

Lymphostimulatory and delayed-type hypersensitivity responses to a candidate leprosy vaccine strain: *Mycobacterium habana*

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Summary Lymphostimulatory and delayed-type hypersensitivity (DTH) immune responses to a candidate antileprosy vaccine *Mycobacterium habana* have been quantified in inbred AKR mice. *M. habana* vaccine in three physical states, live, heat-killed and γ -irradiated, was given intradermally to separate groups of mice and after 28 days these mice were given subcutaneous challenge with heat-killed *M. leprae* and heat-killed *M. habana* in the left hind footpad. Live BCG vaccine alone and in combination with γ -irradiated *M. habana* were also compared similarly. A sufficient degree of DTH response was generated in mice by *M. habana* vaccine in all physical forms against two challenge antigens (lepromin and habanin). The BCG combination with *M. habana* did not increase the DTH response indicating internal adjuvanticity endowed in *M. habana*. The active hypersensitivity of immunized mice was transferable to syngeneic mice by the transfer of sensitized cells from the donor to the recipient mice intravenously. *M. leprae*-infected Rhesus monkey PBMC have shown comparable stimulatory response with *M. habana* (sonicate), and *M. leprae* (sonicate) antigens. The possibility of developing *M. habana* as a candidate antileprosy vaccine is discussed.

Introduction

In mycobacterial diseases, protective immunity is mediated through bacterial kill and/or modulation of the immune response which is essentially cell mediated with two basic cell types: the T-lymphocytes and macrophages interacting through cytokines.^{1,2} The basis of *in vivo* and *in vitro* tests is to examine and measure the nature of the responses of these cells to the immunogens. The most commonly employed test is the delayed type of hypersensitivity response in the skin elicited by an intradermal injection of the immunogen. The *in vitro* tests, measuring the T-cell immunity are lymphocyte proliferation test (LPT) and the leucocyte migration inhibition test (LMIT). LMIT measures the release of lymphokines from antigenically driven lymphocytes and LPT measures DNA synthesis in actively multiplying cells due to release of lymphostimulatory factor(s). The skin DTH response assesses the individuals ability to mobilize the immunocytes to the injection site of the antigen. The DTH response evaluated in the form of a footpad enlargement (FPE) after injection of

the antigen in animals is a way to monitor the test and to prove the indirect efficacy of antileprosy vaccines.^{3,4}

One candidate antileprosy vaccine: *M. habana* (*M. simiae* serovar 1) protects mice against infectious challenge with *M. tuberculosis* H₃₇Rv, *M. leprae* and *M. ulcerans*.⁵⁻⁹ *M. habana* generates a cell-mediated immune response (CMI) which recognizes *M. tuberculosis* and *M. leprae* antigens^{10,11} and shares several immunodominant proteins with them.^{12,13} *M. habana* combines the useful traits of these mycobacteria and may suitably be developed as a vaccine against leprosy.

In this report we present the data of comparative evaluation of *in vivo* DTH response generated by *M. habana* and other vaccine(s) in mice and successful transfer of the active immune hypersensitivity through sensitized cells in syngeneic mice. The *in vitro* lymphostimulatory response of *M. leprae* sensitized monkey cells have also been studied against *M. habana* antigen(s).

Materials and methods

GROWTH AND PREPARATION OF IMMUNIZING ANTIGENS

Mycobacterium habana (*M. simiae* serovar-1, TMC 5135) was obtained from the TMC collection centre Saranac Lake, New York. The strain of BCG (Madras) was procured from the Tuberculosis Research Centre, Madras and freeze-dried preparations of armadillo derived *M. leprae* were obtained through Dr R. J. W. Rees of the National Institute for Medical Research Mill Hill, London. Mycobacteria other than *M. leprae* were maintained separately on Lowenstein-Jensen medium and their bulk growth was obtained in Sautons liquid medium. A live culture of BCG was used as vaccine, while *M. habana* vaccine was treated by γ -irradiation of the culture at 300 K rads from a ⁶⁰Co source. *M. habana* was also used in live and heat-killed states for immunization. Freeze-dried *M. leprae* preparations was reconstituted in normal saline at the required concentration.

