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Induction of Cell-Mediated Immunity to *Mycobacterium leprae* in Guinea Pigs

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Guinea pigs immunized with intact or disrupted armadillo-grown human *Mycobacterium leprae* administered in aqueous or oil vehicles were tested with various dilutions of *M. leprae* suspended in saline, water-soluble *M. leprae* extract, purified protein derivative, and a water-soluble extract of normal armadillo tissue. The results demonstrated the following. (i) Under no conditions was any skin test reactivity found to normal armadillo tissue extract. (ii) Positive sensitization to both *M. leprae* and its water-soluble extract was achieved by sensitizing guinea pigs with *M. leprae* suspended in Hanks solution or saline. Autoclaved *M. leprae* in Hanks solution or saline inoculated intradermally was an effective immunogen. Oil suspensions or emulsions were effective at sensitization, but appeared to be no better and, in general, slightly weaker, than simple inoculation in aqueous suspension. (iii) Living BCG failed to reveal a significant adjuvant effect on sensitization to *M. leprae*. However, cord factor appeared to potentiate slightly the sensitization to *M. leprae* in aqueous suspension. (iv) The minimum dose required for sensitization with *M. leprae* in aqueous suspension was 55 μg of purified bacilli. (v) Animals inoculated with *M. leprae* in saline or with *M. leprae* together with BCG showed positive skin test reactivity to the first skin test application made fully 1 year after the initial sensitization. The efficacy of autoclaved, irradiated *M. leprae* in aqueous, oil-free medium suggests a relatively safe approach to human vaccination studies.

The possibility for developing a vaccine against leprosy has become a feasible one due to two significant recent findings: first, the availability of significant amounts of human lepra bacilli from the armadillo and, second, methods for their purification from leprous tissues. To evaluate the potential usefulness of such purified armadillo-grown human *Mycobacterium leprae*, it is first necessary to demonstrate that it retains the capability of inducing delayed-type hypersensitivity in experimental animals under appropriate conditions which could be used in humans and that such *M. leprae* preparations fail to sensitize to tissue components. As part of the IMMLEP Program of the Special Program for Research and Training in Tropical Diseases of the World Health Organization, we have been able to study the conditions required to fulfill these requirements in guinea pigs. A variety of regimens in terms of dose, route of administration, adjuvants, and specificity were studied, and the results indicate that armadillo-grown and purified *M. leprae* have an ability to engender high levels of delayed-type hypersensitivity in normal guinea pigs in the absence of oil adjuvants under conditions in which no sensitization to armadillo tissues can be found.

MATERIALS AND METHODS

Animals. Albino guinea pigs weighing 450 to 600 g were sensitized in groups of four to six animals.

Antigens. Soluble extract prepared from normal armadillo liver, purified *M. leprae* obtained from armadillo tissue, and soluble *M. leprae* antigen (lots A14, AB19, and 22) were supplied by P. Draper and R. J. W. Rees. The method used for the extraction and purification of *M. leprae* is described in the report of the Second IMMLEP Task Force meeting (9). BCG was obtained from the Trudeau Institute (2×10^8 viable bacilli per ml). The source of purified protein derivative (PPD) was the Ministry of Food, Fishery and Agriculture, Weybridge, Surrey, England. Integral lepromin, at a concentration of 1.4×10^8 bacilli per ml, was obtained from A. Dhople and J. Hanks (Johns Hopkins School of Hygiene and Public Health, Baltimore, Md.), and *Mycobacterium vaccae* was from J. L. Stanford (Middlesex Hospital Medical School, London, England).

Vehicles. The various vehicles used for immunizing guinea pigs were Hanks solution, saline, and incomplete Freund adjuvant (IFA; Difco Laboratories; bacilli suspended directly in oil or suspended in saline

and emulsified in oil). Other adjuvants used were biodegradable adjuvant (adjuvant 65) with peanut oil (prepared by the method of Peck et al. [6]), cord factor, and muramyldipeptide (MDP) obtained from E. Lederer (Centre National de la Recherche Scientifique, Institut de Chimie des Substances Naturelles, Gif-Sur-Yvette, France).

Animals received 0.5 mg of *M. leprae* either in four footpads (oil suspensions) or intradermally (0.1 ml/site) in five sites on the flank above the foreleg.

