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Foot Pad Enlargement as a Measure of Induced Immunity to *Mycobacterium leprae*[1]

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We have reported previously that *Mycobacterium leprae* is immunogenic in mice, especially when injected intradermally ([8, 11]). Moreover, unlike other mycobacteria so far studied, *M. leprae* does not lose any of its immunogenicity when it is killed ([15]); instead it gains activity moderately, at least when it is killed by heat. In previous work, we judged immunogenicity by protection against infectious challenge with *M. leprae* and by the persisting enlargement of the lymph node draining the intradermal vaccination site. In the work reported here, we have used foot pad enlargement (FPE) as a measure of the immune response. The eliciting antigens injected into the foot pad have been suspensions of intact *M. leprae* or products of the disruption of *M. leprae*. Condensed versions of this work have been presented ([9, 11]).

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MATERIALS AND METHODS

The *M. leprae* suspensions for immunization and for FPE tests were prepared from the livers of experimentally infected armadillos. The precautions taken to minimize confusion with feral infections have been described ([13]). The short-trypsin (ST) procedure ([10]) used for purification consists of centrifugal washing of the bacilli, followed by a mild trypsin treatment (0.125% trypsin of 1:250 purity for 5 min at 37°C at pH 7.4–7.6), followed again by centrifugal washing. The suspending medium was usually phosphate buffered saline (pH 7.3) with 0.05% Tween 80 (PBS-Tw) or Hanks Balanced Salt Solution with 0.05% Tween 80 (Hanks-Tw). Some ST preparations of *M. leprae* were further purified by the two phase polyethylene glycol:dextran system adapted to *M. leprae* by Draper ([7]). Because the yield of bacilli in the upper (polyethylene glycol) phase was low (10–30%), the lower phase was diluted 1:2 or more with PBS-Tw and the bacilli recovered and washed by centrifugation; the trypsin digestion described above was then repeated, and the bacilli were washed and again subjected to the two phase separation. The *M. leprae* from the two upper phases were

washed and combined; they are called 2P purified.

M. leprae (unheated) were disrupted by treatment in the Mickle apparatus with glass beads (0.17 mm in diameter, 1.0 g beads/ml bacterial suspension, 10 mm amplitude, 60 cycles per second, 10 min duration, prechilled suspension, cup and shaker arm). Counts of acid-fast bacteria indicated greater than 99% disruption. The cell wall and protoplasmic fractions were separated by centrifugation at 20,000 × *g* for 30 min. The resuspended sediment and the carefully removed supernatant were centrifuged again for further purification before use.

Heat treatment of the various antigens was carried out on the final (use) dilution for 30 min at the temperature shown.

The normal armadillo liver preparation was a simple suspension (not centrifuged) diluted to contain five times the protein (Lowry) of the armadillo-derived *M. leprae* suspension with which it was compared. The human-derived *M. leprae* suspension was prepared from biopsy specimens of lepromatous patients' skin by the ST method.

At the start of each experiment, mice of the same age were distributed five to a cage and the cages assigned in the experiment by random number. Individual mice in each cage were distinguished by marks with picric acid. There were usually ten mice per group. Intradermal injections ([12]) were given in a volume of 0.01 ml in the right flank. The volume of the foot pad injections was 0.03 ml. By either route the dose of antigen, unless otherwise stated, was 1×10^7 *M. leprae*, and 30 gauge needles were used. Female CFW mice were used throughout, and they were 7 to 13 weeks old at the time of first injection.

The thickness of the foot was measured with dial calipers. FPE was the thickness at the designated time minus that just before injection. For corrected FPE (corr. FPE), the mean FPE produced by injection of the antigen in the (unimmunized) normal controls was subtracted. The size of the lesion at the site of the intradermal injection and the size of the regional (inguinal) lymph node were also measured in most experiments ([12]). Unless otherwise stated, the FPE reported is that at 48 or 72 hr, depending on which interval had the greater corr. FPE.

The procedure for infectious challenge has been described ([15]). Briefly 5000 *M. leprae* in mouse passage were inoculated into the right hind foot (RHF). Counts of *M. leprae* in pools of four unvaccinated mice were carried out at 28 day intervals, and when the growth curve passed 10^6 (at about 6 months), harvests of eight individual mice were carried out in all vaccinated groups and in the two control groups. The harvests were repeated in 90 days.

Differences between groups are assessed by the two sample rank test because the harvests in individual mice are usually not distributed normally, and the FPEs often were not. Unless otherwise stated, the p values reported are for a two tailed test.

RESULTS

In experiment 1, 20 mice were immunized intradermally with *M. leprae*, and they along with 20 previously unimmunized mice were challenged by single foot pad or intradermal injections of *M. leprae* (Fig. 1). After the intradermal challenge, the previously immunized mice had some acceleration of regional lymph node enlargement. There was also some increased swelling at the injection site, but this local swelling was hard to read at this stage because the vertical component was slight. FPE after foot pad challenge revealed the most distinct differences between immunized and control mice. There was almost no overlap between the two groups; the p values for the differences were thus usually less than 0.001.

