Fernandez and Mitsuda Reactivity in Guinea Pigs Sensitized with Heat-killed *Mycobacterium leprae*: Persistence and Specificity of Skin Reactivity to Soluble and Particulate Antigens[1]

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Recent studies indicate that both mice ([19]) and guinea pigs ([11]) can be sensitized by the introduction of a dense saline suspension of heat-killed leprosy bacilli into the tissues. The resulting immune response to the dead *Mycobacterium leprae* appears to be superior to that achieved with other mycobacterial vaccines, with the possible exception of live BCG ([18]). In particular, little or no cross-protection could be observed with *M. vaccae* or *M. nonchromogenicum* despite taxonomic and immunologic evidence ([21]) that they were both antigenically related to the leprosy bacillus ([20]). This lack of immunogenicity shown by live *M. vaccae* and *M. nonchromogenicum* could be explained by their inability to establish persistent systemic infections in the vaccinated mice or guinea pigs ([18, 25]). Available clinical and epidemiological data suggest that the same is probably equally true for man ([20]). However, it may not be necessary to use live mycobacterial vaccines to sensitize the host to the relevant *M. leprae* antigens. It has been known for many years that killed mycobacteria can induce both delayed hypersensitivity and antituberculous immunity in appropriately vaccinated animals, provided that large enough amounts of antigen(s) were introduced into the tissues ([11, 22]). Thus, guinea pigs could be sensitized by injecting a large dose (500–1000 μg dry weight) of saline-suspended heat-killed *M. leprae*, *M. tuberculosis*, *M. nonchromogenicum*, or *M. vaccae* into normal guinea pigs. Subsequently, a persistent state of skin hypersensitivity developed in all four groups. However, the response to the *M. leprae* suspension was always quantitatively superior to that observed for the other three test mycobacteria.

MATERIALS AND METHODS

Animals. Specific pathogen-free, inbred strain 2 guinea pigs weighing 250–350 g were obtained from the Trudeau Animal Breeding Facility, Saranac Lake, New York, U.S.A. They were housed two to a cage and fed sterile commercial guinea pig chow and chlorinated water *ad libitum* ([3, 5]).

Bacterial suspensions. *M. tuberculosis* H37Rv (TMC #102), *M. nonchromogenicum* (TMC #1481), and *M. vaccae* (TMC #1526) were obtained from the Trudeau Mycobacterial Culture Collection, Saranac Lake, New York, U.S.A. The organisms were grown as surface pellicles on Proskauer and Beck synthetic liquid medium incubated at 27°C in Roux bottles. The pellicle was removed by filtration, washed thoroughly with sterile saline, inactivated by heating at 100°C for 30 min, and the suspension lyophilized without further washing. The dried bacteria were suspended in 0.05% Tween 80-saline at a concentration of 500 μg dry weight per ml and homogenized with a teflon pestle for 30 sec (Tri-R Instruments, Rockville Center, New York, U.S.A.) Finally, the suspension was exposed to 5 sec of ultrasonic vibration (50% power output) before being standardized microscopically to 2×10^9 acid-fast bacilli (AFB) per ml using auramine-stained smears examined under ultraviolet illumination ([4]).

***M. leprae* suspensions.** Human *M. leprae* were obtained from wedge biopsies taken from untreated Burmese lepromatous patients. The tissue samples were generously

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donated by Dr. A. M. Dhople, Johns Hopkins University School of Hygiene and Public Health, Baltimore, Maryland, U.S.A. Briefly the tissues were autoclaved at 115°C for 10 min and homogenized in 10 g batches in 0.3 M sucrose-phosphate buffer containing 0.25 M EDTA (pH 7.2). The resulting homogenate was centrifuged at 300 × *g* for 5 min to remove residual tissue pieces, and the supernatant fluid was centrifuged at 15,000 × *g* for 10 min at 4°C. The cell pellet was resuspended in 1% Triton X-100 in 0.3 M sucrose-EDTA buffer and allowed to stand at room temperature for 1 hr to lyse the residual human cell-membrane fragments. No attempt was made to digest the homogenate with proteinases or to treat the cells with sodium hydroxide ([15]). The *M. leprae* were concentrated by centrifugation at 15,000 × *g* for 10 min at 4°C, and the pellet washed twice with sucrose-EDTA buffer before being resuspended in 0.1 M Tris-HCl buffer (pH 7.2) and incubated overnight at 37°C with 10 μg of collagenase per ml (Worthington Biochemicals, Freehold, New Jersey, U.S.A.) in the presence of 10^{-4} M calcium chloride and 0.05% sodium azide. The *M. leprae* were washed, resuspended in 0.05% Tween-saline, and standardized microscopically using the method of Shepard and McRae ([16]). The suspension was adjusted to a final concentration of 2×10^9 AFB per ml which represents approximately 1 mg dry weight per ml ([4]).