Skin tests. The animals were tested with 6, 0.6, and 0.06 μg of soluble extract from armadillo tissue, purified *M. leprae*, soluble *M. leprae* antigen (A14, AB19, or 22), and PPD injected intradermally on the flank per 0.1 ml. Diameters of induration of the test sites were measured at 2, 24, and 48 h, and unless otherwise indicated, 24-h data are shown. Thickness was graded by the following scale: 0, ±, +, ++, +++, where + was considered a positive reaction (3).

In vitro stimulation of peripheral blood lymphocytes with mitogens and antigens. Mononuclear cells were isolated from heparinized blood of *M. leprae*-immunized guinea pigs over Ficoll-Hypaque gradients and cultured in 10% guinea pig serum at a density of 2×10^5 cells per 0.2 ml in microtiter plates in the presence and absence of optimal doses of concanavalin A (20 μg/ml), phytohemagglutinin (20 μg/ml), *M. leprae* (10 μg/ml), PPD (20 μg/ml), and lepromin (1:10). Plates were cultured for 3 and 4 days, and 18 h before harvest, 1 μCi of [^{3}H]thymidine was added.

Results are expressed as a stimulation index (experimental counts per minute/control counts per minute).

Enumeration of antigen-sensitive cells. Enumeration of antigen-sensitive T-cells was carried out by the virus plaque assay (2). This technique measures the ability of antigen-sensitive T-cells to permit replication of vesicular stomatitis virus after they are activated by the specific antigen. In brief, after peripheral blood mononuclear cells had been cultured in the presence and absence of mitogens or antigens in culture tubes for 3 days, they were infected with vesicular stomatitis virus at a multiplicity of 10 plaque-forming units per cell for 2 h. The excess virus was removed and neutralized with anti-vesicular stomatitis virus. Each sample was plated in three dilutions on L-cells. Plates were overlaid with 1% agar in minimal essential medium containing 6% fetal calf serum and incubated for 2 days. At the end of the incubation period the plates were fixed and stained to count plaques. The results are expressed as plaque-forming cells per 10^3 cells in stimulated cultures above the background in unstimulated control cultures.

RESULTS

Comparison of sensitization of guinea pigs with intact or sonically treated *M. leprae* in aqueous or oil vehicles. The initial series of studies compared the ability of a constant amount of *M. leprae* (0.5 mg) to induce delayed-type hypersensitivity in normal guinea pigs. To assess the degree and specificity of sensitization, animals so immunized were tested intradermally approximately 1 month after sensitization with intact bacilli, a water-soluble *M. leprae* extract (A14), tuberculin PPD, and a soluble extract of armadillo liver. In the initial series, each guinea pig was tested with 6, 0.6, and 0.06 μg intradermally, and reactions were measured at 2, 24, and 48 h. As Fig. 1 and 2 show, no skin test reactivity was detectable in this or any subsequent experiments to soluble armadillo tissue extract, indicating that, even though *M. leprae* is likely to be as effective an adjuvant as *Mycobacterium tuberculosis*, the bacilli are sufficiently pure that too little armadillo antigen remains to engender detectable sensitization. When intact bacilli were used for sensitization, significant sensitization after a single immunization was achieved both to *M. leprae* and to the soluble A14 *M. leprae* extract at 6 and 0.6 μg when the immunizing antigen was given in the absence of an oil vehicle or in an oil emulsion. These results were confirmed in four independent experiments. In addition, it is important to note that sensitization to armadillo-