During the 50 day period of observation, the difference in FPE between the primary (previously unimmunized) and secondary (previously immunized intradermally) groups remained fairly distinct, presumably as a reflection of the poor immunization resulting from the challenge injection of *M. leprae* via the foot pad route. After intradermal injection the small difference in lymph node size between the primary and secondary group disappeared in about a week. The difference in the local skin reaction after the challenge injection disappeared after 20 days, presumably as a reflection of the immunizing effect of the intradermally injected *M. leprae* used to

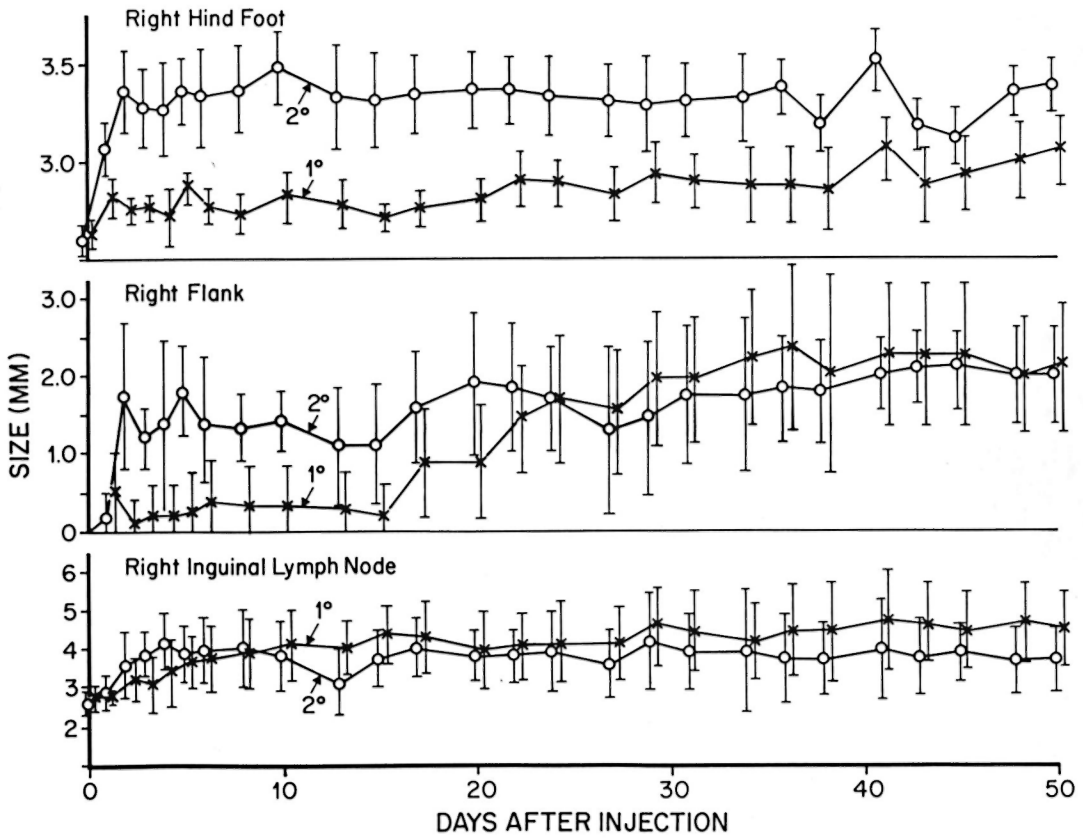

FIG. 1. The time courses of enlargements of foot pads, flank (intradermal) sites, and regional (inguinal) lymph nodes after foot pad or flank (intradermal) injection of *M. leprae* into normal mice (1°) and mice that had been immunized intradermally with *M. leprae* 28 days before (2°). The injected dose in all cases was 1×10^7 *M. leprae* prepared by the ST method, suspended in Hanks Balanced Salt Solution with 0.1% bovine albumin, and heated to 60° for 30 min. The symbols indicate the group averages and the vertical bars the standard deviations.

challenge the animals. After about 20 days the local skin reaction became thicker and easier to read in both the primary and secondary groups.

In the remaining experiments, FPE after the first peak did not persist regularly, but the peak always occurred at 2–3 days after challenge. Consequently, only the reading at this interval is reported. The differences in average size of lymph nodes appeared to parallel the differences in FPE; because the differences in lymph node size were less distinct, they are not reported.

In experiment 2 (Fig. 2), the effects of various treatments of the eliciting antigen were studied. Fourteen days before challenge, mice had been immunized with a suspension that had been heated to 60°C. The values shown in Fig. 2 are those at 48 hr after challenge when corr. FPE was maximal. From the p values the following conclusions could be drawn: a) With unheated antigens there was no statistically significant difference between PBS and Hanks as suspending medium. b) In Hanks, heating to 100°C or 121°C increased the antigen's activity (B vs E, $p < 0.002$). Heating to 60°C or 80°C had a lesser effect that was of borderline significance. c) Phenol or Tween was not harmful to antigen that had been heated to 60°C. d) Disruption of the bacilli increased the activity somewhat (B vs J, $p < 0.01$). Heating the disrupted product did not change its effectiveness.