Immunization procedures. Groups of guinea pigs were injected subcutaneously into the nape of the neck with 1.0×10^9 (ca. 500 μg) of heat-killed *M. leprae, M. tuberculosis, M. nonchromogenicum,* or *M. vaccae* in 0.5 ml of sterile 0.05% Tween-saline ([11]). Control animals were injected with 0.5 ml of sterile saline. Some of the guinea pigs were given an identical booster injection of the homologous suspension some three months later.

Skin test antigens and testing procedures. Whole-cell antigen (WCA) was prepared from heat-killed *M. leprae, M. tuberculosis, M. nonchromogenicum,* and *M. vaccae* by suspending $1–2 \times 10^8$ AFB per ml of 0.05% Tween-saline (ca. 50 μg dry weight) as described previously ([25]). The presence of residual human tissue antigens in the *M. leprae* preparations resulted in some skin reactivity to human skin extracts injected into appropriate control animals. However, the human skin antigen responses were small enough to be disregarded in animals immunized with the Triton X-100 treated preparations (E. P. Elliston, personal communication).

Cytoplasmic protein antigens (CPA) were prepared by sonic disruption of approximately 1 mg dry weight of whole cells at 0°C over a 30-min period in 30-sec bursts ([25]). The residual whole cells were spun down at 15,000 × *g* for 10 min and the supernatant fluid subjected to ultracentrifugation at 100,000 × *g* for 90 min. The protein content of the supernatant fluid was standardized to 100 μg of protein per ml ([25]).

Cell wall protein antigen (CWA) consisted of the outer membrane proteins prepared from *M. leprae* by Dr. T. M. Buchanan, Seattle, Washington, U.S.A., using the method described by Caldwell, *et al.* ([2]). The CWA was standardized at a concentration of 100 μg of protein per ml of diluent.

Skin test antigens were injected intradermally into freshly shaven flank or belly skin in a volume of 0.1 ml of saline. Normal and saline controls were always included in each test. The injection sites were examined at 3 hr and 24 hr for erythema and induration and at 48 hr and 72 hr for induration (measured as increases in skin-fold thickness using Schnelltäster dial gauge calipers[5]). Whole cell antigen (WCA) test sites were also examined for erythema and induration at weekly intervals for up to six weeks. Central necrosis, especially in animals which had received two doses of immunogen, was noted in the *M. leprae*-sensitized animals tested with 10 μg of lepromin. Increases of ten or more Schnelltäster units (> 1.0 mm) in skin-fold thickness (after subtraction of control values) were significant at the 1% level for five replicate determinations per time point.

Statistical analysis. Differences between mean swelling reactions were evaluated by means of the Student *t* test ([26]).

RESULTS

Skin reactivity following injection with heat-killed mycobacteria. Guinea pigs were immunized with 10^9 heat-killed *M. leprae, M. tuberculosis, M. nonchromogenicum,* or *M. vaccae* and skin tested with 10 μg of the homologous CPA or WCA 30 days later.