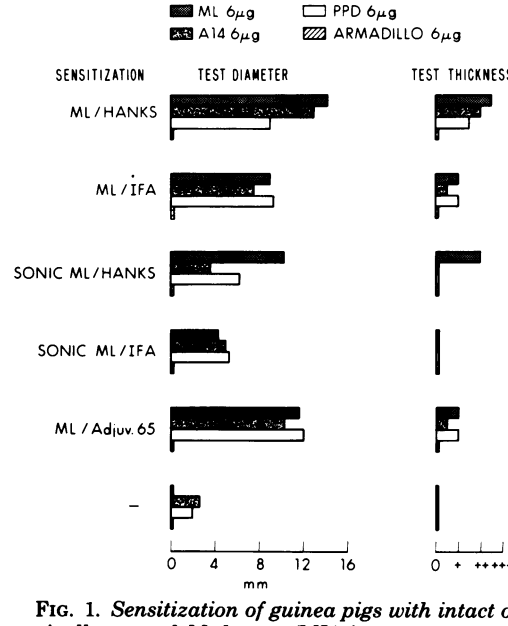

FIG. 1. *Sensitization of guinea pigs with intact or sonically treated M. leprae (ML) in aqueous or oil vehicles. Each guinea pig received 0.5 mg of intact or disrupted (sonically treated for 5 min at 30-s intervals) M. leprae in four footpads. The first three groups were immunized with M. leprae suspended in Hanks solution or IFA. The other two groups received M. leprae emulsified in IFA or adjuvant 65. Animals were tested intradermally with 6 μg of intact M. leprae per 0.1 ml, water-soluble M. leprae extract (A14), PPD, or soluble extract of armadillo liver 1 month after immunization, and reactions were measured at 24 h. Each bar represents the mean of reactions obtained with four to six guinea pigs.*

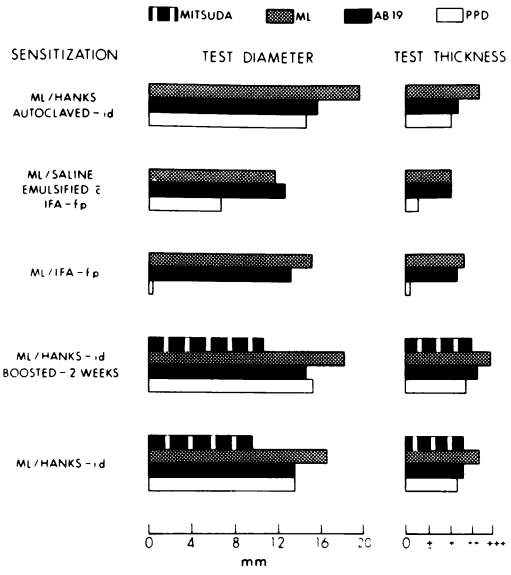

FIG. 2. *Sensitization of guinea pigs with M. leprae (ML) in aqueous or oil vehicles. Animals were immunized with 0.5 mg of intact M. leprae suspended in Hanks solution or IFA or with a saline suspension of M. leprae emulsified with IFA. A group of guinea pigs was also immunized with M. leprae autoclaved at 15 lb/in² for 15 min. Each guinea pig received 0.5 mg of M. leprae either in four footpads (fp) or intradermally (id) in five sites (5 intradermal injections of 0.1 ml each) on the anterior flank above the foreleg. In addition to skin testing with M. leprae, soluble antigen AB19, and PPD, animals in two groups were skin tested with 0.1 ml of human Mitsuda lepromin (1.4 × 10⁸ bacilli per ml).*

grown *M. leprae* in Hanks solution induced positive delayed-type reactivity not only to armadillo-grown bacilli, but also to human integral lepromin (Fig. 2). In contrast to virtually all other mycobacteria previously studied, it appears that *M. leprae* suspended in Hanks solution gave stronger sensitization than *M. leprae* suspended in an oil or oil and water emulsion.

Sonically disrupted bacilli emulsified with IFA were ineffective in sensitizing guinea pigs to any of the antigens, whereas a suspension of disrupted bacilli in Hanks solution engendered some degree of sensitization to *M. leprae*. When a variety of adjuvant protocols were compared, there was little difference in the sensitization with *M. leprae* suspended in IFA or emulsified in biodegradable adjuvant 65. In any case, sensitization with bacilli suspended either in Hanks solution or saline was invariably as good and generally slightly stronger.

Last, although the bacilli are killed by radiation, for any human vaccine it is likely to be preferable to have the bacilli sterilized at a later time by autoclaving, and the effect of autoclaving on the ability of *M. leprae* to sensitize guinea pigs was examined. The results clearly indicated that autoclaved bacilli, again in contrast to experience with other mycobacteria, were as good or better sensitizers than bacilli not subjected to autoclaving, as Shepard et al. have found in mice (8).

As a result of these experiments, we infer that the optimal procedure for sensitization of guinea pigs to *M. leprae* is intradermal inoculation of autoclaved intact bacilli suspended in aqueous media in multiple sites.

