Immunoprophylaxis against *Mycobacterium leprae* infection with subunit vaccines

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Summary We have investigated the effect of subunit vaccines against infection with *Mycobacterium leprae*, employing DNA plasmids as the vaccine vectors, and the immunodominant 35 kDa protein of *M. leprae* as the candidate antigen. A DNA vaccine that expresses the *M. leprae* 35 kDa protein both stimulated interferon-γ (IFNγ)-secreting T cells in mice, and demonstrated protection against *M. leprae*-infection of mice.

Introduction

During the last 15 years, implementation of multidrug therapy (MDT) for leprosy has been very effective, resulting in a dramatic fall of prevalence.[1] However, the new-case detection rate in the countries with the major disease burden has not decreased, suggesting that transmission of *Mycobacterium leprae* will continue for some time. A combination of MDT applied to active cases with immunization against infection by *M. leprae* in leprosy endemic regions may be the most effective, long-term control measure that will lead to eradication of the disease. Immunization with *M. bovis* BCG has been partially effective; a recent controlled trial of BCG against *M. leprae* infection in Malawi demonstrated 50% protection by a single administration.[2] Although meta-analysis of a number of trials of BCG indicated a protective efficacy of approximately 50% against pulmonary tuberculosis, and 85% against disseminated tuberculosis,[3] BCG did not confer protection against tuberculosis in the Malawi trial.[2]

There has been renewed interest in new subunit vaccines against tuberculosis, both to increase protective efficacy, and to avoid the use of a live vaccine in potentially immunodeficient subjects. Moreover, it may be possible to develop vaccines containing a limited number of antigens, which will permit continued skin testing with other mycobacterial components, to detect subsequent infection by *M. tuberculosis*. Approaches to anti-*M.*

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tuberculosis subunit vaccines include the use of single or combinations of secreted proteins in adjuvant,[4] and DNA vaccines, in which mycobacterial genes are expressed under the control of a eukaryotic promoter.[5,6] These were moderately protective against experimental *M. tuberculosis* infection, and a combination of DNA vaccines expressing three different secreted proteins was as effective as BCG against pulmonary tuberculosis in mice.[7]

We have investigated the effect of subunit vaccines against *M. leprae* infection, employing DNA plasmids as the vaccine vectors. The immunodominant 35 kDa protein of *M. leprae* was selected as the candidate antigen. The gene encoding the 35 kDa protein is present in *M. leprae* and *M. avium*, but not in members of the *M. tuberculosis* complex,[8] and monoclonal antibodies specific for the *M. leprae* protein formed the basis of a serological test that detects *M. leprae*-specific antibodies in patients with leprosy, but not tuberculosis patients.[9] The recombinant 35 kDa protein, which is recognized by more than 90% of leprosy patients, elicits a strong T-cell response in tuberculoid leprosy and an antibody response in lepromatous leprosy, as well as delayed-type hypersensitivity in *M. leprae*-sensitized guinea pigs.[10] The DNA vaccine expressing the *M. leprae* 35 kDa protein both stimulated interferon-γ (IFNγ)-secreting T cells in mice, and demonstrated a significant protective effect against *M. leprae*-infection of the mouse footpad.

Materials and methods

The genes encoding the *M. leprae* and *M. avium* 35 kDa proteins[10,11] were amplified and cloned into the vector pJW4303 downstream of the early/intermediate promoter of cytomegalovirus, yielding DNA-Mav35 and DNA-ML35.[12] The gene-sequences were confirmed by double-stranded sequencing, and expression of the proteins was demonstrated by transient transfection of COS7 cells and immunoblotting of cellular homogenates. The immunogenicity of the vaccines was tested by immunizing C57BL/6 (H-2^b) and outbred Swiss albino mice with three intramuscular injections of 100 μg of the purified DNA vaccines or the control vector pJW4303 at intervals of two weeks. BCG-immunized animals were administered 10^6 CFU of BCG (CSL) intradermally. Antigen-specific lymphocyte proliferation and IFNγ-responses of splenocytes and lymph node cells were measured as previously described.[12] Serum antibody responses to the 35 kDa protein were measured by ELISA using recombinant 35 kDa protein as antigen.[10] The protective effect of DNA-ML35 was tested by inoculating immunized Swiss albino mice with 10^4 *M. leprae* into each hind footpad 4 weeks after the last immunization. Control mice were administered the DNA vector alone or phosphate buffered saline (PBS). *M. leprae* were harvested from the footpads of control mice after 6 months and each month thereafter until the organisms were found to have multiplied to a level of at least $3{\cdot}16 \times 10^5$ acid-fast bacilii (AFB) per footpad. The AFB were counted as previously described.[13]