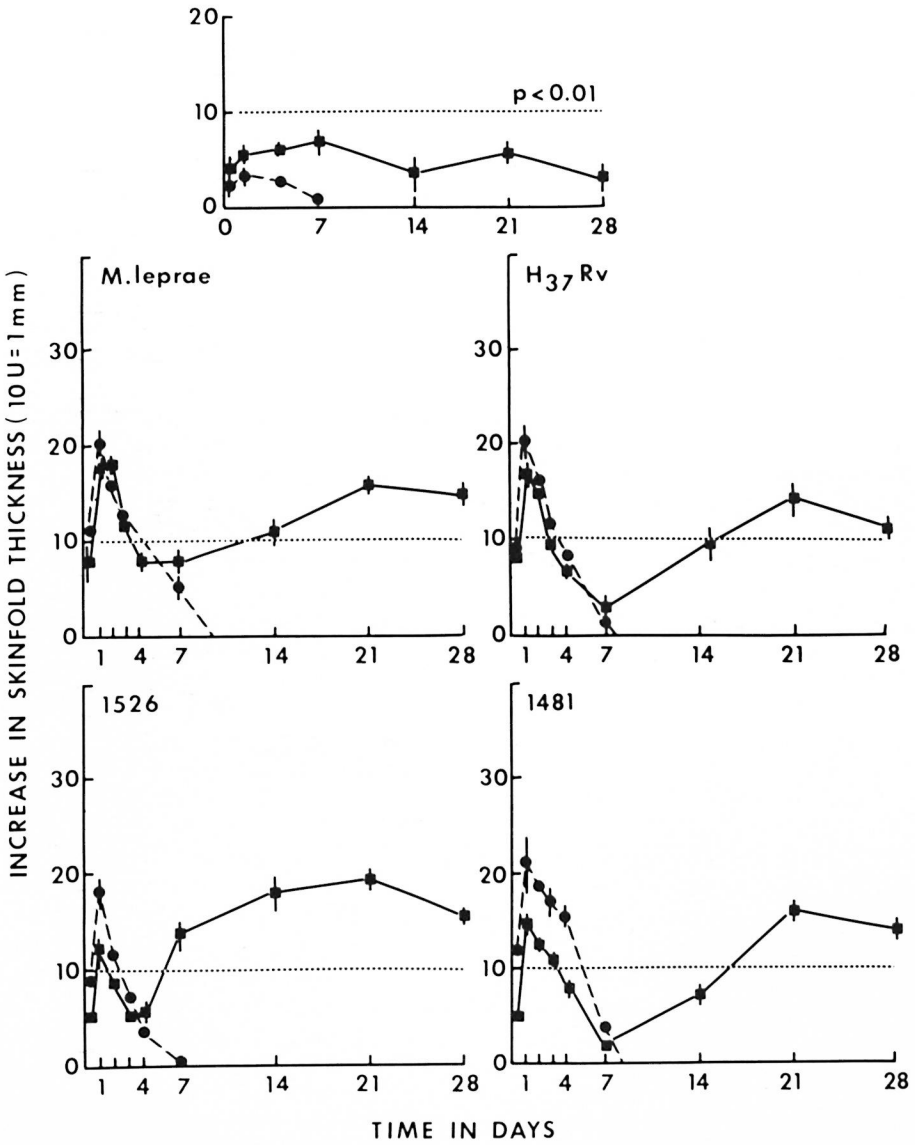

FIG. 1. Increases in skin-fold thickness in strain 2 guinea pigs immunized with heat-killed *Mycobacterium leprae*, *M. tuberculosis* H37Rv, *M. nonchromogenicum* #1481, or *M. vaccae* #1526 30 days prior to skin testing with 10 μg of the homologous CPA (---●---) or WCA (—■—) in 0.1 ml of diluent. The vertical lines represent ±SEM for four determinations. Control guinea pigs received saline only in place of the mycobacterial vaccine. Control CPA and WCA values, as shown in the uppermost panel, represent the *M. leprae* values which are generally larger than the values for the other three test antigens although still below the limit of significance ($p < 0.01$) represented by the broken horizontal line.

The resulting skin swelling responses are shown in Figure 1. In every case, the 3 hr swelling responses to the CPA were substantially lower than those observed at 24 hr, quickly falling to nonsignificant levels by 48 hr. None of the unvaccinated controls developed significant responses to either the CPA or the WCA preparations (Fig. 1).