In experiment 3 (Fig. 3) the effect of various treatments of the immunizing antigen

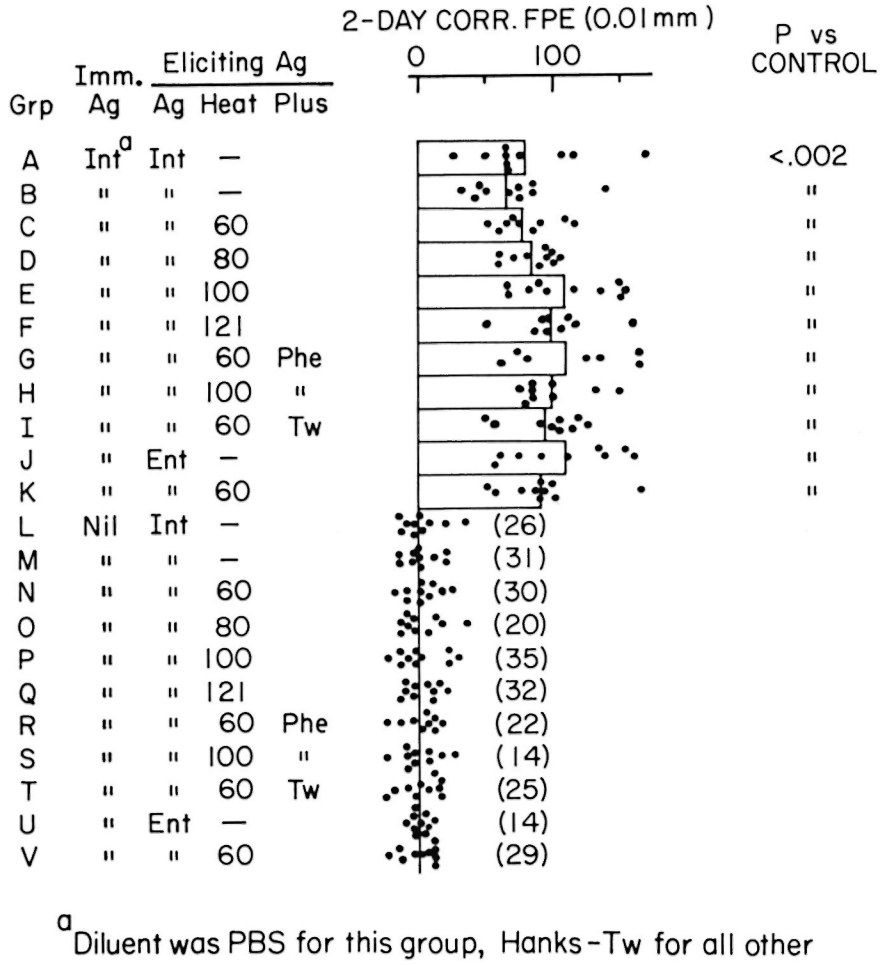

FIG. 2. Effect of various treatments of the eliciting antigen. ST-purified *M. leprae* were heated for 30 min to the temperatures indicated, and phenol (Phe) or Tween 80 (Tw) was added to concentrations of 0.5% and 0.05%, respectively. The mice were immunized intradermally at 0 days with intact organisms (Int) in Hanks, heated to 60°C for 30 min, and the eliciting suspensions, either intact bacilli or the entire disrupted product (Ent), were given in the foot pad at +14 days. The eliciting suspension for group A was suspended in PBS; all other antigens were suspended in Hanks. Corrected FPE, 2 days later, is indicated by dots for individual mice and bars for the group average. The average uncorrected FPE in the controls is shown by the figures in parentheses. The p value is for the difference from the control (unimmunized mice receiving the same eliciting antigen). Grp = group. Imm. Ag = immunizing antigen.

was studied. In addition, to assess specificity, mice immunized with *M. leprae* from armadillo liver were tested with normal armadillo liver and with *M. leprae* from human skin. The p values allowed the following conclusions. a) There was no significant difference between Hanks and PBS as a suspending medium in this test. In other tests Hanks has been equivalent or superior to PBS. b) Heating the antigen to 121°C increased its immunogenicity somewhat (B vs G, $p < 0.05$). Other experiments have given similar results. c) Phenol or Tween was not harmful in the heated antigen. d) Corrected FPE was not seen in mice that were challenged with a suspension of normal armadillo liver (I vs O). e) FPE was caused by challenge with an *M. leprae* suspension prepared from human tissue (J vs P). f) A suspension of *M. leprae* that had been further purified by the two phase polymer system was fully potent as an immunizing or as an eliciting antigen.