Comparison of route of immunization and the effect of multiple sensitization inoculations. As Fig. 3 shows, comparison of the intradermal route with the footpad inoculation route for sensitizing guinea pigs with intact bacilli suspended in aqueous suspension indicated that, although both were effective, the intradermal route appeared to be somewhat more effective. When animals sensitized to 0.5 mg were boosted at intervals of 1 week to 1 month after initial sensitization, only a very slight increase in degree of skin test reactivity was observed, suggesting that a single inoculation was effective in inducing delayed-type hypersensitivity in guinea pigs.

Use of living BCG and other mycobacterial products as adjuvants. Because of the well-known sensitizing and adjuvant properties

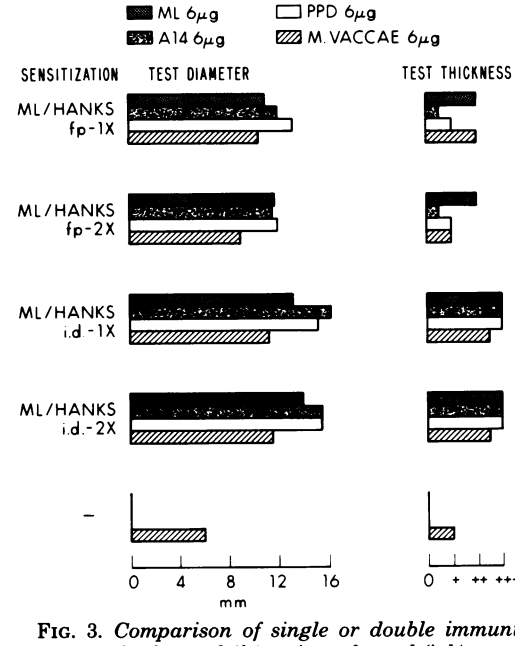

FIG. 3. *Comparison of single or double immunization by the footpad (fp) or intradermal (i.d.) route. Guinea pigs immunized on two occasions received the second injection 1 week after the first. ML, M. leprae.*

of living BCG and the possibility that an admixture of living BCG and killed *M. leprae* might be an adjuvant which could be used in humans, a study was made of the ability of *M. leprae* together with BCG to sensitize guinea pigs. As Fig. 4 shows, guinea pigs immunized with *M. leprae* together with BCG (either 10^6 or 10^7 viable organisms) showed a significant degree of specific sensitization, but the levels were no better than those of animals immunized with *M. leprae* inoculated intradermally in aqueous medium alone. Indeed, in other experiments not shown here, there was some evidence of decreased sensitization when BCG was given together with *M. leprae*. Thus, in guinea pigs, *M. leprae* appears to be a significantly strong sensitizer such that addition of BCG fails to enhance significantly the degree of sensitization achieved, at least when tests are made at 1 month after sensitization.

It is clear that animals immunized with *M. leprae* alone showed marked cross-sensitization to tubercle antigens and to PPD, as well as to *M. vaccae* (Fig. 3 and 4). Thus, it must be concluded that *M. leprae* shares sufficient cross-reactive antigens with certain other mycobacteria that specific sensitization was not found. In this regard, it was quite remarkable that the soluble *M. leprae* antigen preparations were *M. leprae* specific in skin tests, demonstrating no skin reactivity in guinea pigs immunized with BCG.

Cord factor and MDP previously have been demonstrated by Lederer and his associates to be effective adjuvants (1, 4). Consequently, guinea pigs were sensitized with mixtures of *M. leprae* and cord factor or MDP. The results (Fig. 5) indicate that cord factor enhanced sensitization to *M. leprae*, as detected by reactivity to the soluble skin test antigen AB19, while MDP had no effect on sensitizing ability of *M. leprae*. In addition, neither adjuvant induced sensitization to itself. In any case, these results indicate that cord factor might be a useful adjuvant for sensitization to *M. leprae*.

From these studies with guinea pigs, optimal sensitization would appear to be achieved by intradermal inoculation of autoclaved *M. leprae* suspended in aqueous medium in multiple sites, possibly in the presence of cord factor as an adjuvant.