Injection of 10 μg of the corresponding WCA's into the immunized animals resulted in an early Fernandez-type response (peaking at 24–48 hr) for both the *M. leprae* and *M. tuberculosis* preparations. The *M. vaccae* and *M. nonchromogenicum* WCA skin responses were barely significant at that time (Fig. 1). The 24–48 hr swelling re-

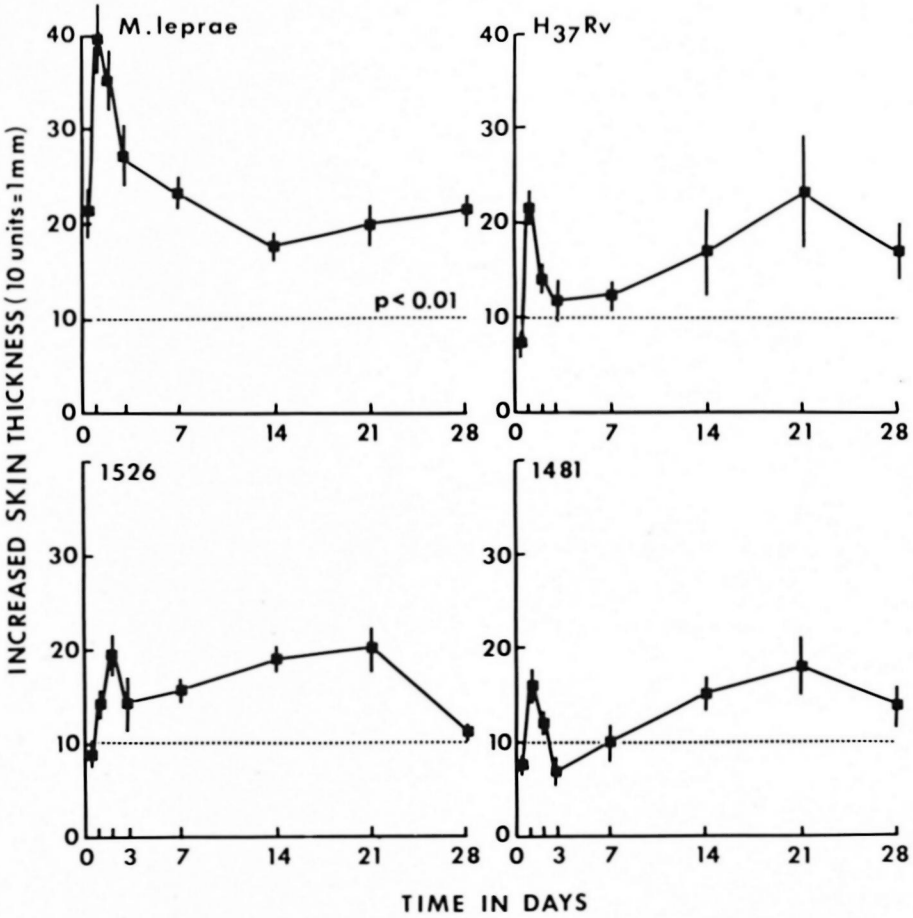

FIG. 2. Increases in skin-fold thickness in the four groups of guinea pigs following boosting with 500 μg of the homologous organism 30 days prior to skin testing with the corresponding WCA preparations. (See Figure 1 legend for further details.) Control WCA values were similar to those shown in Figure 1.

sponses declined to near zero levels by day 7, followed by a second, Mitsuda-type response which peaked around day 21 of the test period. Both the *M. vaccae* and *M. nonchromogenicum* sensitized guinea pigs showed lower, although still significant, Mitsuda-type responses when tested with their homologous WCA preparations (Fig. 1).

Effect of antigenic boosting on skin reactivity. The four groups of immunized pigs were restimulated with an identical dose of mycobacteria given some three months after their primary sensitization. A substantial local inflammatory reaction developed at the secondary *M. leprae* and *M. tuberculosis* injection sites in the neck region, eventually resulting in some local scarring extending to as much as 2 cm in diameter for the *M. leprae* preparation. The local response at the *M. nonchromogenicum* and *M. vaccae* depot sites was relatively much smaller, resulting in little or no localized necrosis or scarring.

