

SPECIFICITY OF THE DHARMENDRA ANTIGEN AS TESTED IN GUINEA-PIGS¹

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In previous studies (¹¹⁻¹³) the two commonly-used types of lepromin produced distinct differences in their patterns of response when tested intracutaneously in guinea-pigs. The classical Mitsuda-Wade lepromin had the greater sensitizing capacity, and produced a more prolonged test response. The Dharmendra antigen produced a less persistent reaction in sensitized animals, and showed a slight but probably unimportant decrease in cross reaction with tissue antigens.

These findings in guinea-pigs confirm accepted concepts of how these antigens behave in humans (^{6, 7, 9}). Dharmendra's original clinical trials showed that his "defatted" bacillary antigen produced maximal numbers of positive 48-hour (Fernandez) reactions (^{1, 2, 3, 4, 10}). The Mitsuda-Wade lepromin, of course, produces the greatest number of positive "late" (or Mitsuda) reactions when read at 3 weeks. In a subsequent comparative study Dharmendra (⁵) claimed to be able to separate tuberculoid and lepromatous patients almost equally well whether he used his own antigen and read for early reactions, or if he used Mitsuda lepromin and read for the late reaction. However, there continues to be a vague uneasiness that the two tests may have a different immunologic significance, and that their results should not be generally equated (¹⁶).

MATERIALS AND METHODS

Experimental procedures were in general identical with those that have been previously described (^{11, 12}). Cutaneous hypersensitivity was produced in 8 groups of 9 guinea-pigs by two intracutaneous injections of 0.1 cc. of the respective antigens, with an interval of one month. One month after the second sensitizing injection, cross-testing was done for reactivity to a battery of 8 antigens, with 0.1 cc. of each being injected intracutaneously in two parallel rows down the backs of the animals. Calibration of syringes, techniques of injection, and measurement of skin reactions were according to WHO-BCG campaign standards, with the diameter of reactions being recorded daily for the first ten days and then every two or three days for the rest of the month.

Particular attention was paid to the elimination of observer-error bias by double-blind reading. The guinea-pigs were randomized and renumbered after injection, and the sites of injection were rotated, so that the reader never knew the immunization group a guinea-pig came from, or which antigen had been injected at a particular site. Profiles of mean skin reactions for each group were charted on graph paper, both for test capacity (reactions to each antigen being compared with others in a particular immunized group), and also for immunizing capacity (the reaction to a single antigen being compared in the various immunized groups). Figure 1 shows the results of tests in the non-

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sensitized control group, and indicates that a reaction may be considered positive if it exceeds 3.5 mm. at 48 hours, 1.5 mm. at 10 days and 1 mm. at 20 days. Significance was tested by calculating standard errors of differences between means on the 2nd, 10th and 21st days. It is probable, however, that two curves which are consistently different indicate valid differences even though statistical tests on individual days may not be significant.

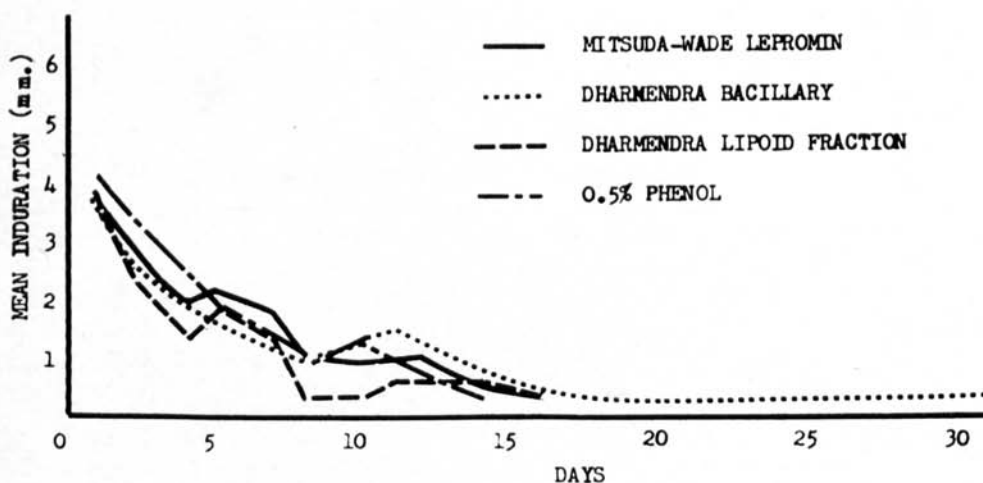


FIG. 1. Reactions in unimmunized control guinea-pigs to *M. leprae* antigens.

ANTIGENS

1. *Mitsuda-Wade lepromin (M-L)*.—This antigen was prepared in this laboratory and standardized by our standardized-drop technique⁽⁸⁾ for equivalent number of bacilli against a batch of lepromin prepared by Wade.

2. *Dharmendra bacillary antigen (DBA)*.—Autoclaved lepromatous tissue, 5 gm., was ground by hand with mortar and pestle, with aliquots of 4-5 cc. of chloroform. After each grinding the chloroform was pipetted off and a smear made from the residue. After 5 hours of grinding very few acid-fast bacilli remained. The 68 cc. of pooled aliquot of chloroform contained many bacilli and little tissue. The chloroform was evaporated and the waxy paste residue dissolved in ether. After centrifugation at 2500G for 10 minutes, the supernatant was removed and the residue resuspended in ether and recentrifuged. The pooled ether supernatant was saved for making the Dharmendra lipid antigen. The sediment was transferred to a mortar, a few drops of 0.1 N NaOH were added, and grinding started. Progressive grinding and dilution in 80 cc. of 0.5 per cent phenol-saline produced a fine suspension in which many poorly staining bacilli could be seen. The dilution contained very nearly the same number of organisms as the Wade lepromin.

A further observation of interest is that when a smear was made of this Dharmendra lepromin after more than 3 months' storage in a

refrigerator, the bacilli had regained their acid-fast staining capacity. They now stained clearly as normal-appearing mycobacteria rather than the bacillary ghosts observed soon after preparation.

3. *Dharmendra lipoid fraction (DLA)*.—The pooled ether supernatant removed from the previous preparation was evaporated to leave an abundant waxy residue containing relatively few recognizable bacilli. A few drops of 0.1 N NaOH were added, and by grinding with a mortar and pestle a fine suspension in 280 cc. of phenol-saline was prepared.