In addition, since *M. leprae* fails to grow in guinea pigs, it is not possible to test the important question of the correlation between delayed-type hypersensitivity and protection against infection in guinea pigs, and such results in animal models will have to derive from either mice or armadillos. With these reservations in mind, however, the studies with guinea pigs have indicated that optimal conditions for achieving

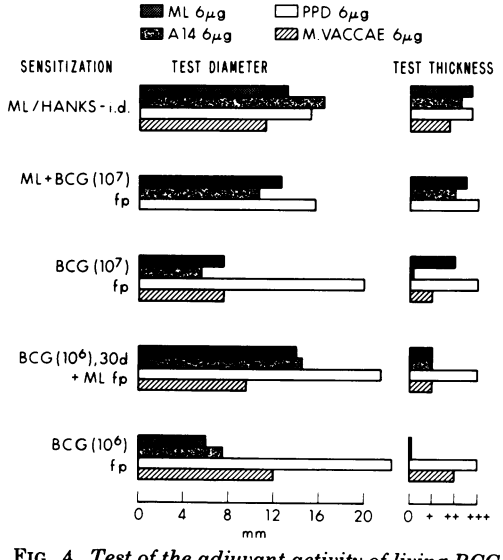

FIG. 4. *Test of the adjuvant activity of living BCG injected together with killed M. leprae (ML). Guinea pigs were immunized with 10^6 or 10^7 BCG and 0.5 mg of M. leprae (either admixed with BCG or given 30 days later) by footpad (fp) inoculation. i.d., Intradermal.*

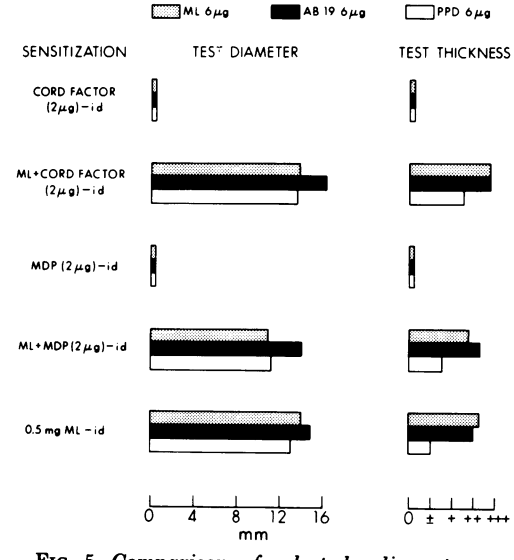

FIG. 5. *Comparison of selected adjuvants, cord factor, and MDP as vehicles. Animals were immunized intradermally (id) with 0.5 mg of M. leprae (ML) in 2 μg of cord factor or MDP per 0.5 ml and skin tested 1 month later with M. leprae, AB19, and PPD.*

delayed-type hypersensitivity to *M. leprae*-specific antigens appear to be those conditions which could be considered for use in a normal human population.

Dose response relationship. Guinea pigs were sensitized by the intradermal inoculation in saline of six different doses of purified *M. leprae*, and skin tests were performed 2 months later with 6 and 0.6 μg of *M. leprae*, the soluble AB22 extract of purified *M. leprae*, and PPD. The results indicate that as little as 55 μg of *M. leprae* induced positive reactions to both 6 and 0.6 μg (data not shown), with clear-cut induration in essentially all animals. Lower doses were essentially ineffective (Fig. 6).

Duration of sensitization. Groups of six animals were immunized with *M. leprae* in Hanks solution, adjuvant 65, or IFA or *M. leprae* together with BCG. The animals were maintained without further contact with *M. leprae* antigens for a period of 1 year and then skin tested with the above battery of antigens. The results (Fig. 7) show that at the first skin test 1 year after sensitization there was positive reactivity to *M. leprae* and that soluble antigen was particularly strong in groups inoculated with *M. leprae* suspended in Hanks solution and in those animals inoculated with *M. leprae* together with BCG. Immunization with 0.5 mg of *M. leprae* in Hanks solution given on only one occasion showed comparable, indeed slightly better sensitization, when tested for the first time at 1 year (18.5-mm induration to *M. leprae*; 17-mm induration to AB22). Animals inoculated with BCG alone or with *M. leprae* in IFA showed weak erythematous reactions without induration. These results indicate that intradermal inoculation of *M. leprae* in aqueous suspension is capable of inducing long-lasting sensitization to antigens of the lepra bacillus.

