Clin. exp. Immunol. (1981) **44**, 501–506.

Induction of delayed-type hypersensitivity to *Mycobacterium leprae* in healthy individuals

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(Accepted for publication 2 January 1981)

SUMMARY

Delayed-type hypersensitivity (DTH) to a soluble *Mycobacterium leprae* skin test antigen (SML) was successfully induced in healthy volunteers following immunization with 2×10^8 killed armadillo-derived *M. leprae*. No better sensitization was obtained by a mixture of live BCG and killed *M. leprae*. The relative specificity of the DTH reaction to SML has been demonstrated in this study, since little cross-reactivity was observed to PPD, after immunization with BCG or *M. leprae* alone, or combined. Moreoever, armadillo-derived *M. leprae* readily induced a specific hypersensitivity with the time course DTH response associated with protective immunity, suggesting that this bacterial preparation may be a candidate for an effective anti-leprosy vaccine.

INTRODUCTION

Immunity to mycobacterial and other intracellular-type bacterial and parasitic infections is mediated by cellular, rather than by humoral, defence mechanisms. Moreover, the features of cell-mediated immunity (CMI) are particularly well developed in leprosy where, for example, the lesions in patients with high-resistant, tuberculoid-type disease present all the histopathological features of CMI; the patients manifest delayed-type hypersensitivity (DTH) in their skin reactions to leprosy antigens, and *in vitro* to leprosy antigens in tests including lymphocyte transformation (LTT) and leucocyte migration inhibition (LMIT) (Godal *et al.*, 1971; Bjune *et al.*, 1976). On the other hand, in patients with low-resistant or lepromatous-type leprosy, these features of CMI are absent (Godal *et al.*, 1971).

In general, protective immunity to mycobacterial infections, as manifested by CMI, is closely related to DTH reactivity to mycobacterial antigens (Mackaness, 1967; Lenzini, Rottoli & Rottoli, 1977; Patel & Lefford, 1978a). However, there are exceptions to this association as demonstrated by Youmans (1975), where in mice, acquired immunity to experimental tuberculosis infections existed in the absence of DTH, and by Rich & McCordock (1929) who showed that desensitization to tuberculin in man did not reduce the immunity of patients with pulmonary tuberculosis. Unfortunately these accepted but unexplained dissociations between protective immunity and DTH in tuberculosis and the continuing failure to identify a specific antigen responsible for protective immunity against tuberculosis in spite of many years of intensive studies, provide no general guidelines for developing a specific vaccine against leprosy. Furthermore, for leprosy there is still no skin test antigen available with a specificity comparable to that of PPD for tuberculosis. Therefore, in leprosy it is not possible to identify individuals at particular risk to infection or

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0099-9104/81/0600-0501$02.00

individuals who have already encountered but overcome infection with *M. leprae* or to identify patients actually incubating the infection.

Nevertheless, induction of DTH to *M. leprae* derived from infected armadillos has been achieved in guinea-pigs (Mehra & Bloom, 1979; Rees & Lowe, 1981). Moreover, Patel & Lefford (1978a, 1978b) induced with killed *M. leprae* (also from armadillos) CMI with concomitant DTH to an *M. leprae* antigen (also from armadillos) in mice. Convit *et al.* (1979) reported conversion of Mitsuda reactivity in man after repeated inoculations of a mixture of BCG and *M. leprae* (also armadillo-derived). Finally, Shepard, Walker & Van Landingham (1978) and Shepard *et al.* (1980) have demonstrated that killed *M. leprae* (derived from man and from armadillos) have given significant protection against *M. leprae* infections in mice.

Taken together these studies show that both DTH and protective immunity can be produced with armadillo-derived, killed *M. leprae*, indicating that this preparation may be a candidate for a vaccine against leprosy in man.

Therefore, we set out to investigate whether a specific DTH skin reaction to *M. leprae* antigen could be induced in healthy volunteers using this same killed *M. leprae* preparation. The efficacy and specificity of killed *M. leprae* alone and with live BCG to induce DTH was compared with BCG alone.