4. *Dharmendra combined antigen (DCA)*.—In attempt to reconstitute the lepromin components, equal proportions of the Dharmendra bacillary and lipoid antigens were combined. It was recognized that this produced a 50 per cent dilution of the bacillary antigen, which would have to be taken into account in testing the adjuvant effect of the lipoid antigen.

5. *Guinea-pig spleen (GPS)*.—One gram of uncontaminated guinea-pig spleen was ground in a mortar with 20 cc. of saline. After filtration through a fine nylon mesh (provided by Dr. Wade for preparing Mitsuda lepromin), the residue was reground in saline and again filtered. Phenol, 0.5 per cent was added and the preparation was autoclaved.

6. *Guinea-pig spleen-Dharmendra lipoid antigen (GPS-DLA)*.—Equal proportions of the above two antigens were combined to test for the adjuvant effect of the Dharmendra lipoid antigen.

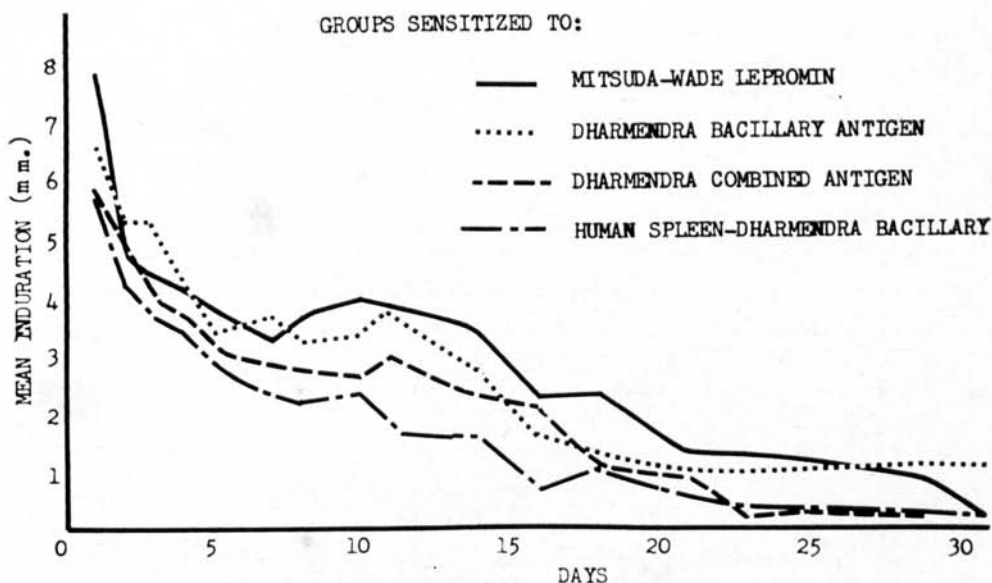


FIG. 2. Cross reactions to the Dharmendra bacillary antigen in guinea-pigs sensitized to *M. leprae* antigens, comparing particularly the adjuvant effects of Dharmendra lipoid fraction and human tissue when combined with Dharmendra's antigen.

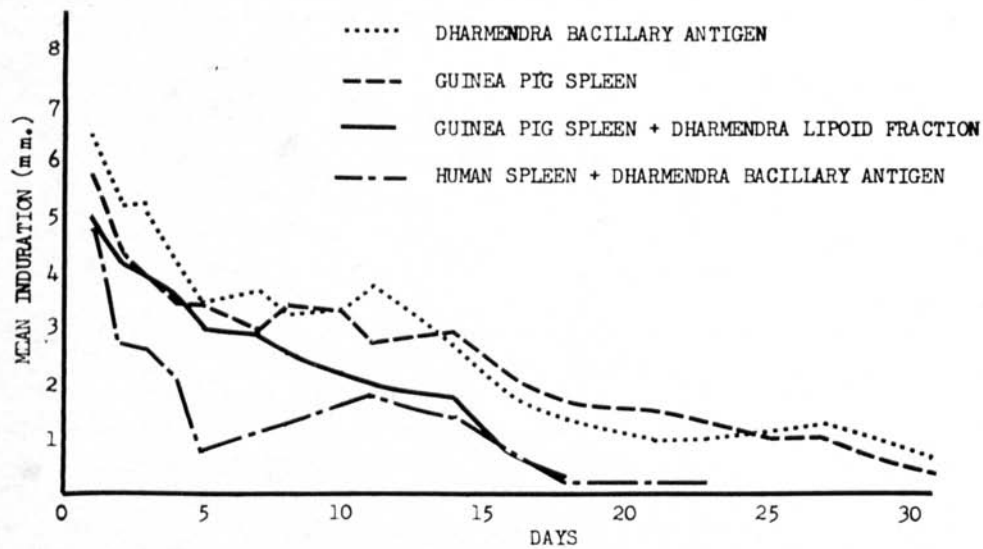


FIG. 3. Cross reactions in guinea-pigs sensitized to the Dharmendra bacillary antigen, when tested with tissue and *M. leprae* antigens.

7. *Human spleen-Dharmendra bacillary antigen (HS-DBA).*—Normal human spleen was prepared according to the method described above for guinea-pig spleen and added to an equal amount of Dharmendra bacillary antigen. Quantitatively, this preparation represented approximately a 50 per cent dilution of standard Mitsuda-Wade lepromin.

8. *Phenol control.*—Phenol, 0.5 per cent, in saline.

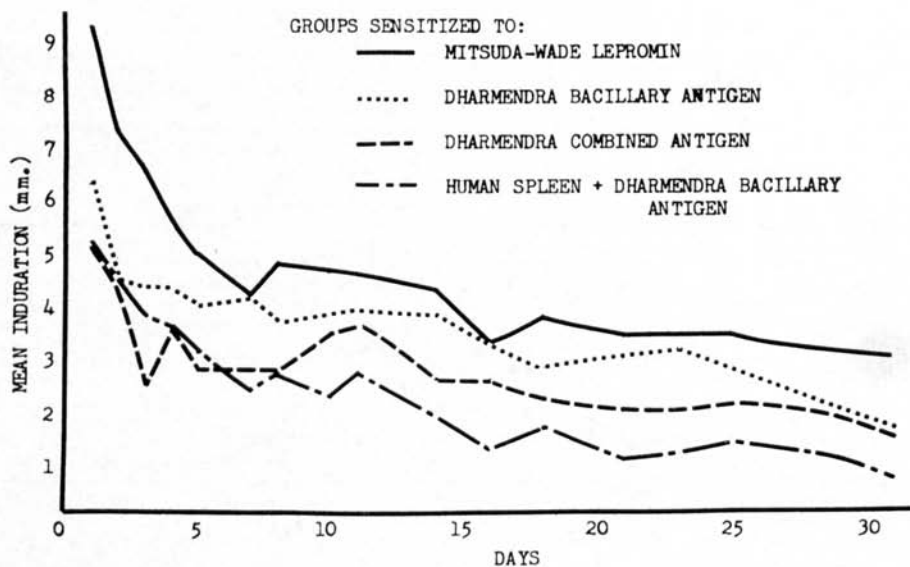


FIG. 4. Reactions to Mitsuda-Wade lepromin in guinea-pigs sensitized to tissue and *M. leprae* antigens.