CHALLENGE ANTIGENS

Integral habanin was prepared from mid-log culture of *M. habana* by standard procedures as applied for the preparation of integral lepromin (WHO protocol). The Mitsuda lepromin was obtained through Professor Noordeen, Chief Medical Officer, WHO, Geneva.

DOSE OF VACCINE AND ANTIGENS

Mice were immunized i/d with 6.27×10^8 organisms ($63.3 \mu\text{g}$ protein/animal) of *M. habana* in either live, heat-killed or γ -irradiated states while the dose of BCG and *M. leprae* were 6.0×10^5 and 2.0×10^7 bacilli per animal respectively (recommended optimal doses). The challenge dose of each antigen (habanin and lepromin) was 3×10^7 bacilli per footpad.

THE EXPERIMENTAL ANIMALS

Inbred AKR mice maintained in the Institute's animal house were used. The animals were kept on a pellet diet of Hindustan Lever Ltd, with water *ad libitum*. They were divided in groups on a similar weight basis. All vaccinations were done intradermally on the back and 28

days postvaccination the animals were challenged with eliciting antigens in the hind feet. The footpad thickness was measured prior and after challenge with a Schnelltaster (made in Germany) and corrected footpad enlargements (FPE) were calculated according to Shepard's method.⁴

ADOPTIVE TRANSFER OF IMMUNITY

Sensitized splenic cells from vaccinated mice were obtained by extirpation of the spleen and mincing and passing through a metal sieve. The method of Patel & Lefford¹⁴ was followed with slight modifications. The viability of the cells was determined by staining with Trypan blue. 1×10^8 viable splenic cells obtained from actively sensitized (vaccinated) mice were given *i/v* to separate groups of syngeneic mice for passive sensitization. After 24 h these passively sensitized mice were given a S/C challenge in the hind footpad with homo and heterologous eliciting antigens and the FPE was measured as described earlier.

LYMPHOCYTE TRANSFORMATION TEST (LTT)

Peripheral blood mononuclear cells (PBMC) were separated from heparinized blood of normal (tuberculin and lepromin negative) and *M. leprae* infected Rhesus monkeys by density gradient centrifugation (Lymphoprep-Nyegard S.Co.OSLO, Norway). These cells were washed thrice and their viability was determined by staining with Trypan blue (93% viable). The cells were finally suspended at 2×10^6 cells/ml in RPMI-1640 supplemented with 10% foetal calf serum (GIBCO, Grand Island, New York, USA), glutamine and antibiotics (streptomycin 100 $\mu\text{g/ml}$, penicillin 100 units/ml and gentamycin 20 $\mu\text{g/ml}$). 100 μl of this suspension were dispensed in each well giving a concentration of 2×10^5 cells/well. The antigens used were *M. leprae* and *M. habana* sonicate (soluble fraction at 2.5, 5.0 and 10 $\mu\text{g/well}$ and mitogen PHA-P (Sigma Chemical Co., USA) at 0.5, 1.0 and 2.0 $\mu\text{g/well}$. Control cultures received no mitogen/antigen. The cultures on day 4 were pulsed with 0.5 μCi of [³H]-thymidine (specific activity of 18 mCi/u mole) obtained from Bhabha Radiation and Isotope Technology, Bombay, India. Harvesting of the cells was done on day 5 through a semiautomatic harvester (PHD cell harvester, Cambridge Technology Inc., Cambridge, Massachusetts, USA) using glass fibre filters (GF/C Whatman, England) and radioactivity was counted in a liquid Scintillation counter (LKB Finland) using a toluene based scintillation cocktail.