In experiment 4 (Fig. 4) the effect of dis-

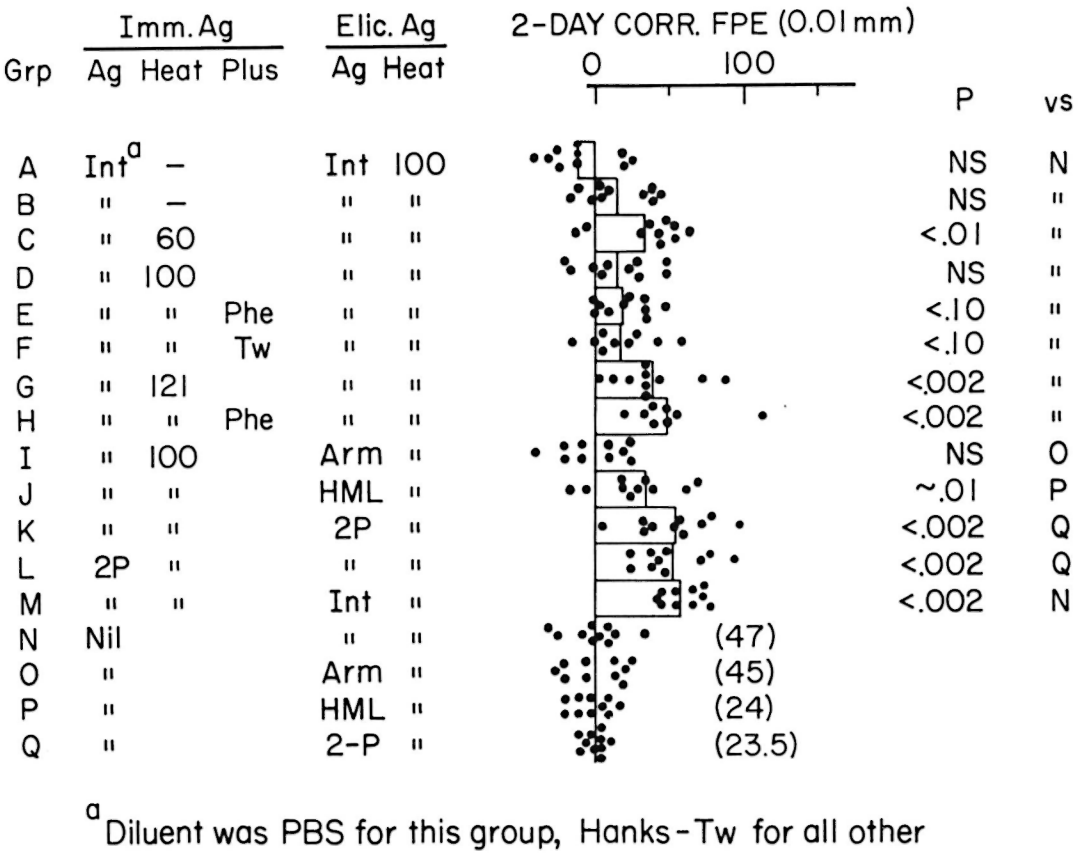

FIG. 3. Effect of various treatments of the immunizing antigen. ST-purified *M. leprae* were heated for 30 min as indicated, phenol or Tween-80 was added, and the suspension was injected intradermally at 0 days. The eliciting antigen (Elic. Ag) was injected into the foot pad at 14 days. Int = intact bacilli. Phe = phenol. Tw = Tween 80. Arm = suspension of normal armadillo liver. HML = *M. leprae* from a human biopsy specimen prepared by the ST procedure (all other *M. leprae* were from armadillo liver). 2-P = ST-prepared *M. leprae* from armadillo liver that were further purified by two-phase polymer separation. The corrected FPE, 2 days later, is indicated by dots for individual mice and bars for group averages. Grp = group. Imm. Ag = immunizing antigen.

ruption of the *M. leprae* was studied. Purified (2P) *M. leprae* were disrupted and separated by centrifugation into the cell-wall and protoplasmic fractions. Neither fraction, whether heated or unheated, was immunogenic; combinations of the two fractions were not immunogenic either. The cell wall fraction had more eliciting activity than the protoplasmic fraction (K vs M, $p < 0.002$; L vs N, $p < 0.002$). Heated intact *M. leprae* caused a moderate reaction in normal animals (average 21 units) as usual; this reaction was decreased by the prior injection of the unheated cell-wall fraction ($p < 0.05$). Similar results with disrupted products have been seen in other experiments. When the protoplasmic fraction was mixed with intact bacilli before injection, the resultant sensitization was somewhat greater (I vs B, $p < 0.10$; J vs B, $p < 0.01$). Later work has shown that the disrupted products may be immunosuppressive when administered before the immunizing antigen (manuscript in preparation).

The relationship between the dose of immunizing antigen and the dose of eliciting antigen was investigated in experiment 5 (Table 1). Three doses were used—4.4×10^7, 2.2×10^7, and 1.1×10^7 *M. leprae*. Only with the lowest dose of immunizing (0 day) antigen did the corr. FPE increase with the dose of eliciting (28 day) antigen. Similarly, only with the lowest dose of eliciting antigen did the enlargement increase with the dose of immunizing antigen. The results suggest that it would be difficult to

TABLE 1. *Effect of antigen dosage on foot pad enlargement (FPE). The suspension used throughout was intact, 2P-purified bacilli that had been heated to 100°C for 30 min. There were five mice per group.*

Dose of imm. Ag.[a]	Dose of eliciting antigen		
	4.4×10^7	2.2×10^7	1.1×10^7
	2 day corr. FPE (10^{-2} mm)		
4.4×10^7	30	23	44
2.2×10^7	36	38	36
1.1×10^7	50	28	23
	3 day corr. FPE (10^{-2} mm)		
4.4×10^7	41	27	41
2.2×10^7	52	34	30
1.1×10^7	62	20	9

[a] Immunizing antigen.

discern increased antigenic activity if the immunizing and eliciting antigens contained more than 1×10^7 *M. leprae*.

Similar results were obtained in another experiment (results not shown) that involved doses of 1×10^7 and 3×10^7 in the immunizing and eliciting (28 day) antigens. In this experiment groups of mice also received the entire disrupted product of 1×10^7 and 3×10^7 *M. leprae*. In these latter groups the corrected FPE was less than with the intact bacilli, and the differences between the groups were not significant.