RESULTS

1. *Dharmendra bacillary antigen*.—A. Effectiveness as a sensitizing agent: Dharmendra's bacillary antigen was not as effective as the Mitsuda-Wade lepromin even when tested in animals given homologous sensitization (Fig. 2). No difference was observed up to the 8th day, but from the 10th to the 25th days the group immunized by the Wade lepromin consistently showed slightly larger reactions. The differences were not, however, significant at the P 0.5 level on either the 10th or 21st days. It is worth noting that in most groups in which the Dharmendra bacillary antigen was used as a test agent, a distinctly bimodal response was produced. An unexplained observation is that guinea-pigs sensitized with Dharmendra bacillary antigen showed relatively strong reactions to the two antigens containing guinea-pig spleen (Fig. 3).

Of particular interest in providing support for the quantitative validity of these observations is a comparison between the sensitizing capacity of the Dharmendra bacillary antigen alone and when it was combined with either Dharmendra lipid fraction or human spleen, so

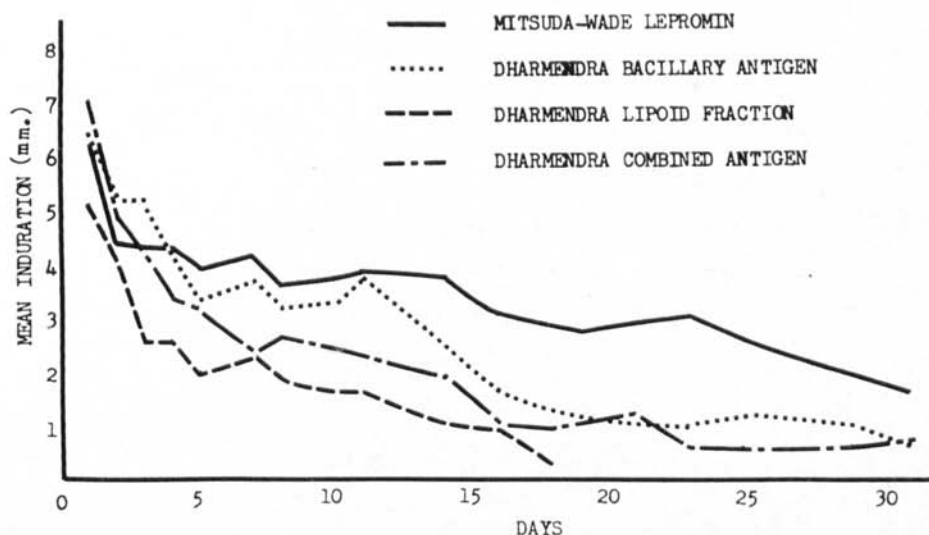


FIG. 5. Cross reactions in guinea-pigs sensitized to the Dharmendra bacillary antigen, when tested with *M. leprae* antigens.

that the amount of bacillary antigen was reduced by half. Tests with the Mitsuda-Wade lepromin showed that reactions in guinea-pigs sensitized to Dharmendra bacillary antigen alone were consistently higher than in those sensitized to Dharmendra combined antigen, and these again gave stronger reactions than when human spleen was combined with Dharmendra bacillary antigen (Fig. 4). With Dharmendra bacil-

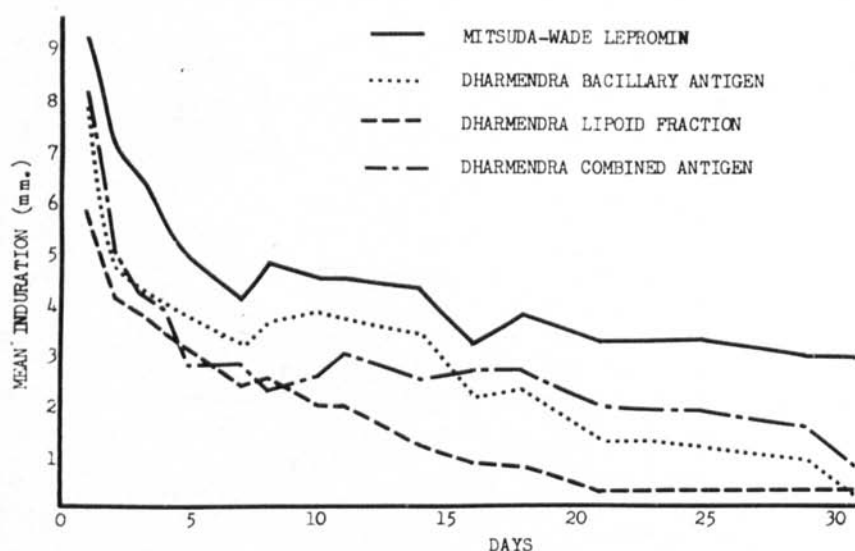


FIG. 6. Cross reactions in guinea-pigs sensitized to Mitsuda-Wade lepromin, when tested with *M. leprae* antigens.

lary antigen as the test agent, similar differences were observed (Fig. 2) which were consistent although not statistically significant on individual days.

B. Effectiveness as a test antigen: In guinea-pigs sensitized to the Dharmendra bacillary antigen, sensitivity to the same antigen was as high for the first ten days as response to the Mitsuda-Wade lepromin (Fig. 5). At 21 days the reactions to Dharmendra bacillary antigen had decreased, so that the differences were highly significant (three times the standard error of the differences between the means). In the group of guinea-pigs sensitized to the Mitsuda-Wade lepromin the same relationship between the early and late portions of the curves was observed, although the Mitsuda-Wade lepromin response was relatively higher throughout (Fig. 6).

The important consideration is that the Dharmendra antigen had not been purified sufficiently to give minimal or no reactions in guinea-pigs sensitized by tissue antigens. When tested in animals sensitized to guinea-pig spleen, the Dharmendra antigen produced reactions which were consistently, though not significantly, larger than those to Mitsuda-Wade lepromin (Fig. 7). Evidence for a relative reduction of the tissue component in Dharmendra bacillary antigen was provided, however, in a series of experiments reported in detail elsewhere⁽¹³⁾, in which groups of guinea-pigs were sensitized with purified bacillary suspensions.