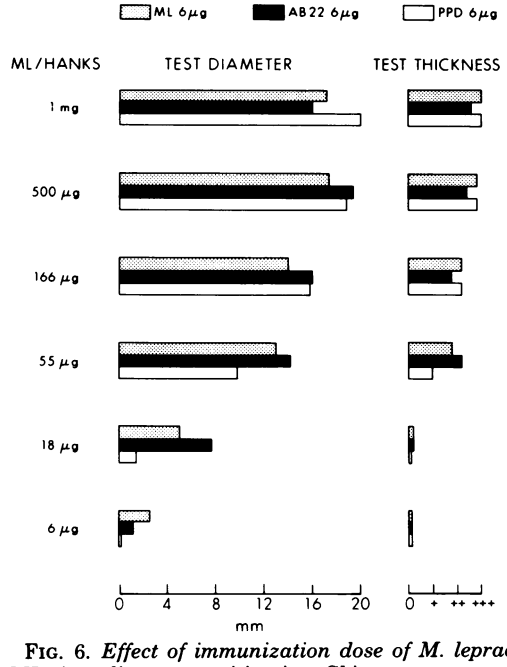

FIG. 6. *Effect of immunization dose of M. leprae (ML) in saline on sensitization. Skin tests were applied 2 months after sensitization.*

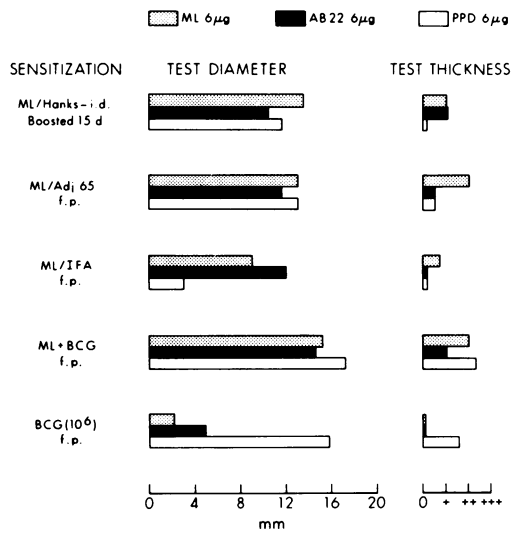

FIG. 7. *Duration of sensitization to M. leprae (ML). Reactions of the first skin test applied 1 year after sensitization are shown. i.d., Intradermal; f.p., footpad.*

Histology of the skin test sites. Figure 8 shows the histology of representative skin test sites of unimmunized guinea pigs and animals immunized with *M. leprae* intradermally and skin tested for the first time 1 year later with 6 μg of purified *M. leprae*, soluble *M. leprae* antigen (AB22), and PPD.

The subepidermal region in immunized animals showed marked cellular infiltration of predominantly mononuclear cells at the *M. leprae* and PPD test sites, although some polymorphonuclear cells were present, particularly in the PPD site. Cellular infiltrate at the AB22 test site was less intense but almost entirely mononuclear in composition. Clearly the histological picture is consistent with classical delayed-type hypersensitivity reactions.

In vitro assay of antigen responsiveness. Antigen-sensitive cells in the peripheral blood of guinea pigs immunized with *M. leprae* were assessed by: (i) incorporation of [^{3}H]thymidine by the stimulated lymphocytes and (ii) enumeration of antigen-sensitive cells by the virus plaque assay. Table 1 shows the means of stim-