Mehra and Bloom have reported that cord factor (CF) increases the immunogenicity of *M. leprae* in guinea pigs ([5]). In experiment 6 (Table 2) the following factors were varied: dose of immunizing *M. leprae* (1×10^6 or 1×10^7), route (RHF, SC, or ID), diluent (incomplete Freund adjuvant [IFA] or Hanks-Tw), presence or absence of CF in the IFA. The CF used was a highly purified preparation from Prof. E. Lederer, and it was dissolved in IFA before being mixed with the aqueous suspension of *M. leprae*. At 28 days after the immunizing injections, groups of ten mice were chal-

TABLE 2. *Effect of cord factor (CF) on the immunogenicity of* M. leprae *(ML). Heat-killed (100°C, 30 min), 2P-purified* M. leprae *in incomplete Freund adjuvant (IFA) or Hanks-Tw (H-Tw), with or without 10 μg CF, was injected in the right hind foot (RHF), subcutaneously (SC) in the flank, or intradermally (ID) in the flank 28 days before challenge. Foot pad enlargement (FPE) is shown 3 days after LHF challenge with 1×10^7 heat-killed 2P-purified* M. leprae. *Protection (Prot.) against infectious challenge is shown in the 6 month and 9 month harvests. Protection = log (average harvest in controls)-log (average harvest in the vaccinated group). For the unimmunized control (group S), the uncorrected FPE and the logs of the average harvests are shown in parentheses.*

Group	Log dose of ML	CF	Diluent	Route	FPE, 3 day, corr.		Protection against infection			
							6 months		9 months	
					10^{-2} mm	p (vs S)	Prot.	p (vs S)	Prot.	p (vs S)
A	7.0		IFA	RHF	−13.5	<.05	0.12	NS	0.10	NS
B	6.0		IFA	RHF	−15.5	NS	−.17	NS	−.17	NS
C	Nil	+	IFA	RHF	2.4	NS	−.08	NS	.17	NS
D	7.0	+	IFA	RHF	−6.0	NS	.15	NS	.58	<.05
E	6.0	+	IFA	RHF	−12.5	NS	−.15	NS	−.14	NS
F	Nil		IFA	RHF	−10.0	NS	−.46	<.05	.08	NS
G	7.0		IFA	SC	−6.0	NS	−.18	NS	.53	.05
H	6.0		IFA	SC	−11.5	NS	.21	NS	.03	NS
I	Nil	+	IFA	SC	−8.5	NS	.39	<.02	.31	NS
J	7.0	+	IFA	SC	−11.0	<.02	.69	<.002	.28	NS
K	6.0	+	IFA	SC	−9.0	NS	.07	NS	.67	<.01
L	Nil		IFA	SC	−14.0	NS	.44	<.02	.18	NS
M	7.6		H-Tw	RHF	−0.5	NS	−.02	<.10	.12	NS
N	6.0		H-Tw	RHF	−3.5	NS	.32	<.05	.19	NS
O	7.6		H-Tw	SC	−3.5	NS	1.13	<.002	.54	<.02
P	6.0		H-Tw	SC	−7.0	NS	.57	<.01	.80	<.01
Q	7.6		H-Tw	ID	29.5	<.002	1.57	<.002	1.28	<.002
R	6.0		H-Tw	ID	−7.0	NS	.51	<.01	.59	<.02
S	Nil				(37)	—	(5.98)	—	(6.13)	—

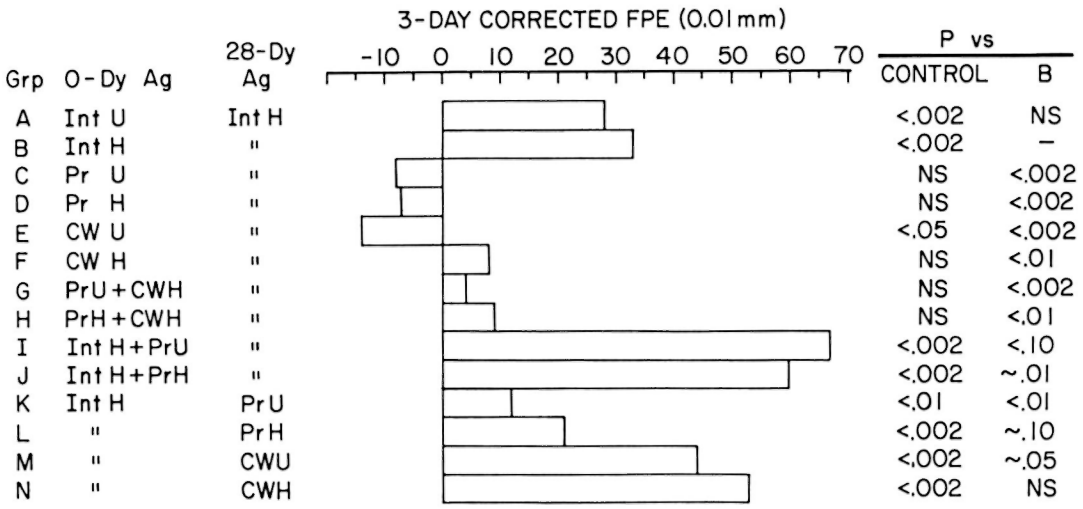

FIG. 4. The effect of disruption of *M. leprae* on its antigenic activity. After physical disruption of 2P-purified *M. leprae*, the product was separated by centrifugation into a protoplasmic fraction (Pr) or cell-wall fraction (CW). Int = intact bacilli. H = heated at 100°C for 30 min. U = unheated. The foot pad challenge was given at 28 days. Grp = group. Dy = day. Ag = antigen.

lenged with heat-killed *M. leprae* for FPE observations, and groups of 30 were given living *M. leprae* as an infectious challenge.