Other tests indicated that bacilli which had been subjected to 6 hours' digestion with a mixture of bile and pancreatin were relatively much freer of tissue proteins than Mitsuda-Wade lepromin, although

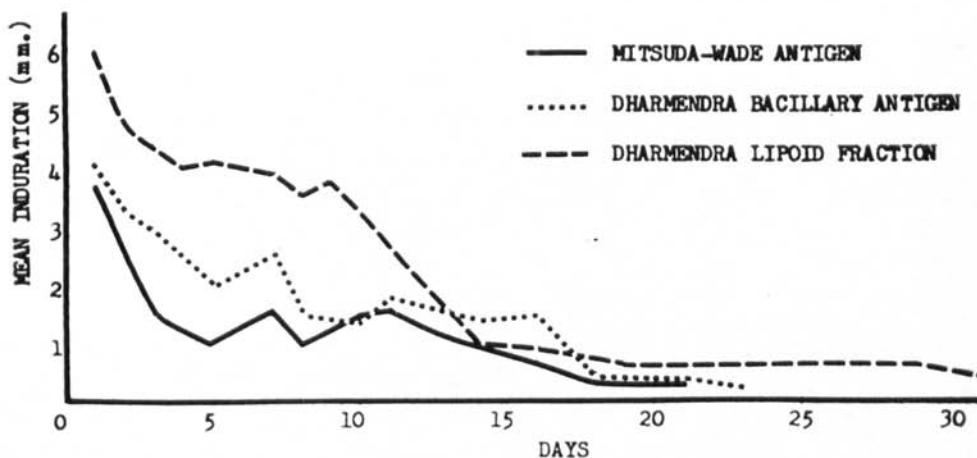


FIG. 7. Cross reactions in guinea-pigs sensitized to guinea-pig spleen, when tested with *M. leprae* antigens.

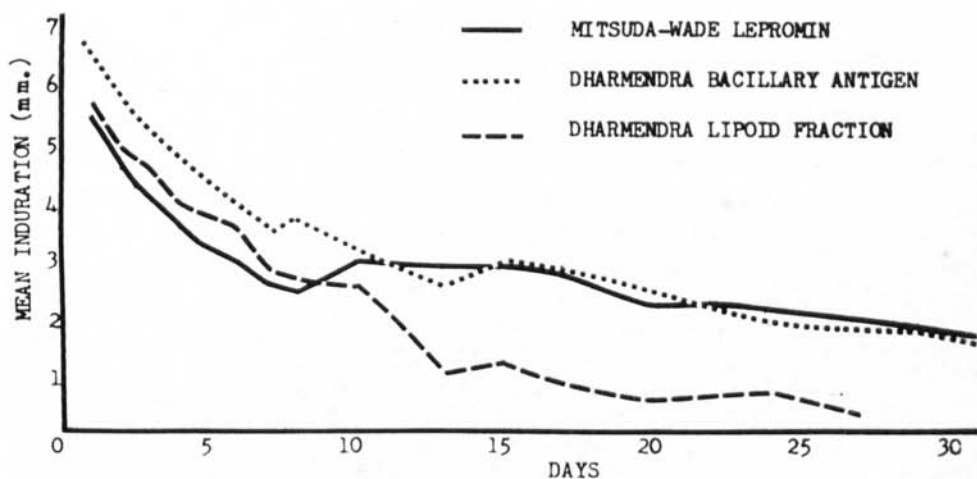


FIG. 8 Cross reactions in guinea-pigs sensitized to bile-pancreatin lepromin, when tested with *M. leprae* antigens.²

this prolonged digestion did significantly reduce the antigenicity of the bacilli. Fig. 8 shows that, in guinea-pigs sensitized to bile-pancreatin lepromin, up to the 10th day Mitsuda-Wade lepromin produced a smaller reaction than Dharmendra bacillary antigen. On the 2nd day this difference was statistically significant at a P of 0.002.

By contrast, in the group sensitized to Mitsuda-Wade lepromin (Fig. 9) the two curves were superimposed for the first 10 days, with Mitsuda-Wade lepromin then producing a more persistent reaction. The relatively higher response to Dharmendra bacillary antigen in this test as compared with the earlier experiment (Fig. 6) may be related to

²Second series, after the Dharmendra bacillary lepromin had regained acid-fast staining capacity after 4 months of storage in refrigerator.

the observation that after three to four months' storage in the refrigerator the Dharmendra bacillary antigen contained normal-appearing leprosy bacilli which had regained their acid-fast staining lipid coats.

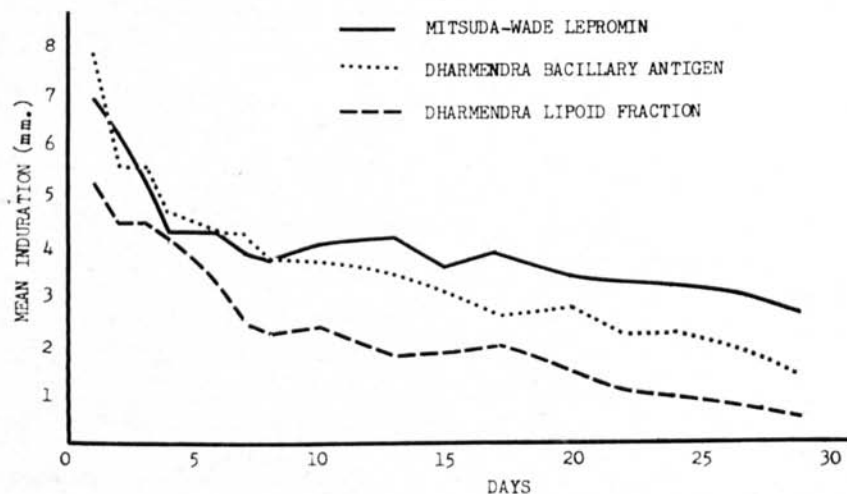


FIG. 9. Cross reactions in guinea-pigs sensitized to Mitsuda-Wade lepromin, when tested with *M. leprae* antigens.

2. *Dharmendra lipid fraction*.—A. Effectiveness as a sensitizing agent: When given alone, the Dharmendra lipid did not serve as an effective sensitizing agent. Even when tested in animals sensitized against itself, the response was consistently smaller than when groups were sensitized with other antigens (Fig. 10).