One month after receiving the second dose of antigen, the four groups of immunized guinea pigs (and an age-matched group of controls) were skin tested with the homologous CPA and WCA preparations. The 3 hr and 24 hr skin responses to the CPA preparations were not greatly increased by the booster injections and are not shown in Figure 2. There was a marked increase in Fernandez reactivity with all four WCA preparations. The later, Mitsuda-type responsiveness also increased substantially in

the *M. leprae* and H37Rv-boosted animals but not in the *M. nonchromogenicum* or *M. vaccae* groups (Figs. 1 and 2).

A remote positivity response ([14]) was observed at the *M. leprae* depot site in the neck at about the time when peak Mitsuda skin reactivity developed at the flank lepromin injection site. No analogous responses occurred at the antigen deposition sites for the other three groups of immunized animals when they were skin tested with the appropriate WCA preparations.

Crossreactivity to the WCA preparations in the sensitized guinea pigs. Guinea pigs sensitized six months earlier with the mycobacterial suspensions were retested for skin reactivity and the resulting swelling process is recorded in Figure 3. Substantial Fernandez (day 2) cross-responsiveness occurred in all four groups to the *M. leprae* and the *M. tuberculosis* WCA preparations. However, the *M. vaccae* and *M. nonchromogenicum* WCA Fernandez responses were relatively weak in both the *M. leprae* and H37Rv sensitized animals. Lepromin and H37Rv WCAs were somewhat less reactive in the *M. nonchromogenicum*- and *M. vaccae*-immunized animals than the corresponding TMC #1481 and TMC #1526 WCA reactions. The late Mitsuda-type skin reactions developed at three weeks to the four WCA preparations indicated that the specific responses were substantially stronger than the heterologous ones. This suggests that there may be less antigenic overlap between *M. leprae* and either *M. vaccae* or *M. nonchromogenicum* (at least in terms of their *in vivo* hypersensitivity responsiveness) than had been implied earlier by the *in vitro* lymphoproliferative studies ([21]).

Persistence of skin reactivity in the antigen-boosted guinea pigs. Guinea pigs sensitized with two doses of heat-killed *M. leprae* were still strongly skin reactive to lepromin some 12 months later (Fig. 4). In particular, the Fernandez reactivity level was quite high. The corresponding Mitsuda skin reactivity seen at 12 months was not increased substantially by the booster injection, but the peak in the late swelling reaction occurred between 14–21 days compared to 21–28 days in the earlier skin tests (Figs. 2 and 4). The tuberculin-type skin responses seen 24 hr after the injection of 10 μg of the cell wall antigen supplied by

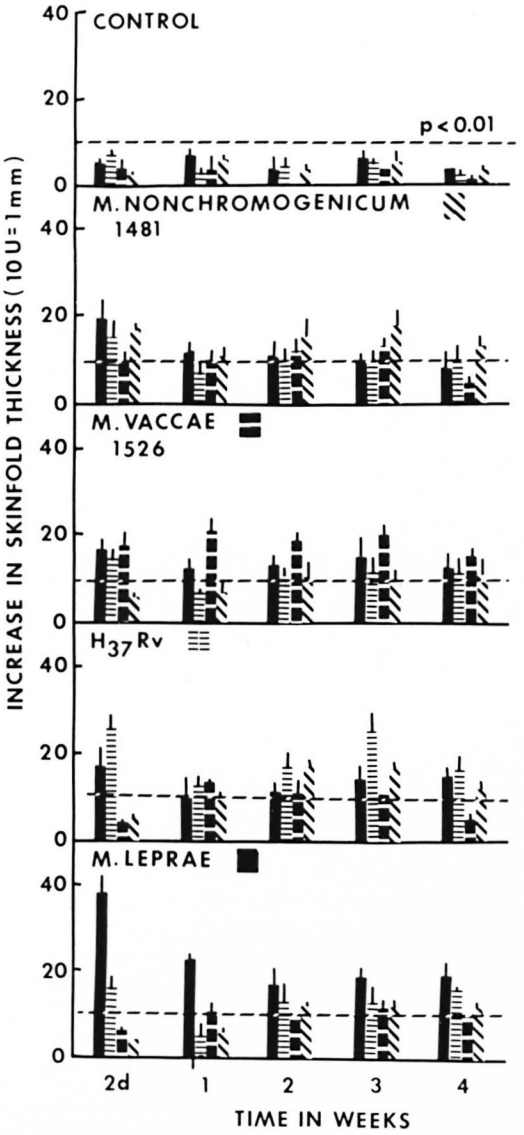

FIG. 3. Crossreactions seen in sensitized or control guinea pigs skin tested with 10 μg of lepromin (▮), *M. tuberculosis* H37Rv WCA (≣), *M. vaccae* WCA (▮) or *M. nonchromogenicum* WCA (▒). Increases of 10 Schnelltäster units or more were significant ($p < 0.01$).