The only significant FPE was seen in mice immunized intradermally with 1×10^7 *M. leprae* in Hanks-Tw. The means for corr. FPE for most of the groups carry a negative sign, presumably because the unimmunized control group average happened to be a little high. Significant protection against infectious challenge in both the 6 month and 9 month harvests was seen only in the groups vaccinated intradermally and subcutaneously with *M. leprae* in Hanks-Tw. In these groups protection was provided by 1×10^6 as well as 1×10^7 *M. leprae*. There was evidence with *M. leprae* in Hanks-Tw that the intradermal route was superior to the foot pad route by FPE (M vs Q, $p < 0.02$; N vs R, P NS) and by protection at 9 months (M vs Q, $p < 0.01$; N vs R, $p < 0.05$). The superiority of the intradermal route over the subcutaneous route was seen only by FPE with the large dose of *M. leprae* (O vs Q, $p < 0.002$). In earlier work we have usually found the intradermal route to be superior to the foot pad route ([8, 11]).

There was a suggestion that moderate protection was afforded by subcutaneous injection of IFA with or without CF (groups I and L); the addition of *M. leprae* in either dose did not change the results significantly. The protection values suggested that Hanks-Tw was a more favorable medium than IFA (at 6 and 9 months, respectively, the p values were as follows: B vs N, < 0.10 and <0.10; H vs P, NS and <0.05; A vs M, NS and NS; G vs O, <0.002 and NS).

The chief disagreement between the FPE and protection results lay in the lack of FPE after subcutaneous immunization with *M. leprae* in Hanks-Tw. This discrepancy is discussed below.

In the next two experiments to be reported, we have used FPE tests as a measure of the immunogenicity of antigens prepared in different ways. The experiments were aimed according to the IMMLEP/WHO efforts to develop a human leprosy vaccine with *M. leprae* grown in armadillos ([2]). The obvious goal is a product that is free of armadillo antigens but highly immunogenic for *M. leprae*. The various purification steps that have been used include washing in the centrifuge, digestion with proteinases, and separation in two phase polymer systems ([7]).

In experiment 7 (Fig. 5) the steps tested were centrifugal washing, short trypsin digestion, and 24 hr treatment with trypsin and chymotrypsin (crystalline, 0.1 mg/ml of

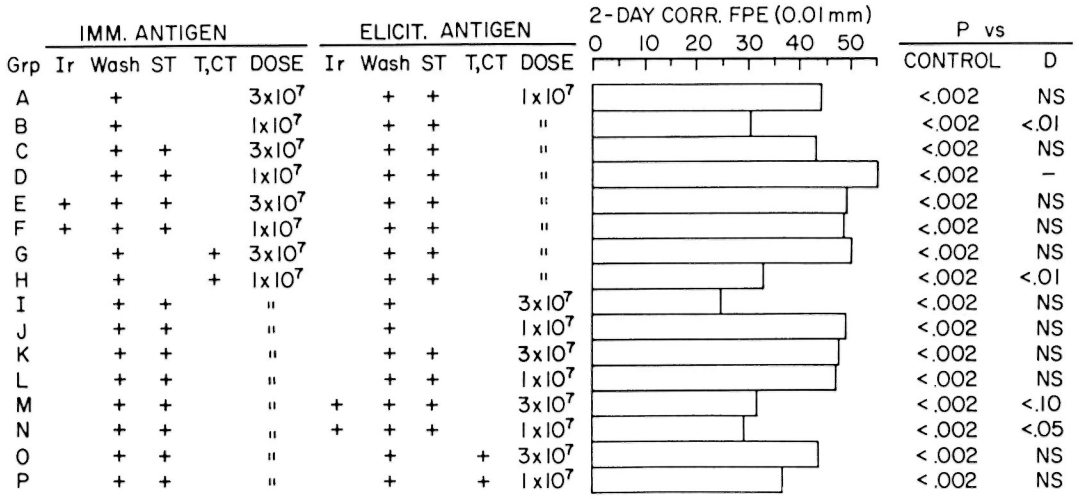

FIG. 5. Effect of irradiation and purification steps on the antigenic activity of *M. leprae*. *M. leprae* were treated as indicated by 0.25 megarads of gamma irradiation (Ir), two washings in the centrifuge (Wash), short trypsin digestion (ST), or 24 hr digestion with trypsin and chymotrypsin (T, CT). All preparations were heated to 100°C for 30 min. The immunizing antigen was given intradermally at 0 days, and the eliciting antigen was given in the left hind foot pad at +28 days. The uncorrected 2 day FPE for each of the controls, corresponding to groups I–P, respectively, was 45.5, 26, 38.5, 21, 37, 33, 45.5, and 47.5. Grp = group. IMM. = immunizing. ELICIT. = eliciting.

each, pH 7.2). The various antigens were used for immunization or elicitation. The dose of bacilli was 3×10^7 or 1×10^7. Two mouse groups, D and L, received the same antigens.

Because of the dose relationships described above, principal emphasis is placed on the results with the lower dose, 1×10^7 bacilli. a) Thus irradiation with 0.25 megarads caused no change in the immunogenicity of the bacilli that were to be purified by the short trypsin treatment (F vs D). b) The short trypsin treatment increased the immunogenicity of washed bacilli (B vs D, $p < 0.01$). c) The 24 hr trypsin and chymotrypsin treatment was harmful to immunogenicity (H vs D, $p < 0.01$). The various treatments had no significant effect on the eliciting activity of the antigens except for the possible exception of irradiation (N vs D, $p < 0.05$).