B. Effectiveness as a test agent: Smaller responses were elicited by the Dharmendra lipid fraction than by any of the other preparations, regardless of whether the groups of guinea-pigs had been sensitized to Mitsuda-Wade lepromin (Fig. 6), Dharmendra bacillary anti-

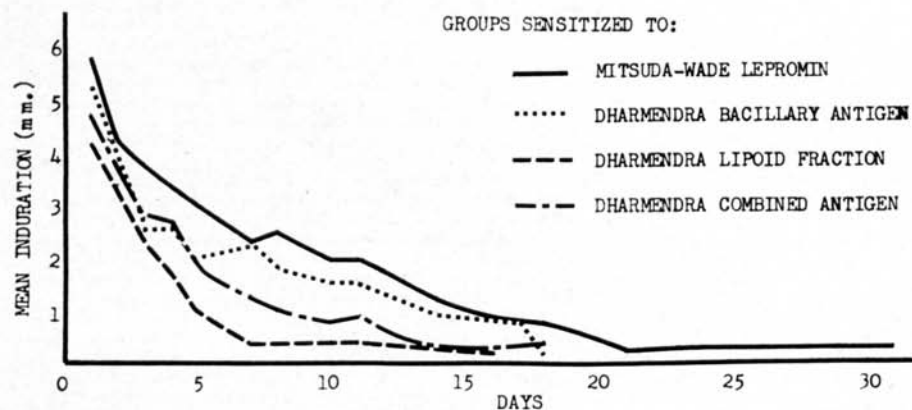


FIG. 10. Reactions to Dharmendra lipid fraction when tested in guinea-pigs sensitized to *M. leprae* antigens.

gen (Fig. 5), or Dharmendra lipid fraction itself (Fig. 11). Most of these differences were significant beyond P 0.01 at the 10th day.

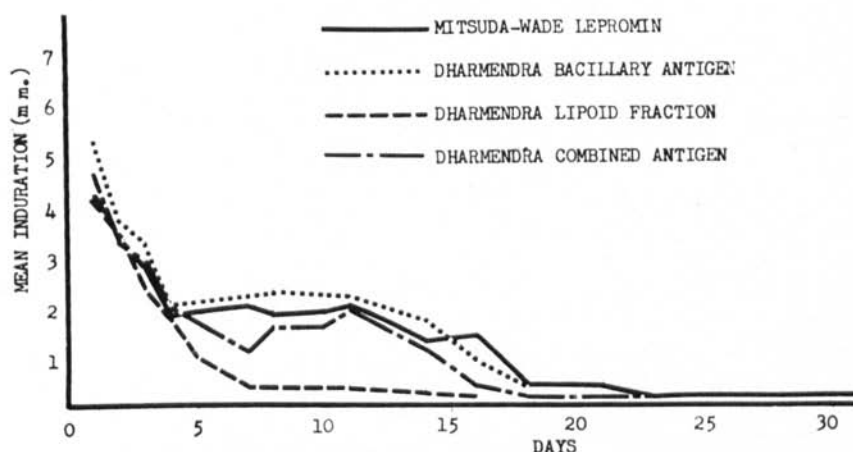


Fig. 11. Cross reactions in guinea-pigs sensitized to the Dharmendra lipid fraction, when tested with *M. leprae* antigens.

Because of the greater reaction in tissue-sensitized guinea-pigs, it seems probable that the method of extraction carries into the Dharmendra lipid fraction a proportion of the tissue antigens as well as dissolved bacillary proteins and lipids (Fig. 7). This is supported by the essentially equivalent early responses to the Dharmendra lipid fraction whether tested in groups sensitized to bacillary products or to tissue antigens (Fig. 12).

3. *Dharmendra combined antigen*.—A. Effectiveness as a sensitizing agent: Reactions to recombined Dharmendra antigen in the homologous group of guinea-pigs were equivalent to those in animals sensitized to Dharmendra bacillary antigen alone (Fig. 13). Both series of responses were smaller than those in the Mitsuda-Wade lepromin

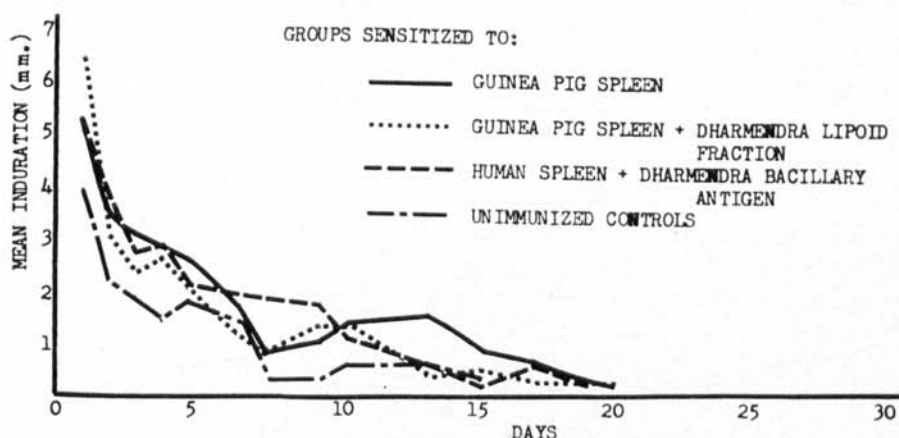


Fig. 12. Reactions to the Dharmendra lipid fraction when tested in guinea-pigs sensitized to tissue and *M. leprae* antigens.

group after the 15th day. In view of the 50 per cent dilution of bacilli in the combined antigen, this suggests that the lipoid had an adjuvant effect. Additional evidence is provided by comparing the sensitizing capacity of the Dharmendra combined antigen with the bacillary antigen diluted by mixing with human spleen antigen. After 10 days the reactions to Mitsuda-Wade lepromin were significantly larger in the Dharmendra combined group than in the human spleen-Dharmendra bacillary antigen group, indicating again that an adjuvant effect had occurred (Fig. 4).