MATERIALS AND METHODS

Healthy volunteers. From a group of 84 healthy female volunteers (age range 17–41 years; average 22·5 years), 29 were selected for the study who were skin test-negative, i.e. less than 5 mm induration at 72 hr, both to PPD (1 TU) and to soluble *M. leprae* antigen, SML (1·0 μg protein). Thirty volunteers were finally taken into the study by including one who was negative to SML but gave 5 mm induration to PPD.

Skin test antigens. Killed *M. leprae* (batch AB/22) was derived from heavily infected armadillo tissues (after exposure to 2·5 mega rad ^{60}Co radiation) from animals inoculated intravenously with *M. leprae* from a lepromatous patient. The bacteria were extracted by enzyme digestion of the host tissue with collagenase, pronase and trypsin followed by separation from host tissues by partition in an aqueous two-phase polymer system according to the method of Draper (see WHO, 1976). The purified *M. leprae* were broken by sonification at 20 kHz, 80 W (indicated), for 20 min using a Dawe Soniprobe type 7532A immersed in iced water. The skin test antigen (designated SML) was prepared from the sonicated material following centrifugation at 105,000 ***g*** for 30 min and filtration through a 0·22- μm membrane filter. The skin test antigen was standardized on its protein content to contain 10 μg protein/ml.

Purified protein derivative (PPD, batch RT23, Statens Serum Institut, Copenhagen, Denmark) was used at a concentration of 10 TU/ml.

Both antigens were injected with a disposable tuberculin syringe (25 g × 16 mm needle) intradermally in the forearm in a volume of 0·1 ml, giving a skin test dose of 1·0 μg protein and 1 TU of SML and PPD respectively. The skin reactions to both antigens were read at 24, 48 and 72 hr as mm of erythema and induration, recorded from the mean of two measurements across the diameter of the sites.

Immunizing bacillary antigens. BCG freeze-dried preparation (Japan BCG Laboratory, Tokyo) was given intradermally in the deltoid region at a dose of $1{\cdot}5 \times 10^6$ viable bacilli in 0·1 ml saline.

M. leprae (batch AB/22) prepared by the method described above under skin test antigens was inoculated similarly at a dose of $2{\cdot}0 \times 10^8$ acid-fast bacilli (AFB) in 0·1 ml saline containing 0·001% Tween 80.

Protocol of the study. The thirty volunteers were randomized into four groups, as set out below:

Group	*Preparation given*	*No. of volunteers*
A	*M. leprae*	7
B	BCG	8
C	*M. leprae* + BCG	8
D	None	7

For group C where BCG and *M. leprae* were given together, the suspension of *M. leprae* at a concentration of $2{\cdot}0 \times 10^9$ AFB/ml was used to reconstitute the freeze-dried BCG immediately prior to inoculation.

Six weeks after immunization all the volunteers were again skin-tested with SML and PPD at the same doses as used initially and their skin reactions read similarly.

Group D was included to determine whether the sequence of skin tests *per se* affected the skin reaction.

Statistical analysis. Statistically significant differences in the immunizing capacities of the single and combined bacillary antigens were computed by Student's *t*-test.

RESULTS

Skin reactions to SML and PPD

Induction of DTH to soluble *M. leprae* antigen (SML) was successful in six out of seven volunteers (group A) who received *M. leprae* alone where the mean induration of the skin reaction increased by 10·4 mm at 72 hr (see Table 1). This result is highly significant ($P < 0{\cdot}005$). All seven remained skin test-negative for PPD at 72 hr. In group B where eight volunteers received BCG only, all were skin test-positive to PPD after 6 weeks, giving an average increase in induration of 10·2 mm ($P < 0{\cdot}001$). Only two individuals in group B showed weakly positive reactions to SML, 6 weeks after BCG.

When BCG and *M. leprae* were given together to group C, seven out of eight volunteers showed a positive DTH reaction to SML ($P < 0{\cdot}001$) and all to PPD ($P < 0{\cdot}001$) after 6 weeks, with a mean increase of induration of the skin of respectively 10·7 and 7·4 mm at 72 hr.