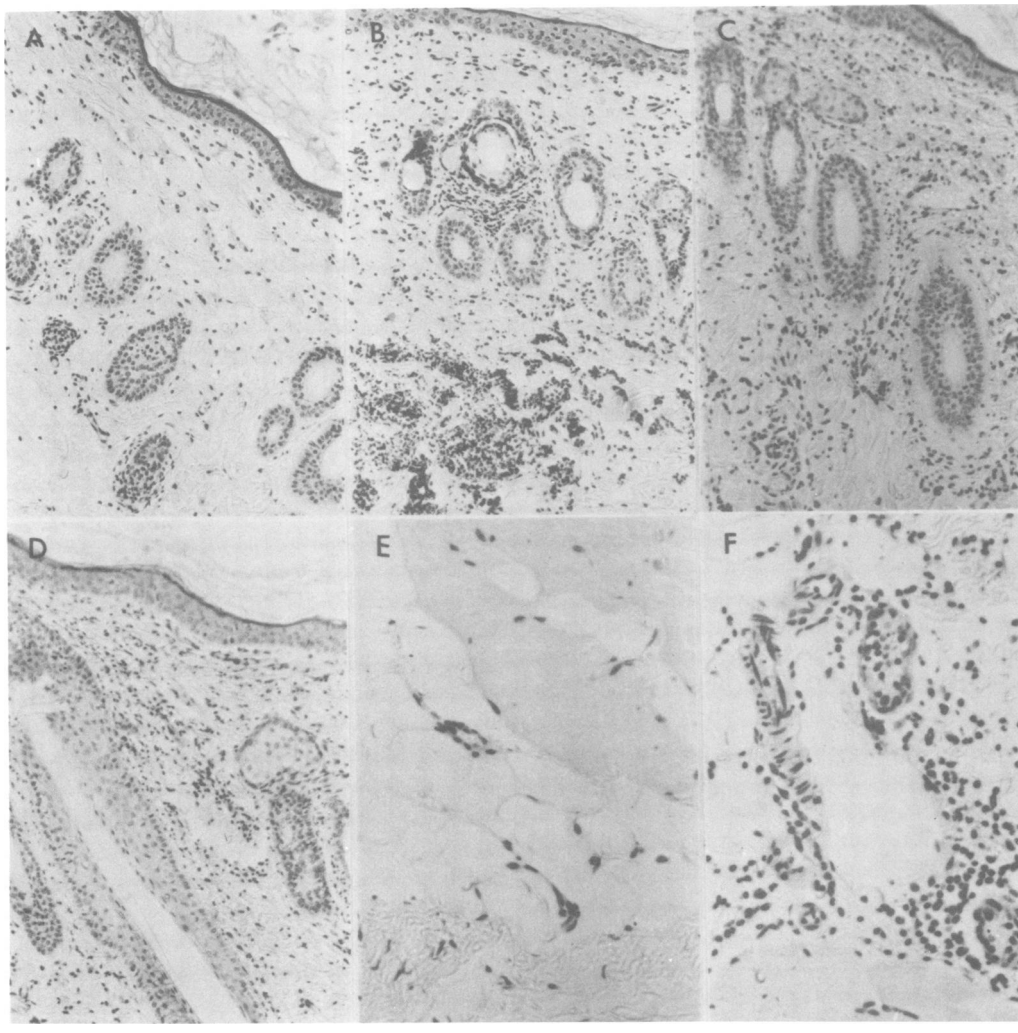

FIG. 8. *Histological appearance of skin test sites of M. leprae-sensitized guinea pigs tested for the first time at 1 year. The immunized animals received 0.5 mg of M. leprae in saline at six intradermal sites. The 24-h reaction skin sections represent: (A and E) normal guinea pig skin; (B) M. leprae (6 μg); (C and F) M. leprae soluble antigen (6 μg); and (D) PPD (6 μg). The M. leprae and PPD sites are infiltrated predominantly with mononuclear cells and with some polymorphonuclear leukocytes, and the soluble antigen sites consist exclusively of mononuclear cells. A through D, ×122; E and F, ×300.*

ulation indexes obtained in 5 to 10 guinea pigs after stimulation with concanavalin A, phytohemagglutinin, *M. leprae*, PPD, and lepromin. *M. leprae* proved to be an exceptionally strong eliciting antigen, more active than PPD and comparable to concanavalin A in the virus plaque assay, with 1.4% of the cells responding.

DISCUSSION

This study was undertaken to explore the possibility of developing a vaccine against leprosy by using purified armadillo-grown killed human *M. leprae*. A variety of immunization protocols were designed to determine conditions which could be used in humans for optimal sensitization to the soluble *M. leprae* skin test antigen and purified *M. leprae* in guinea pigs.

The results indicate that intact bacilli produced significant sensitization to both *M. leprae* and its soluble antigen when given in the absence of an oil vehicle or emulsion. Since these bacilli are obtained from armadillos, the contribution of contaminating armadillo tissue antigens toward skin reactivity was a theoretical concern, but no skin test reactivity was detectable in any of our experiments to soluble armadillo extract.