RESULTS

INDUCTION OF DTH RESPONSE BY DIFFERENT VACCINES

All the three preparations (live, heat killed, and γ -irradiated) of *M. habana* live, BCG, heat-killed *M. leprae* and live BCG+ γ -irradiated *M. habana* were tested for generation of DTH response against homologous and heterologous antigenic challenge. The data are presented in Figure 1. Average FPE with homologous antigen-habanin are 28.33 ± 7.53 ; 31.43 ± 6.90 and 31.66 ± 7.30 respectively in three types of vaccinated animals. With lepromin the FPE response of these groups of animals were 26.67 ± 5.15 , 26.66 ± 8.16 and 28.33 ± 7.53 respectively. In animals vaccinated with live BCG heat killed *M. leprae* and live BCG+ γ -irradiated *M. habana* the responses with habanin were 23.33 ± 5.16 , 21.66 ± 4.08 and

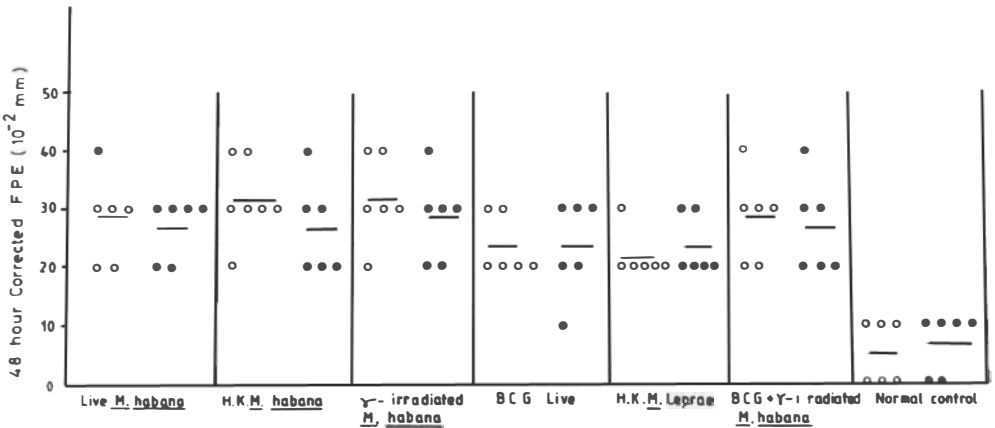


Figure 1. Forty-eight footpad enlargement in mice actively sensitized with different vaccines after challenge with habanin and lepromin. FPE in AKR mice ID route (6.27×10^8 bacilli); \circ → habanin; \bullet → lepromin.

28.33 ± 7.52 and with lepromin the responses were 23.33 ± 8.16 , 23.33 ± 5.16 , 26.66 ± 8.16 respectively. It is apparent that both the sensitins have generated sufficient degree of DTH response in vaccinated animals than unvaccinated controls. The combined vaccination of animals with BCG + *M. habana* did not impart better DTH response than with either vaccine alone.

ADOPTIVE TRANSFER OF IMMUNITY THROUGH SENSITIZED CELLS

Sensitized splenic cells from mice vaccinated with γ -irradiated *M. habana*, heat-killed *M. habana* and heat-killed *M. leprae* were transferred intravenously to normal syngeneic mice. After 24 hours of passive sensitization these groups of animals were challenged with homologous and heterologous antigens. The individual animal data are presented in Figure 1. A considerably higher number of animals developed passive sensitization and had higher FPE values than the controls with both the sensitins (habanin/lepromin). Gamma irradiated *M. habana* group of passively sensitized animals had better response than other groups. The average FPE of respective groups was 23.33 ± 8.16 , 20.00 ± 8.94 and 16.66 ± 8.16 with habanin challenge and 20.00 ± 8.94 , 18.7 ± 8.99 and 20.00 ± 5.77 with lepromin challenge.