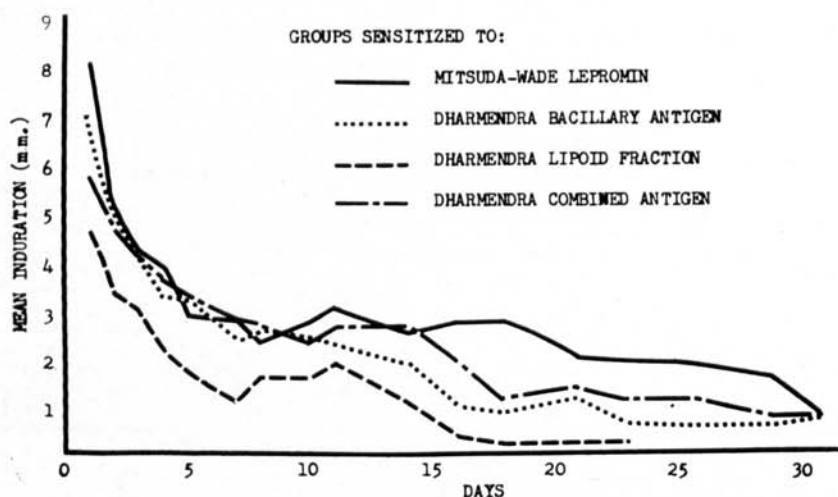


Fig. 13. Reactions to the Dharmendra combined antigen when tested in guinea-pigs sensitized to *M. leprae* antigens.

B. Effectiveness as a test antigen: Addition of Dharmendra lipoid fraction to bacillary antigen caused a relative increase of the late response. This is seen most clearly in the group of guinea-pigs sensitized to Mitsuda-Wade lepromin (Fig. 6), where the bacillary-antigen and the combined-antigen curves cross on the 15th day. When tests were done in guinea-pigs sensitized with either the Dharmendra bacillary antigen or the combined antigen, responses to the homologous antigen were somewhat exaggerated so that rather than crossing, the curves ran together for either the first or the second halves of the observation period (Figs. 5 and 14). In interpreting the reactions to combined antigen, it should be remembered that it contained only a 50 per cent concentration of bacilli.

4. *Tissue antigens.*—A. Effectiveness as sensitizing agents. The groups of guinea-pigs sensitized to tissue antigens developed generalized reactivity to antigens which contained any form of tissue. An exception is the response to Mitsuda-Wade lepromin which was significantly higher in the group sensitized to human spleen-Dharmendra bacillary antigen than in the two groups sensitized with preparations

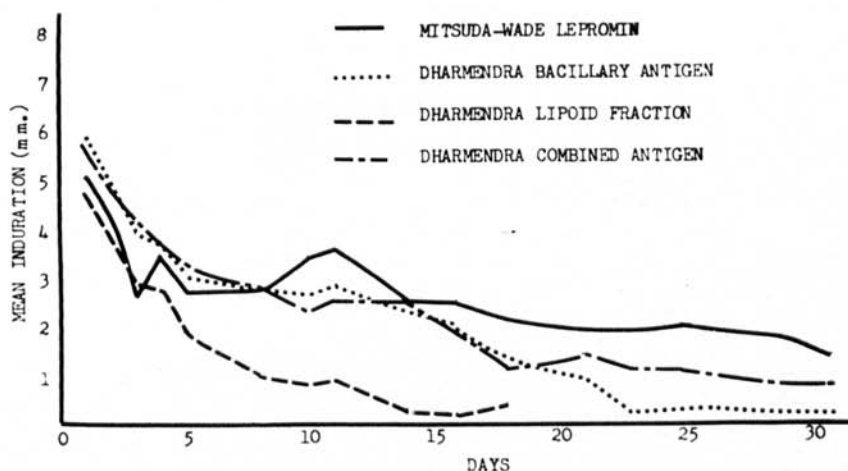


FIG. 14. Cross reactions in guinea-pigs sensitized to the Dharmendra combined antigen, when tested with *M. leprae* antigens.

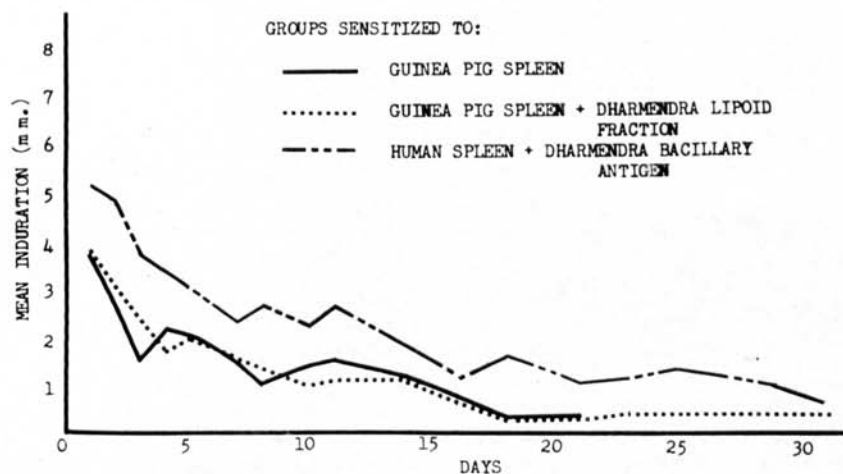


FIG. 15. Reactions to Mitsuda-Wade lepromin in guinea-pigs sensitized to tissue antigens.

containing guinea-pig spleen (Fig. 15). The responses of the tissue-sensitized groups were otherwise essentially the same whether the test preparations contained Dharmendra or tissue antigens (Figs. 16A & B, 17 and 18).

B. Effectiveness as test antigens: The three preparations containing tissue antigens produced almost identical patterns of reactions in most of the groups of animals tested. The reactions sloped steadily down over a period of 10 or more days with little tendency to produce persistent reactions (Fig. 19A & B). The only exceptions to the pattern of uniformly waning reactions was that in the group of guinea-pigs sensitized to guinea-pig spleen-Dharmendra lipoid; the reactions to the two guinea-pig antigens were slightly larger than those to human