Results and discussion

In initial studies, we demonstrated that a DNA-vaccine expressing the *M. avium* 35 kDa protein, which exhibits 95% homology with that of *M. leprae*, stimulated strong T-cell and antibody responses to the 35 kDa antigen in C57BL/6 mice.[12] It was of interest that the high-titre, anti-35 kDa protein antibodies were directed against the same conformational epitopes

on the protein as those recognized by sera of leprosy patients. The DNA-Mav-35-immunized H-2^b mice were protected against intravenous infection with a virulent strain of *M. avium*, which causes progressive infection in mice. There was a 99% reduction of the bacterial load in the spleen, indicating a protective efficacy greater than that of BCG 4 weeks post-challenge.[12] As expected, the DNA-ML35 vaccine was also strongly immunogenic in the C57BL/6 mice, inducing high levels of antigen-specific T cells which released the cytokine IFNγ on restimulation.

To ensure that the protein was recognized in genetically diverse animals, the immunogenicity of the DNA-ML35 vaccine was tested in outbred Swiss Albino mice, which were used to study the protective efficacy of the vaccine. Antibodies which increased in titre with successive vaccine doses were detected in all of the mice. Six weeks after immunization with DNA-ML35, the titre of anti-35 kDa protein IgG was 3·3 $\log_{10}$ compared to 1·09 $\log_{10}$ in the mice administered the control DNA vector. As shown in Table 1, the mice demonstrated anti-35 kDa T cell proliferative and IFNγ responses in the spleen and lymph nodes. There was some variability of the level of the T-cell responses; about 60% of the mice showed high responses comparable to those observed in H-2^b mice, whereas 40% showed lower responses.[14] However, all demonstrated significant, antigen-specific T-cell responses, compared to the mice administered the control vector.

When the mice that had been immunized with DNA-ML35 were challenged with viable *M. leprae* in the footpad, they showed significantly less mycobacterial replication after 7 months than the mice that had been administered either the control DNA vector or PBS, and the level of protection was equivalent to that induced by BCG immunization (see Table 2). This protective effect was observed employing two different strains of *M. leprae*.

To investigate whether DNA vaccines expressing *M. tuberculosis* genes are capable of inducing heterologous protection against *M. leprae* infection, we tested the protective effect of DNA-85B, which encodes the *M. tuberculosis* 85B protein, against the mouse footpad infection with *M. leprae*. The antigen 85 complex includes a group of three secreted proteins

Table 1. Antigen-specific T-cell responses in Swiss Albino mice immunized with DNA-ML35 or control vaccines

Vaccine[a]	Antigen[b] (μg/ml)	Proliferation[c] (Δcpm)	IFNγ response[d] (units/ml)
DNA-ML35	10	5049 ± 805	61 ± 16
	3	3900 ± 479	54 ± 15
	1	3307 ± 461	36 ± 11
DNA-Neg	10	1465 ± 83	6 ± 1
	3	1550 ± 54	5 ± 1
	1	966 ± 36	5 ± 1

[a] Mice were administered 100 μg DNA-ML35 or the empty vector, DNA-Neg, intramuscularly on three occasions at intervals of 2 weeks, and tested 4 weeks after the last injection.

[b] Purified splenocytes were restimulated with recombinant *M. leprae*-35 kDa protein at the concentrations shown.

[c] Proliferative responses are shown as the mean specific incorporation of ^{3}HdT in triplicate wells (±SEM).

[d] IFNγ responses are shown as the mean specific release of IFNγ in triplicate wells (±SEM) at different antigen concentrations.