Dr. Buchanan were barely significant at any time during this study (Fig. 4). The corresponding Fernandez reactions for the other three groups of boosted animals were substantially lower than the *M. leprae* response both at six and 12 months (Fig. 5). However, the response by the four groups of animals to the corresponding CPA prepara-

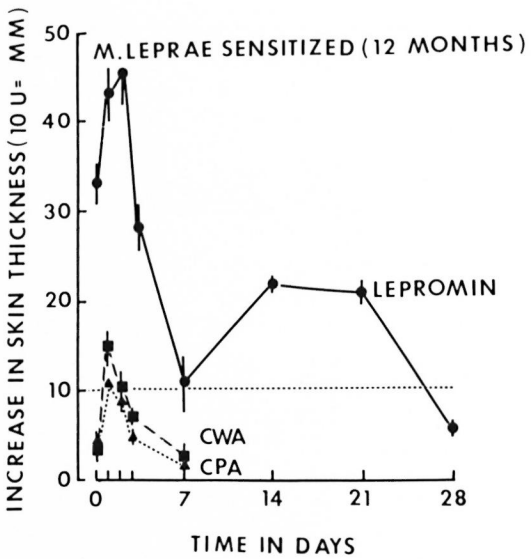

FIG. 4. Skin-fold thickness increases to 10 μg lepromin in guinea pigs sensitized with two doses of heat-killed *M. leprae* and skin tested at 12 months. Skin reactions to 10 μg of *M. leprae*-CPA and a cell-wall protein antigen CWA (Buchanan) were also determined. The dotted line represents the limit of significance for the skin swelling response ($p < 0.01$).

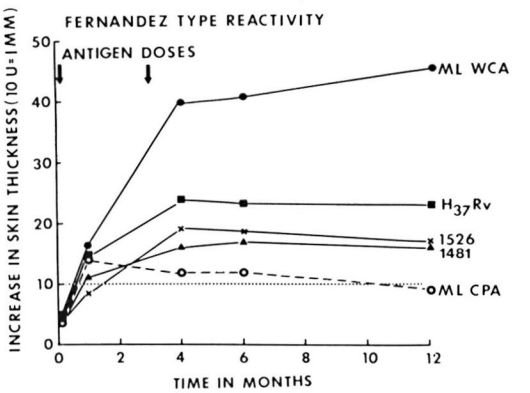

FIG. 5. Persistence of Fernandez-type skin hypersensitivity responses seen in guinea pigs sensitized and boosted with 500 μg heat-killed *M. leprae* (●), *M. tuberculosis* H37Rv (■), *M. nonchromogenicum* #1481 (▲) or *M. vaccae* #1526 (x) and skin tested with 10 μg of the homologous WCA preparation. The level of hypersensitivity to *M. leprae* CPA is represented by the broken line (O). The dotted line represents the limit of significance ($p < 0.01$).

tions were barely significant at any time during the study, and only the *M. leprae* data are shown in the present paper as being typical of the skin swelling responses in all four groups of sensitized animals (Fig. 5).