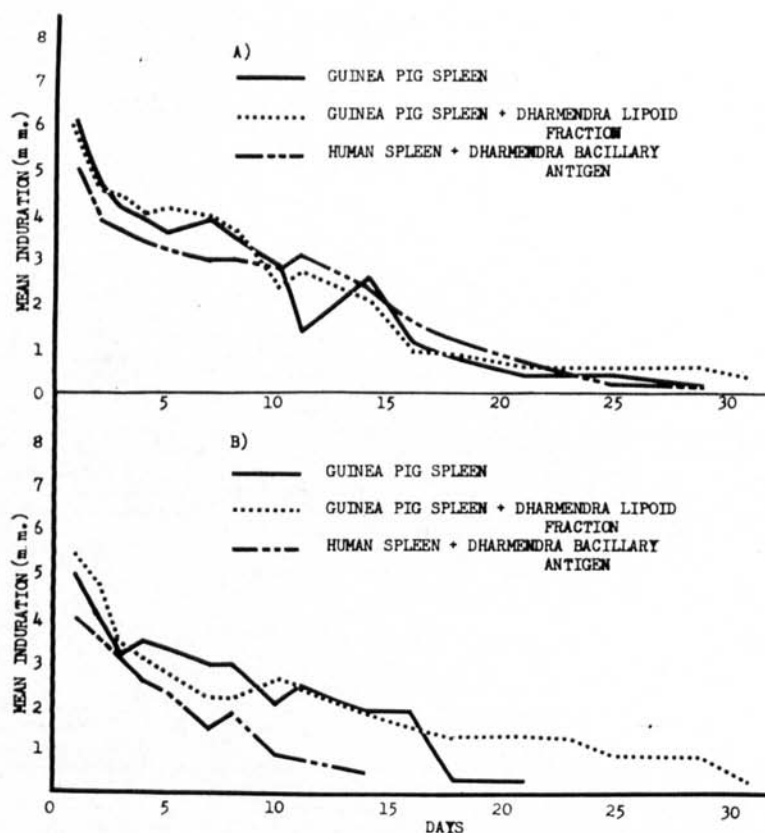


FIG. 16. **A.** Cross reactions in guinea-pigs sensitized to guinea-pig spleen, when tested with tissue antigens. **B.** Cross reactions in guinea-pigs sensitized to the guinea-pig spleen-Dharmendra lipid fraction, when tested with tissue antigens.

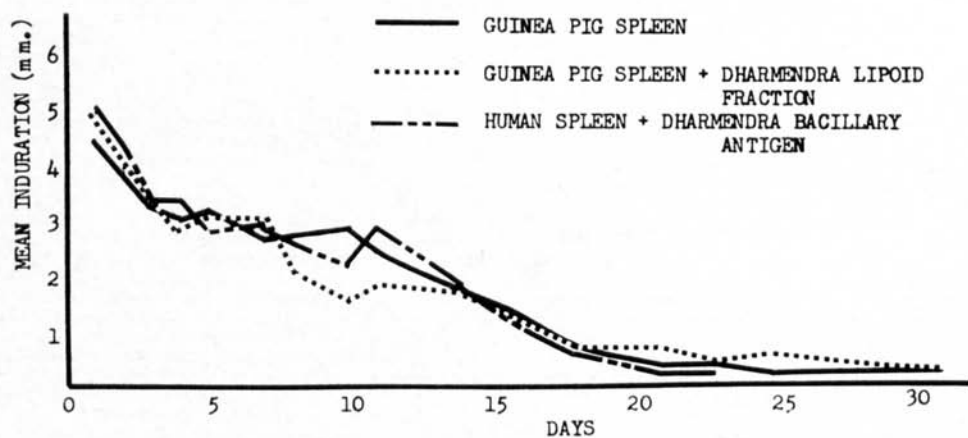


FIG. 17. Cross reaction in guinea-pigs sensitized to the human spleen-Dharmendra bacillary antigen, when tested with tissue antigens.

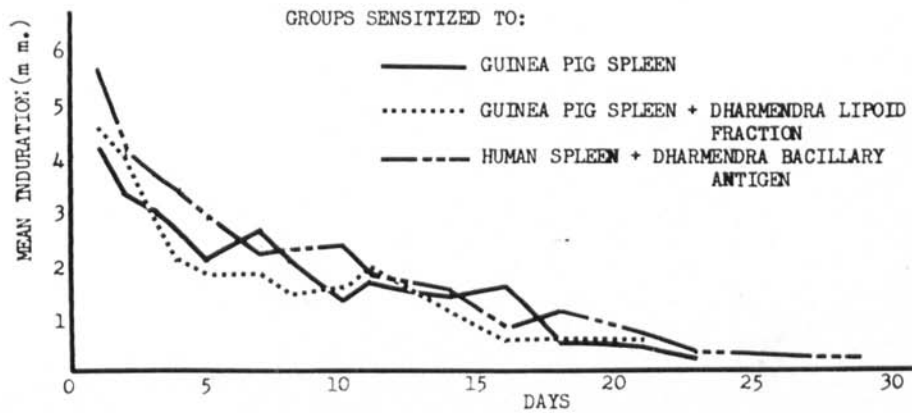


FIG. 18. Reactions to Dharmendra bacillary antigen in guinea-pigs sensitized to tissue antigens.

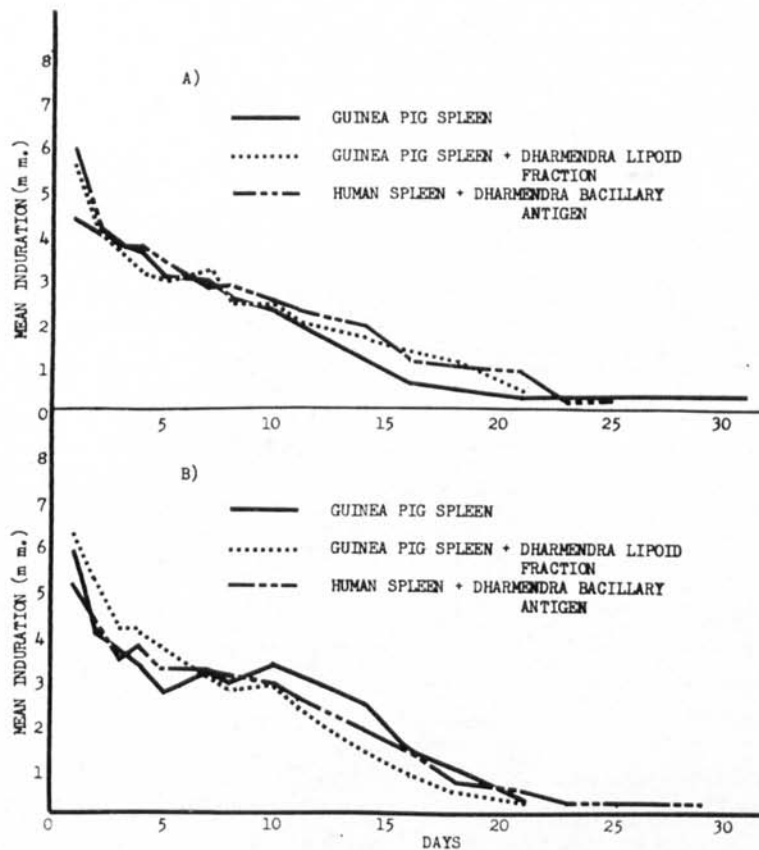


FIG. 19. **A.** Cross reactions in guinea-pigs sensitized to Mitsuda-Wade lepromin, when tested with tissue antigens. **B.** Cross reactions in guinea-pigs sensitized to the Dharmendra combined antigen, when tested with tissue antigens.

spleen (Fig. 16A & B). No homologous bacterial specificity could be detected when the two preparations containing tissue plus a specific Dharmendra antigen were tested in groups of guinea-pigs sensitized to the Dharmendra bacillary and lipid antigens (Fig. 3 and 20).