None of the volunteers showed clinical signs of adverse reactions (lymphadenitis, abscess or immediate-type hypersensitivity) to any of the mycobacterial preparations used. When BCG and *M. leprae* were given together, a slightly larger ulcer at the site of inoculation was observed than when BCG or *M. leprae* were given alone. The ulcers which developed after *M. leprae* inoculation were not distinguishable from BCG ulcers. All ulcers healed within 3 months.

Time–response curve of DTH reactions to SML and PPD

The mean response to SML at 24 hr increased by 3·9 mm 6 weeks after BCG vaccination ($P < 0{\cdot}01$) (see Fig. 1). This indicated that BCG had influenced the SML reaction, possibly due to cross-reactive antigens. To a lesser degree *M. leprae* inoculation appeared to influence the 24-hr reaction to PPD in group A, where the response had increased by 2·6 mm ($P < 0{\cdot}05$); however, when compared with the skin test results of the control group (D), the increase is not significant. At 72 hr the average PPD and SML responses in groups A and B respectively were close to the pre-test values.

The time–response curves of PPD and SML for group C (BCG + *M. leprae*) showed a peak at

Table. 1. Reactions to soluble *M. leprae* protein (SML, 1·0 μg) and PPD (1 TU) in healthy skin-negative individuals immunized with *M. leprae* and BCG, alone or combined, 6 weeks previously

Group (no. of individuals)	Immunization	72-hr skin reaction to: SML		PPD	
		Proportion positive	Mean increase (mm) ± s.e.	Proportion positive	Mean increase (mm) ± s.e.
A (7)	*M. leprae*	6/7	10·4 ± 2·14*	0/7	0·4 ± 0·76
B (8)	BCG	2/8	1·6 ± 1·06	8/8	10·2 ± 1·32*
C (8)	BCG + *M. leprae*	7/8	10·7 ± 1·97*	8/8	7·4 ± 1·27*
D (7)	None (control)	0/7	0·5 ± 0·57	0/7	0·2 ± 0·43

* Significantly different from the control group ($P < 0{\cdot}001$).

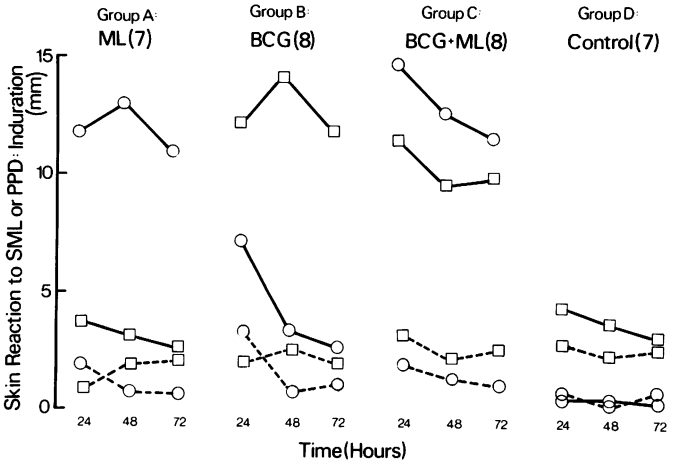

Fig. 1. Mean diameter (mm) of induration of skin reactions to soluble *M. leprae* (SML) (o) and PPD (□) at 24, 48 and 72 hr, before (- - - -) and 6 weeks after immunization (————).

24 hr instead of at 48 hr as in groups A and B. However, the only statistically significant difference was between the 48-hr response to PPD 6 weeks after inoculation in groups B and C ($P < 0{\cdot}001$).

DISCUSSION

Data presented in this paper demonstrate that a delayed skin reaction to SML antigen could be readily induced in healthy individuals following a single intradermal injection with killed *M. leprae* or a combination of *M. leprae* and BCG. However, no better sensitization was obtained by the combination.

The immunizing dose of *M. leprae* used was approximately 80 times higher than that of BCG ($2{\cdot}5 \times 10^6$ AFB, of which $1{\cdot}5 \times 10^6$ were viable). The rationale for using a higher dose of *M. leprae* was based on the finding that five-fold higher doses of armadillo-derived purified *M. leprae* organisms were required to elicit the level of immune responses equivalent to those elicited by crude human-derived preparations for LTTs (Smelt *et al.*, 1978). Thus the dose of $2{\cdot}0 \times 10^8$ purified bacilli derived from armadillos used in the present study was ten-fold greater than the dose ($2{\cdot}0 \times 10^7$) of non-purified bacilli derived from man used in the standard human lepromin skin test.