DISCUSSION

Although it has been widely recognized for many years that heat-killed suspensions of the appropriate mycobacteria can sensitize guinea pigs to tuberculin and to lepromin ([22, 23]), it is only relatively recently that such preparations have also been shown to induce a protective immunity against the specific parasite ([17, 19]). The present study examines the nature of the skin responsiveness which develops when heavy saline suspensions of mycobacteria are injected into normal guina pigs by the subcutaneous route. The persistence of these responses, both in terms of the Fernandez (24–48 hr) and the late Mitsuda (21–28 day) type responses ([12, 17]), correlated with the inherent immunogenicity of the sensitizing organism although an early development of skin reactivity occurred whether the animals were sensitized with the highly virulent *M. tuberculosis* or the avirulent *M. vaccae* and *M. nonchromogenicum* (Fig. 5). It should be noted, however, that the *M. leprae* response was always quantitatively superior to that observed for the other three test organisms, presumably because of its greater adjuvantive and/or immunogenic properties. *M. leprae* contains a mycoside A glycolipid envelope ([9]) which may be responsible for the enhanced immunogenicity shown by the leprosy bacilli in this test system, since this unique glycolipid cannot be found on the cell surfaces of most of the other mycobacteria ([8]).

The level of Fernandez responsiveness seen in the *M. leprae*-immunized animals was substantially higher throughout the study period than that achieved with the other three mycobacteria. This level of Fernandez responsiveness was significantly increased by all four booster injections (Fig. 5), whereas the Mitsuda-type skin responsiveness was only enhanced substantially by the *M. leprae* and *M. tuberculosis* booster injections (Figs. 1 and 2). The latter finding may have only limited immunological significance, however, since the Mitsuda response merely indicates that the host defenses are able to respond to the

mycobacterial antigens introduced into the skin test depot site, but this response may not be relevant as an index of the level of cell-mediated immunity generated by this organism within the immunized host ([13]). On the other hand, the size and persistence of the Fernandez skin reaction bears a much greater potential significance with respect to the protective qualities of the immunogen, since Shepard, *et al.* ([17]) have shown that a close agreement exists between the size of the 48 hr foot pad response seen in appropriately vaccinated mice and the level of immunity expressed against a subsequent foot pad challenge with live *M. leprae.* Such a correlation appears to be consistent with reports that the cellular response associated with the Fernandez skin reaction is predominantly mononuclear in nature ([24]), thus resembling the cellular hypersensitivity reactions seen in *Listeria* and *M. tuberculosis*-immunized animals ([10]). Both the *M. vaccae* and *M. nonchromogenicum* suspensions induced a specific Fernandez reactivity in appropriately sensitized guinea pigs, but there was only limited crossreactivity seen in these animals skin tested with the heterologous WCA preparations (Fig. 3).

The elevated Fernandez-type skin reactivity observed in the *M. leprae*-sensitized guinea pigs coincided with a remote positivity response ([14]) at the original site of implantation of the *M. leprae* suspension, even when such skin tests were carried out nearly 12 months later. No remote positivity responses occurred in the other three test groups of animals, and this appeared to correlate with the stronger Fernandez responses induced by the *M. leprae* vaccine. Maintenance of this high level of Fernandez reactivity could only be achieved at the cost of a severe local inflammatory response at the site of the *M. leprae* deposition and this, in turn, resulted in persistent swelling with substantial scarring. The severity of this localized tissue response to the killed *M. leprae* suspension could well limit the size of the immunizing inoculum to be used in human vaccination trials ([1]). Smaller doses of heat-killed *M. leprae* may be effective without inducing such a severe localized tissue response if they are first mixed with live BCG vaccine, as suggested recently by the studies of Convit, *et al.* ([6]). Comparative studies of various vaccinating regimens of this type would seem to be well worthwhile before widespread vaccination trials using armadillo-grown *M. leprae* suspensions are attempted in human populations.

SUMMARY

Guinea pigs were sensitized with 500 μg dry weight of heat-killed *Mycobacterium leprae, M. tuberculosis* H37Rv, *M. vaccae,* or *M. nonchromogenicum* suspended in saline. Significant Fernandez (peak swelling at 48 hr) and Mitsuda (peaking at 21 days) reactions were observed when all four groups of animals were skin tested with 10 μg of the homologous whole-cell antigen (WCA) preparations one month after sensitization. Some of the guinea pigs were given a booster injection of the homologous suspension three months later and were then retested with the four WCA preparations. The Fernandez (rather than the Mitsuda) reactivity was enhanced by the second immunization and was still substantial when tested eight months after boosting. The Mitsuda-type responses observed 12 months after the primary sensitization peaked earlier than in the first- and four-month tests, regardless of the vaccinating organism. The *M. leprae*-sensitized guinea pigs produced larger Fernandez skin reactions than those seen in the other three groups of sensitized animals, but there was substantial crossreactivity between *M. leprae* and *M. tuberculosis* antigens, as well as somewhat lesser responsiveness in the *M. vaccae*- or *M. nonchromogenicum*-sensitized animals skin tested with lepromin. The present study indicates that saline suspensions of heat-killed *M. leprae* induced a highly persistent state of lepromin hypersensitivity which was quantitatively superior to that observed in animals sensitized with the three other mycobacteria.