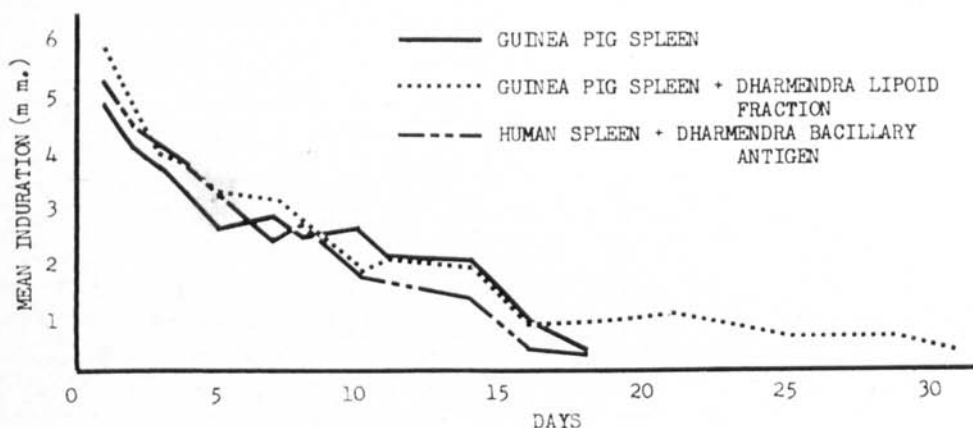


FIG. 20. Cross reactions in guinea-pigs sensitized to the Dharmendra lipid fraction, when tested with tissue antigens.

DISCUSSION

In testing the specificity of response to Dharmendra's antigen, our first interest was to discover whether the soluble tissue antigens had been significantly reduced in respect to sensitizing capacity and to skin test reactivity. Dharmendra antigen produced definite sensitization to tissue antigens (Fig. 3). When tested in guinea-pigs sensitized to guinea-pig tissue antigens, Dharmendra antigen produced slightly stronger reactions than did Mitsuda-Wade lepromin (Fig. 7). We did not have a group of guinea-pigs sensitized to human tissue alone, but our data show a very large degree of cross reaction between tissue antigens from human and guinea-pig sources (Figs. 16 A & B and 17).

Wade (^{14, 15}) has postulated that the Mitsuda (3-week) reaction in human leprosy is due to the release of antigen by the slow and progressive breakdown of bacilli. Confirmatory evidence is the difference observed between the speed of reaction of Mitsuda-Wade lepromin and of Dharmendra lepromin (Figs. 5 and 6). In Dharmendra's antigen the bacilli appear to have been modified so that active antigen components were released more readily and produced earlier reactions than did the bacilli in Mitsuda-Wade lepromin. Bimodal responses were obtained more consistently than with any agent we have tested thus far except tubercle bacilli. It appeared that some of the reactivity which in Mitsuda-Wade lepromin was maintained for 3 or 4 weeks was here accelerated to reach a peak between 10 and 14 days. Grossly, the bacilli in Dharmendra's lepromin appeared to have remained structurally

intact although they had lost much of their acid-fast staining capacity during their intensive exposure to chloroform and ether. A considerable volume of waxy and fatty material was separated in the lipoid fraction. That the bacillary suspension still contained lipid was indicated by the chance observation that bacilli regained their acid-fastness after several months' storage in a refrigerator.

In these studies Dharmendra lipoid fraction did not seem to be a potent adjuvant for producing sensitivity. A slight adjuvant effect is indicated by the better response when the Dharmendra bacillary antigen was combined with the Dharmendra lipoid fraction than when it was combined with the human tissue antigen (Figs. 2 and 4).

SUMMARY

In guinea-pigs sensitized by heated *M. leprae*, Dharmendra's antigen produced a more rapid skin response than Mitsuda-Wade lepromin, with a tendency to bimodal peaking at ten days. Dharmendra's antigen showed as much cross reactivity with tissue antigen as Mitsuda-Wade lepromin when the two were tested in guinea-pigs sensitized to guinea-pig spleen. Dharmendra's antigen also sensitized guinea-pigs to tissue antigens.

Dharmendra's lipoid fraction had a weak adjuvant effect.

As judged by immunologic criteria, the chloroform procedure does not produce satisfactory purification of *M. leprae* suspensions.

RESUMEN

En cobayos sensibilizados con *M. leprae* calentados, el antígeno de Dharmendra produjo una cutirreacción más rápidamente que la lepromina de Mitsuda-Wade, con tendencia a alcanzar su punto álgido binodal a los 10 días. El antígeno de Dharmendra reveló tanta reactividad cruzada con el antígeno de tejido como la lepromina de Mitsuda-Wade al comprobar los dos en cobayos sensibilizados al bazo de cobayo. El antígeno de Dharmendra también sensibilizó a los cobayos a los antígenos de tejido.

La fracción lipóidea de Dharmendra ejerció efecto coadyuvante débil.

A la luz de las pautas inmunológicas, el procedimiento del cloroformo no obtiene una purificación satisfactoria de las suspensiones de *M. leprae*.

RESUMÉ

Chez les cobayes, sensibilisés par *M. leprae* après que celui-ci ait été chauffé, l'antigène de Dharmendra produit une réponse cutanée plus rapide que la lépromine de Mitsuda-Wade, avec une tendance vers un maximum bimodal au huitième jour. Lorsqu'ils, chez des cobayes sensibilisés à la rate de cobaye, le lépromine de Mitsuda-Wade et l'antigène de Dharmendra ont été testés, ce dernier produit a témoigné d'autant de réactivité croisée avec l'antigène tissulaire que la lépromine. L'antigène de Dharmendra a également sensibilisé les cobayes aux antigènes tissulaires.

La fraction lipéidique de Dharmendra a un faible effet adjuvant.

A en juger d'après les critères immunologiques, la méthode au chloroforme ne donne pas de suspensions de *M. leprae* d'une pureté satisfaisante.

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