In experiment 8 (Table 3), the same treatments plus the two phase polymer-partition procedure were tested. Three doses of immunizing antigen were compared (2×10^7, 7×10^6, and 2×10^6) with most preparations. The eliciting antigen was the one most highly purified (the preparation given G, H, I, and J); the eliciting dose was 1×10^7 bacilli. Except for group C (the group given simple washed bacilli in the smallest dose) all the groups had significant FPE. Except for group B (the group given simple washed bacilli in a dose of 7×10^6) none of the groups had FPE that was significantly different from the ST suspension in the same dose. Thus, neither irradiation (0.25 megarads) nor 24 hr digestion with trypsin and chymotrypsin caused detectable damage in this experiment.

DISCUSSION

In exploring the immunogenicity of various preparations of *M. leprae*, the FPE results have been useful to us because they are obtained much more rapidly than infectious challenge results (about 1 month vs 7–10 months). Such a shortened time interval becomes especially valuable when one must await the results of a first experiment before planning the next. We have chosen to use intact bacilli as the challenge (eliciting) antigen for FPE tests because there was no other preparation that seemed as likely to contain a predominance of exposed bacillary surface antigen. The vaccine protection against infection is exerted against a very small population of *M. leprae*, less than 10^4 bacilli in most animals, early in the growth curve, and it seems pos-

TABLE 3. *Effect of irradiation and purification procedures on the immunogenic activity of* M. leprae. M. leprae *were treated as indicated by 0.25 megarads of gamma irradiation (Ir), two centrifugal washings (Wash), short trypsin digestion (ST), 24 hr digestion with trypsin and chymotrypsin (T,CT), or two phase polymer separation (2P) and heat-killed at 100°C for 30 min. The immunizing antigens were given intradermally at 0 days. The eliciting antigen (2P-purified and heated to 100°C for 30 min) was given into the left hind foot pad at +28 days in a dose of 1 × 10⁷ bacilli. The uncorrected 2 day FPE in the control is shown in parenthesis.*

Group	Immunizing antigen						2 day corr FPE	p values			
	Treatment										
	Ir	Wash	ST	T, CT	2P	Dose	(10^{-2} mm)	p	vs	p	vs
A		+				2×10^7	15.5	<.002	T	NS	D
B		+				7×10^6	16	<.02	T	<.02	E
C		+				2×10^6	7	NS	T	NS	F
D		+	+			2×10^7	30	<.02	T	—	
E		+	+			7×10^6	37.6	<.002	T	—	
F		+	+			2×10^6	21.5	<.02	T	—	
G		+	+		+	4×10^7	19.5	<.002	T	NS	H
H		+	+		+	2×10^7	17	<.02	T	NS	D
I		+	+		+	7×10^6	34	<.002	T	NS	E
J		+	+		+	2×10^6	19.5	<.05	T	NS	F
K	+	+	+			2×10^7	21	<.002	T	NS	D
L	+	+	+			7×10^6	26	.002	T	NS	E
M	+	+	+			2×10^6	21.5	<.002	T	NS	F
N		+		+		2×10^7	27.6	<.001	T	NS	D
O		+		+		7×10^6	28.5	.002	T	NS	E
P		+		+		2×10^6	20	<.002	T	NS	F
Q	+	+		+		2×10^7	38	<.002	T	NS	D
R	+	+		+		7×10^6	27	<.002	T	NS	E
S	+	+		+		2×10^6	20	.02	T	NS	F
T	Nil						(28.5)	—			

sible that the specificity is directed against the surface of intact viable bacilli rather than against some interior antigen(s) released after death and disintegration of the organism. Most of the work has been done with heat-killed intact suspensions because they provide better protection against infectious challenge and because they do not produce consistently less FPE than did the disintegrated products of the same number of organisms. The FPE peaked at 2 or 3 days so we have chosen to report these results; earlier responses might reflect the presence of antibody, and later responses might indicate the immune response to the challenge antigen itself. Patel and Lefford ([6]) have shown that the injection of 100 μg of irradiation-killed *M. leprae* into the foot pads of mice induces cell-mediated immunity to antigens of *M. leprae*, as indicated by several approaches, including FPE in response to a soluble product of *M. leprae* that was the centrifugal supernatant of physically disrupted bacilli (what we have called the protoplasmic fraction). There are some technical differences between their work and ours, however. In their work the *M. leprae* before and after disruption had been purified by a prolonged enzymatic digestion; the *M. leprae* had been killed with 2.5 megarads of gamma irradiation; immunization was by the foot pad route; and the dose (100 μg) was about 25 times ours. (With a similar preparation of such antigen we found 2.5×10^9 *M. leprae*/mg after the bacteria were declumped by the chloroform method ([1])).

In the work reported here and in other unpublished work we have found agreement, in general, between FPE results and protection against infectious challenge. Dissociation was seen here in experiment 6 where the subcutaneous route was seen to be ineffective for FPE but effective for protection. Likewise, a dose of 1×10^6 *M. leprae* ID was not effective as judged by

FPE, but it was as judged by protection. A simple explanation for these apparent discrepancies may lie in the timing of the response; the response in FPE in this experiment was exerted about 1 month after immunization whereas that in protection against infectious challenge can be exerted several months later. Thus protection is observed with vaccine administered *after* challenge whether the vaccine is living BCG [14] or heat-killed *M. leprae* (unpublished results). It seems possible also that vaccine protection detects a smaller degree of sensitization; thus, tests on experimentally infected mice in the plateau phase, when they are immune to challenge [3, 4, 12], have revealed only small amounts of FPE (unpublished results).