The relative specificity of SML when compared with PPD has been clearly shown in this study, since *M. leprae* or BCG did not induce conversion to PPD or SML respectively. This is consistent with our earlier report (Smelt *et al.*, 1978) which showed little cross-reaction between *M. leprae* antigens and BCG or PPD in the lymphocyte transformation test in leprosy. It is also in agreement with the often-observed specific non-responsiveness of lepromatous leprosy patients to *M. leprae* (Godal *et al.*, 1971). However, guinea-pigs immunized with killed *M. leprae* showed strong cross-reactive delayed skin reactions to PPD (Mehra & Bloom, 1979; Rees & Lowe, 1981). Our results are also in direct contrast with the serological data of Harboe *et al.* (1977). These workers showed extensive cross-reaction between antigens of armadillo-derived *M. leprae* and other mycobacterial species. On the other hand, even with the serological analysis, it is clear that there exist *M. leprae*-specific antigens (Abe, 1970; Abe, Minagawa & Yoshino 1972) or specific antigenic determinates in armadillo-derived *M. leprae* (Harboe *et al.*, 1978). The differences between the serological data and our cellular results (both DTH and LTT) may be due to a fundamental difference between the mechanisms of activation of T and B lymphocytes which lead to cellular and humoral immunities respectively (Greaves & Bauminger, 1972; Möller, 1976; Liew, Russell & Brand, 1979). An alternative explanation for the differences may be found in the assay systems used. Crossed immunoelectrophoresis and DTH represent two distinct analyses probably having different degrees of sensitivity.

Lenzini *et al.* (1977) described three different DTH skin reactions to PPD in patients with various levels of resistance to *M. tuberculosis* infection. The typical delayed hypersensitivity reaction to PPD in high-resistant tuberculosis patients peaked at 48 hr with strong induration persisting up to 72 or 96 hr. In our routine observations, patients with inactive tuberculoid (TT, TT/BT and BT) leprosy who are responsive to SML antigens also show this time–response curve (Smelt, unpublished). The DTH reaction induced by *M. leprae* or BCG in the present study (Fig. 1, groups A & B) is clearly of this type, thus suggesting that the delayed skin reaction induced may be related to protective immunity.

Lenzini *et al.* (1977) also demonstrated a Jones–Mote-type hypersensitivity in low-resistant tuberculosis patients. This is typified by an early reaction of mainly erythema and oedema, peaking at 24 hr. A similar time–response curve is seen in borderline lepromatous patients to SML antigen (Smelt, unpublished). The DTH response to SML following immunization with BCG (Fig. 1, group B) was significantly higher at 24 hr than at 48 or 72 hr, indicating a Jones–Mote type of reaction. This reaction was likely to be induced by antigens of BCG that cross-reacted with SML. This interpretation is consistent with the time course of the DTH reactions following immunization with a mixture of BCG and *M. leprae* which peaked at 24 hr instead of 48 hr.

The observation that armadillo-derived *M. leprae* readily induced specific DTH reactions which have a time–response course akin to the protective form of delayed hypersensitivity suggests that this antigen preparation may be a candidate for an effective anti-leprosy vaccine. However, it remains uncertain whether killed *M. leprae* can induce DTH, and perhaps protective immunity, in individuals particularly susceptible to leprosy.

We thank the volunteers involved in this study for their cooperation. Dr A. H. M. Smelt was supported by the Netherlands and British Relief (LEPRA) Associations and the Government of the Netherlands. The Leprosy Research Unit, Sungei Buloh, is jointly sponsored by the Malaysian Ministry of Health and the (British) Medical Research Council.

Soluble *M. leprae* antigen (SML) was supplied by the Immunology of Leprosy (IMMLEP) component of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases and purified *M. leprae* from armadillos was funded by LEPRA.

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