Comparison of various adjuvants showed only

TABLE 1. *Stimulation of peripheral blood lymphocytes by mitogens and antigens*

Mitogen/antigen	Stimulation index[a]	Virus plaque-forming cells (ΔV-PFU/10^3 cells)[b]
Concanavalin A	9.03 ± 2.74[c]	13.16 ± 2.80[c]
Phytohemagglutinin	9.67 ± 4.65	8.95 ± 1.05
M. leprae	4.37 ± 0.67	14.88 ± 3.76
PPD	2.81 ± 0.56	9.40 ± 2.88
Dharmendra lepromin	3.00 ± 0.42	ND[d]

[a] [^{3}H]thymidine incorporated counts in the control cultures ranged from 306 to 1,806 (mean ± standard error, 949 ± 104.84).

[b] ΔV-PFU, Virus plaque-forming units above the background. Background in unstimulated control cultures was 4.27 ± 1.3 plaque-forming units per 10^3 cells (mean ± standard error).

[c] Mean ± standard error of responses obtained in 5 to 10 guinea pigs immunized with *M. leprae*.

[d] ND, Not determined.

small differences among the sensitization with *M. leprae* suspended in IFA, *M. leprae* suspended in saline and emulsified in IFA, or *M. leprae* emulsified in adjuvant 65. They were all effective in sensitization but, in fact, were no better than bacilli suspended in Hanks solution or saline. Other studies also showed that irradiated *M. leprae* in aqueous suspension produced greater delayed-type hypersensitivity response than did a water-in-oil emulsion in mice (5).

The possibility of using living BCG as an adjuvant was also explored. Guinea pigs immunized with *M. leprae* together with BCG (10^6 or 10^7 viable organisms) showed significant sensitization, but again the level was no better than in guinea pigs immunized intradermally with *M. leprae* alone. Thus, *M. leprae* appears to be a remarkably strong immunogen for cell-mediated immunity, and addition of BCG fails to enhance the sensitization significantly. Similarly, the absence of an adjuvant effect of BCG on *M. leprae* in immunization has been observed in mice (7).

Animals immunized with *M. leprae* alone showed quite strong reactivity to PPD, indicating marked cross-sensitization to other mycobacterial antigens. Numerous attempts to establish *M. leprae* specificity by carrying out a series of skin test antigen dilution studies failed; there were no dilutions of antigen at which PPD reactivity was absent and reactivity to *M. leprae* or the soluble antigen was retained. Thus, it must be concluded that *M. leprae* has sufficient antigenic cross-reactivity that sensitization to unique antigens of *M. leprae* and cross-reacting antigens shared with *M. tuberculosis* occurs simultaneously. On the other hand, the soluble *M. leprae* skin test antigen of Draper and Rees was remarkably *M. leprae* specific and demonstrated no skin reactivity in guinea pigs immunized with BCG alone. This specificity of the soluble *M. leprae* extract for sensitization with *M. leprae* suggests that this may well be a useful test antigen for *M. leprae* sensitization in humans.

In vitro studies on lymphocyte transformation and the virus plaque assay for enumerating activated T-cells indicated that *M. leprae* in saline was an excellent sensitizer and an effective eliciting antigen. Of interest is the fact that the number of antigen-reactive cells found in peripheral blood (1.4%) is comparable to that found in guinea pig lymph nodes sensitized with *M. tuberculosis* in adjuvant (2).

Even though the bacilli we received were killed by irradiation, it might be preferable to use bacilli sterilized by autoclaving before use, and our results indicate that immunization with autoclaved *M. leprae* was, surprisingly, as good or better than that with bacilli not subjected to autoclaving, a phenomenon also observed by Shepard et al. in mice (8).

Last, animals immunized with *M. leprae* in various vehicles and skin tested 1 year later for the first time demonstrated positive reactivity to *M. leprae* and soluble antigen. Reactions were particularly strong in guinea pigs inoculated with *M. leprae* suspended in Hanks solution and in those inoculated with *M. leprae* along with BCG. Thus, intradermal inoculation of *M. leprae* in aqueous suspension is not only capable of producing high levels of reactivity but also induces sensitization that persists for long periods of time.

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