Dissociation in the other direction has been seen in mice immunized with suspensions of cultivable mycobacteria. Some cultures in the *M. avium/M. intracellulare/M. scrofulaceum* complex have sensitized mice to FPE with heated, intact *M. leprae* without protecting them against infectious challenge with *M. leprae* [13]. In these cases, of course, the specificity in the FPE reaction may be directed against antigens that are irrelevant to protection against infectious challenge. Perhaps perfect agreement between these two types of tests should be expected only when a single antigen is responsible for protection against infectious challenge and that antigen is available in purified form for FPE tests.

Much of the information about immunity to *M. leprae* in man has been obtained with intradermal injection of suspensions of intact, heat-killed *M. leprae*. The FPE results we have used here would be analogous to the Fernandez test, which is usually read at 2 days. The Mitsuda test is read at about 28 days and would appear to be analogous to the intradermal results in experiment 1 (Fig. 1) where the reaction at the site of injection increased after 20 days, presumably as the result of the immunogenic effectiveness of this route of injection and the persistence of the antigen. We have pointed out earlier [13] that, in terms of the results obtained here, persons with positive Mitsuda reactions would consist of two groups, those with preexisting cell-mediated immunity (CMI) to *M. leprae* before the test and those just acquiring CMI to *M. leprae* as a result of the injection of intact, heat-killed *M. leprae* in the form of Mitsuda antigen.

The 24 hr digestion with 0.1 mg/ml trypsin and chymotrypsin was sometimes harmful to the immunogenicity of *M. leprae*. The short trypsin procedure and the further purification by the two phase polymer procedure was not detectably harmful, however. Irradiation in the dosage used was not harmful either. Results reported early [13] had shown heat-killed 2P-purified *M. leprae* to be highly effective in providing protection against infectious challenge.

SUMMARY

Foot pad enlargement (FPE) has been used as a measure of induced immunity to *M. leprae*. FPE peaked at 2–3 days, but it sometimes persisted for 4 weeks or more. Both as the inducing and eliciting antigen, heat-killed *M. leprae* were effective, and the optimum dose was about 1×10^7 bacilli. Higher doses were associated with flattening of the dose-response curve. Disrupted bacilli were not effective in immunizing mice, but they elicited FPE responses in mice immunized with intact bacilli. Cord factor was not found to have adjuvant activity for *M. leprae*. In immunization, the intradermal route was confirmed to be more effective than the foot pad route; the subcutaneous route was effective in providing protection against infection. FPE tests were used to investigate the steps of standard purification procedures for *M. leprae* in armadillo livers. A trypsin-chymotrypsin digestion step was found to be harmful to immunogenicity in one of two experiments.

RESUMEN

El engrosamiento del cojinete o almohadilla plantar del ratón fue usado como medida de la inmunidad inducida por el *M. leprae*. El engrosamiento plantar alcanzó su máximo entre el segundo y tercer día pero algunas veces persistió por 4 semanas o más. El *M. leprae* muerto por calor fue efectivo tanto en la inducción como en la reestimulación de la respuesta inmune y la dosis óptima fue de aproximadamente 1×10^7 bacilos. Las dosis mayores a la óptima estuvieron asociadas con el aplanamiento de la curva de dosis-respuesta. Los bacilos rotos no fueron adecuados para la inmunización de los ratones aunque sí lo fueron para estimular el engrosamiento plantar en los animales inmunizados con el bacilo intacto. El factor cuerda no demostró tener actividad adyuvante para el *M. leprae*.

Se confirmó que para la inmunización, la ruta intradérmica fue más efectiva que la ruta intra-plantar; la ruta subcutanea fue eficaz para conferir protección contra la infección. Las pruebas del engrosamiento plantar se usaron para investigar las etapas de los procedimientos "estandard" de purificación del *M. leprae* a partir del hígado de armadillos. Se encontró que un paso de digestión con tripsina-quimotripsina afectó la inmunogenicidad del bacilo en uno de dos experimentos.

RÉSUMÉ

On a utilisé la technique d'épaississement du coussinet plantaire (FPE) pour mesurer l'immunité induite à *M. leprae*. Le maximum d'épaississement suvenait après 2 ou 3 jours, mais il persistait parfois pour quatre semaines et plus. *M. leprae* tué par la chaleur s'est révélé actif tant comme antigène d'induction que comme antigène de stimulation de l'immunité. La dose optimum se situait aux environs de 1×10^7 bacilles. Des doses plus élevées entrainaient un aplatissement de la courbe doses-réponses. Les bacilles désintégrés ne présentaient pas d'activité pour l'immunisation des souris. Ils entrainaient cependant des réponses d'épaississement du coussinet plantaire chez les souris immunisés par des bacilles intacts. Le "cord factor" ne s'est pas révélé un adjuvant actif pour *M. leprae*. Pour l'immunisation, il a été confirmé que la voie intradermique était plus efficace que l'inoculation par le coussinet plantaire. La voie sous-cutanée était efficace pour fournir une protection contre l'infection. Les épreuves d'épaississement du coussinet plantaire ont été utilisés pour investiguer les étapes nécessaires pour mettre au point une procédure standard de purification de *M. leprae* dans les foies de tatous. Il est apparu que la digestion intermédiaire par la trypsine-chymotrypsine endommageait le processus d'immunogenicité dans l'une des deux expériences qui ont été pratiquées.

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