RESUMEN

Se sensibilizaron cobayos con 500 μg (peso seco) de *Mycobacterium leprae, M. tuberculosis* H37Rv, *M. Vaccae,* o *M. nonchromogenicum* muertos por calor y suspendidos en salina. Cuando los animales se probaron en la piel con 10 μg del antígeno homólogo completo, un mes después de la sensibilización, se observaron tanto reacciones tipo Fernández (pico de induración a las 48 hs) como reacciones tipo Mitsuda (pico a los 21 días). Algunos cobayos recibieron una inyección adicional dela suspensión homóloga tres

meses más tarde y entonces se volvieron a probar con las cuatro preparaciones antigénicas. La reactividad tipo Fernández (más que la tipo Mitsuda) resultó incrementada por la segunda inmunización y fue todavía evidente 8 meses después de la reestimulación. Las respuestas tipo Mitsuda observadas 12 meses después de la sensibilización primaria alcanzaron su pico antes que las respuestas inducidas después del primer y del cuarto mes, independientemente del organismo utilizado en la vacunación. Los cobayos sensibilizados con *M. leprae* produjeron reacciones dérmicas tipo Fernández más grandes que las observadas en los otros 3 grupos de animales sensibilizados. Hubo, sin embargo, una reactividad cruzada substancial entre *M. leprae* y *M. tuberculosis*, así como una respuesta ligeramente reducida en los animales sensibilizados con *M. vaccae* o con *M. nonchromogenicum* cuando se probaron intradérmicamente con lepromina.

RÉSUMÉ

Des cobayes ont été sensibiliés par 500 μg (valeur en poids sec) de *Mycobacterium leprae*, de *M. tuberculosis* H37Rv, de *M. vaccae*, ou de *M. nonchromogénicum*, en suspension saline. Toutes ces mycobactéries avaient été tuées par la chaleur. Lorsque les quatre groupes d'animaux ont été soumis à une épreuve cutanée par 10 μg d'antigène homologue préparé à partir de cellules entières (WCA), un mois après la sensibilisation, on a observé de réaction significatives de Fernandez, avec un engorgement maximum après 48 heures, et de Mitsuda, dont le maximum se situait au 21ème jour. Quelques-uns de ces animaux ont reçu une injection de rappel de la suspension homologue trois mois plus tard; ils ont alors été soumis à nouveau à une épreuve avec les quatre préparations de WCA. Plus que la réaction de Mitsuda, c'est la réactivité du type Fernandez qui a été renforcée par la seconde immunisation; cette réactivité était encore notable lorsque les animaux ont été testés huit mois après leur rappel. Les réponses du type Mitsuda, observées douze mois après la première sensibilisation, survenaient plus tôt qu'elles n'avaient été observées après les épreuves pratiquées après un et quatre mois, et ceci quel que soit l'organisme utilisé pour la vaccination. Les cobayes sensibilisés à *M. leprae* ont montré des réactions cutanées de Fernandez plus étendues que celles notées dans les trois autres groupes d'animaux sensibilisés. On a cependant relevé une réactivité croisée très nette entre les antigènes de *M. leprae* et ceux de *M. tuberculosis*, de même qu'une réponse quelque peu moindre chez les animaux sensibilisés par *M. vaccae* et par *M. nonchromogenicum*, lorsqu'ils étaient soumis à une épreuve à la lépromine. Cette étude indique que des suspensions salines de *M. leprae* tué par la chaleur induisent un état très persistant d'hypersensibilité à la lépromine, qui est quantitativement supérieure à celle qui est observé chez des animaux sensibilisés par les trois autres mycobactéries.

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