Table 2. Protective effect of immunization with DNA-ML35 and BCG against mouse footpad infection with a clinical isolate of *M. leprae*

Vaccine[a]	Log_{10} AFB/footpad[b] (mean + SEM)
BCG	5·22 + 0·11[c]
DNA-ML35	5·11 + 0·16[c]
DNA-Neg	5·67 + 0·19
PBS	5·74 + 0·13

[a] Mice were administered 100 μg DNA-ML35 or the empty vector, DNA-Neg, intramuscularly, or BCG or PBS intradermally on three occasions at intervals of 2 weeks. Four weeks after the last injection, the mice were inoculated with 10^4 *M. leprae* in each hind footpad.

[b] The geometric mean of the $logarithm_{10}$ number of AFB ± SEM harvested per footpad 7 months after inoculation.

[c] The probability, determined by the Mann-Whitney *U*-test, that the results from this group of mice were drawn from the same population as were those from the mice administered PBS was < 0·05.

of 30–32 kDa, which elicit strong immune responses in tuberculosis and leprosy patients.[15] DNA vaccines expressing antigen 85A[6] and antigen 85B[7] induce partial protective immunity against *M. tuberculosis* infection in mice, and the *M. tuberculosis* 85B antigen shows greater than 75% homology at the amino acid level with the *M. leprae* 85B protein. Therefore, we immunized Swiss albino mice three times at 2-weekly intervals with intramuscular injections each of 100 μg DNA-85B and challenged them with two different strains of *M. leprae*. The organisms multiplied in DNA-85B immunized mice to only about 20% of their number in control mice (Roche, P. R., submitted for publication).

We are currently investigating means of increasing the effect of these subunit vaccines against *M. leprae* infection. One approach is to co-immunize with the mycobacterial DNA vaccine and a plasmid expressing cytokines, which may act as adjuvants to increase the cellular immune response to the mycobacterial antigen.[16] Interleukin-12 (IL-12), which is produced by macrophages and dendritic cells, causes deviation of $CD4^+$ T cells to a Th1-like pattern of cytokine production. We have prepared a novel vector that expresses both the P35 and P40 chains of murine IL-12. When mice were co-immunized with DNA-Mav35 and the IL-12-producing plasmid, there was an increase of the antigen-specific production of IFNγ by lymph-node cells as well as increased clearance of *M. avium* following intravenous challenge, compared to mice immunized with DNA-Mav35 alone (Martin, E., submitted for publication). This increased protective effect is encouraging, and we are now testing whether co-immunization with DNA-ML35 and the IL-12-expressing plasmid will increase the level of protection against footpad infection with *M. leprae*.

These results suggest that effective subunit vaccines can be developed against *M. leprae* infection. One *M. leprae* antigen expressed as a DNA vaccine was moderately effective, but we envisage that a combination of antigens would be incorporated into a future vaccine, to ensure that individuals in a genetically diverse population could respond to at least one of the HLA Class II-restricted epitopes on the antigens. The inclusion of IL-12 or other cytokines

may provide more defined patterns of adjuvant activity, particularly of a Th1-like pattern. A number of other immuno-enhancing strategies are being tested, including the targeting of the DNA-encoded vaccine antigen to antigen-presenting cells, and the combination of different types of vaccine vectors to prime and boost for the required type of immune response to the vaccine antigen.[17]

How might subunit vaccines be employed against *M. leprae* infection? It is more likely that a subunit vaccine for tuberculosis rather than one for leprosy will be developed and tested in humans in the foreseeable future. If this vaccine is to be used in populations with high levels of transmission of both *M. tuberculosis* and *M. leprae*, it will be important to incorporate some *M. leprae*-specific antigens into the vaccine, as well as proteins shared by the two mycobacteria, to provide heterologous protection. This will ensure that the protective benefit of anti-tuberculosis vaccine also assists in the control of leprosy. The *M. leprae* 35 kDa protein and the *M. tuberculosis* antigen 85B are two appropriate candidates for this role, but a broader panel of antigens may be eventually utilized.

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