.18 (3), 0.089 (2), <0.089 | 0.18 | 0.0107 |
| ML membrane | 4.44, 3.46, 2.84, 2.66, 2.04, 1.69, 1.33, 1.15, 0.98, 0.80 | 2.04 | 0.00034 |
| ML membrane in FIA | 0.44 (2), 0.27 (2), 0.18 (3), <0.089 (3) | 0.18 | 0.0073 |
| HKML ($2 \times 10^7$) | 1.60, 0.89, 0.80, 0.44, 0.36, 0.27, 0.18, 0.089 (2), <0.089 | 0.32 | 0.00003 |
| HKML ($2 \times 10^7$) in FIA | 0.89, 0.62, 0.36 (2), 0.27, 0.18, 0.089, <0.089 (3) | 0.22 | 0.00003 |

[a] CW, cell wall; ML, *M. leprae.*
[b] *P*, the probability that these results were drawn from the same population as those of the corresponding control, determined by means of the Mann-Whitney U test.
[c] Number of footpads with indicated result; in all other cases, only a single footpad yielded the indicated result.

control mice were similarly inoculated in both hind footpads. Because the quantity of organisms harvested in the preparation of the first inoculum was insufficient to inoculate all of the mice, this inoculum was used to inoculate the mice of the groups listed in Table 1 and a second inoculum was prepared and used to inoculate the mice of the two groups shown in Table 2, along with a small number of additional untreated control mice. *M. leprae* from the inoculated mice were harvested and enumerated by Shepard's method (16, 20) (Tables 1 and 2). Between 120 and 143 days after inoculation, the mice of all of the groups were sacrificed and individual harvests of *M. leprae* were performed from the inoculated footpads by Shepard's method (16, 20) (Tables 1 and 2). The purpose of the statistical analysis applied to the resulting data was to measure the likelihood that the groups of results being compared were actually derived from a single population of results. A nonparametric technique, the Mann-Whitney U test (24), which is a technique for comparing two independent samples, was employed, because parametric techniques such as Student's *t* test require the assumption that the results are normally distributed, an assumption that is not justified in this case. On the other hand, the power of this technique is approximately equal to that of Student's *t* test.

The results demonstrate that the cell wall skeleton, whether suspended in PBS or emulsified in FIA, was not protective. On the other hand, both the cytosol and membrane fractions as well as the smaller dose of HKML conferred protection, defined as a significantly smaller number of *M. leprae* per footpad, whether the materials had been suspended in PBS or emulsified in FIA. The comparison of the two doses of HKML is particularly interesting. Although different inocula were employed, making a direct comparison impossible, it is clear that the dose of $2 \times 10^9$ HKML completely inhibited multiplication of *M. leprae*. By way of explanation, if a harvest yields $0.089 \times 10^5$ acid-fast bacilli (AFB) per footpad, only one organism has been observed in 40 microscope fields (employing 12.5× oculars and a 100× oil-immersion objective); similarly, if only 2 AFB are observed in 40 microscope fields, the harvest has yielded $0.18 \times 10^5$ AFB per footpad. These small yields do not differ significantly from that of the inoculum of 5,000 *M. leprae* per footpad and are occasionally encountered if mice are inoculated with $5 \times 10^3$ HKML. On the other hand, the dose of $2 \times 10^7$ HKML was not shown to be any more effective than any of the effective fractions employed.

A number of workers have studied the protective properties of various organisms and their components upon *M. leprae* infection of immunocompetent mice. Most often employed in these studies have been BCG and *M. leprae* itself. In early publications (18, 21–23) Shepard reported that 5,000 viable BCG administered either subcutaneously or intracutaneously into the flank or into the left hind footpad conferred protection against challenge with *M. leprae* in the right hind footpad 1 or 2 months later, as did much larger numbers of heat-killed organisms. Protection was enhanced by emulsifying the organisms in FIA and by repeated vaccination (17, 19). Vaccination with viable BCG also conferred protection if the vaccine was administered intracutaneously in the flank within the first 3 months after *M. leprae* challenge (21–23, 27–29). Although Shepard was at first unable to demonstrate protection against *M. leprae* challenge in the left hind footpad after initial infection with *M. leprae* in the right hind footpad, several workers subsequently reported the presence of homologous immunity in such animals, provided the second challenge was carried out after a sufficiently long interval (11, 12). Subsequent studies with cell wall preparations of BCG or *M. leprae* were contradictory (7, 8), probably because methods of preparation had not been standardized and because the degree of contamination of cell wall proper with soluble protective antigens varied among preparations. Nowadays, with a more detailed understanding of the chemistry of the mycobacterial cell wall (2), subcellular fractions of *M. leprae* are prepared in concordance with this knowledge (13). For instance, cell walls are prepared

TABLE 2. Results of harvests of *M. leprae* from mice inoculated with the second inoculum

| Material | No. of AFB per footpad ($10^5$) | | $P^a$ |
|---|---|---|---|
| | Individual harvests | Median | |
| Control | 9.50, 3.91, 3.82, 2.31, 1.78 | 3.82 | |
| HKML ($2 \times 10^9$) | 0.089, <0.089 (9)[b] | <0.089 | 0.00055 |
| HKML ($2 \times 10^9$) in FIA | 0.18, <0.089 (11) | <0.089 | 0.00022 |

[a] See footnote *b* to Table 1.
[b] See footnote *c* to Table 1.

by centrifugation of disrupted bacilli at 27,000 × *g* followed by sucrose density gradient centrifugation (13), yielding a consistent profile of cell wall-associated proteins. Cell walls (known as MLCwA) prepared in this way have consistently been shown to be protective (7, 8, 14). In this present work, we removed the cell wall-associated proteins by extraction with sodium dodecyl sulfate and have now shown that the residual cell wall skeleton or core, a covalent complex composed of peptidoglycan, mycolic acids, and arabinogalactan (2), is ineffective. In light of the known role of mycobacterial lipids and carbohydrates to evoke a T-cell response through the CD-1-restricted pathway (1, 25) and other means (26), it is important to be able to state that the dominant lipoglycoconjugate, the mycolyl-arabinogalactan-peptidoglycan complex, is not protective in the leprosy mouse model. Presumably, the presence of weakly T-cell-reactive mycolic acids and arabinogalactan cannot compensate for the virtual absence of highly immunogenic proteins.

The results clearly indicate that the variable protection associated with cell walls in the past had resulted from the associated soluble proteins. Indeed, in the present work, the cytosolic and membrane proteins were again shown to be protective. Like the cell wall skeleton, these were prepared in a consistent, reproducible manner by centrifugation of the initial 27,000 × *g* supernatant at 100,000 × *g* to obtain soluble cytosol and insoluble, translucent membranes, respectively (13). Thus, maximal protection against *M. leprae* challenge of the mouse is conferred by a wide spectrum of cell proteins, such as cytosolic, membranous, and cell wall-associated proteins, and not by the core framework of the mycobacterial cell, the mycolyl-arabinogalactan-peptidoglycan complex. In extending these studies to recombinant products, fused recombinant proteins, successful in conferring protection against tuberculosis (3, 4), are more promising than individual recombinant proteins, which have consistently failed to confer protection in this model.

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