LYMPHOSTIMULATORY RESPONSE

It is evident that optimal dose PHA-P is $1 \mu\text{g/ml}$ since this dose has generated maximum response in normal as well as *M. leprae* sensitized monkeys. The other two doses of PHA-P (one less, other higher) have also responded well but less than the optimal dose of $1 \mu\text{g/ml}$. The lymphostimulatory responses with leprosin and habanin have also indicated that $0.5 \mu\text{g/ml}$ dose of both the antigens are optimum. Habanin has generated the LP response closer to PHA-P where as homologous response with leprosin and habanin have also indicated that $0.5 \mu\text{g/ml}$ dose of both the antigens are optimum. Habanin has generated the LP response closer to PHA-P where as homologous response with leprosin is of a lower order.

Discussion

The delayed-type hypersensitivity response is one of the several parameters used to test the indirect efficacy of cell-mediated immunity (CMI) to candidate vaccines. The test is conducted by inoculating the vaccine strain intradermally in mice and challenging the vaccinated mice S/C in the footpad after 3–4 weeks with heat-killed *M. leprae*. The footpad enlargement (FPE) is measured after 24 and 48 hr of challenge. Sensitized PBMC either by production of disease and/or by artificial *in vitro* sensitization when allowed to react with the specific antigen(s) secrete lymphocyte stimulatory factor(s) which stimulate the sensitized cells to proliferate profusely. This cell division is measured in terms of DNA synthesis which increases many fold. This test is specific in recognizing the antigenic relatedness/similarity between species/strains.

M. habana (*M. simiae*) an atypical mycobacterium, has afforded protection against *M. leprae* and *M. tuberculosis* infectious challenges: in mice.^{5–8} It generates strong cell-mediated immune responses, possesses several common and specific immunodominant protein epitopes with *M. leprae*, and *M. tuberculosis* and BCG.^{10–13}

This study was undertaken to elucidate, if *M. habana* stimulates DTH responses in mice which could be recognized by *M. leprae* antigens and whether *M. habana* antigen(s) could produce lymphostimulatory response in PBMC(s) obtained from *M. leprae* sensitized Rhesus monkeys.

M. habana vaccine in all the physical states (i.e. live, heat-killed and γ -irradiated) was able to generate DTH response against the homologous antigen habanin and the heterologous antigen lepromin. The DTH responses stimulated by heat-killed and γ -irradiated *M. habana* were apparently better than that stimulated by live vaccine although there were no statistically significant differences. This finding correlates well with a similar findings with heat-killed *M. leprae* by Shepard *et al.*¹⁶ This may possibly be due to exposure of other antigens normally at some inaccessible regions after gamma irradiation or heat treatment. The DTH response stimulated by live BCG was inferior to those stimulated by other vaccines, although a heat-killed preparation of BCG was not included. It has been proved beyond doubt by several investigators that killed BCG does not sensitize mice.¹⁶ The combined vaccination with live BCG and γ -irradiated *M. habana* did not increase sensitization more than either vaccines alone, which may possibly be due to internal adjuvanticity in *M. habana*. In animals, heat-killed *M. leprae* in combination with BCG does not enhance sensitization,^{17,18} although in contrast to this, BCG in combination with heat-killed *M. leprae* in man provides an adjuvant effect.^{19,20} This property of *M. habana* and *M. leprae* seems to be similar in animals. Data for humans in respect of *M. habana* vaccine are not available at present, which is in progress. Heat-killed *M. leprae* was a poor sensitizer of mice than *M. habana* in our hands, whether tested against habanin or lepromin.

Like *M. leprae* (γ -irradiated), BCG and other candidate leprosy vaccines, *M. habana* also produces adoptive immunity which is transferable to syngeneic mice with actively sensitized cells.

M. habana antigen(s) have also stimulated strong lymphostimulatory responses, comparable to those stimulated by *M. leprae* antigen(s) in PBMC(s) from *M. leprae* sensitized Rhesus monkeys. This indicates sharing of common epitopes between these two mycobacteria.

Since *M. habana* possesses several immunogenic, it could be developed as a vaccine for the prophylaxis/immunotherapy of leprosy.

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