IMMUNOLOGICAL IDENTIFICATION OF FOOT-PAD ISOLATES AS MYCOBACTERIUM LEPRAE BY LEPROMIN REACTIVITY IN LEPROSY PATIENTS

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The demonstration of the causative role of an organism in leprosy presents difficulties not present in other diseases. An acid-fast bacterium, not cultivable by present techniques, is found in the patients' lesions; it is designated *Mycobacterium leprae.* The histopathology of the disease and its drug sensitivity are compatible with the notion that the disease is caused by a mycobacterium. By the inoculation of mouse foot-pads a non-cultivable mycobacterium can be isolated with regularity from clinical specimens containing *M. leprae* and apparently not from other specimens (**1, 2**). A relationship between the dose of *M. leprae* inoculated and the incubation period of the experimental disease further relates the isolate to *M. leprae.* The experimental disease in mice is similar to the natural disease in that it is very slow in development and there is a large, round cell response without necrosis, but it is dissimilar in that the nerves are not often involved. Antibody studies have not been helpful because of the widespread cross-reactions among mycobacterial species.

Fortunately the lepromin reaction in leprosy patients has a unique specific immunological aspect that may be applied to test an isolate from the disease; this was pointed out by Hayashi 30 years ago (3). Lepromin is prepared from lepromas and contains large numbers of *M. leprae.* The tissue is boiled or autoclaved, ground in a mortar with saline, and filtered through gauze (4). After injection into the skin there may be an early (Fernandez) reaction with a maximum at about 48 hours, and a late (Mitsuda) reaction with a maximum at 14 to 28 days. The specific aspect of the test lies in the lack of reaction by patients with the lepromatous form of the disease, a more severe type in which bacilli in the tissues are numerous. In contrast, most patients do react who have the tuberculoid disease, a different and milder clinical form in which bacilli are few. The test of a purported isolate of *M. leprae* can be made stringent by several measures, all of which were followed in this study. (*a*) A large group of patients, preferably adults, should be tested, since some persons, especially children, fail to react to leprosy and other mycobacterial antigens (5). If children are included in the study, a correlation between two antigens could arise from age differences of the subjects rather than from immunological relationships between the mycobacteria. (*b*) The late (Mitsuda) reading should receive primary attention, since the early reactivity of some antigens may be unpredictably low. (*c*) Whole bacillary antigens should be employed. Antigens purified by extractions with organic solvents (e.g.,

Dharmendra antigens) have much less late reactivity (6). Also extracts of certain non-leprosy organisms have been prepared that give more early reactions in tuberculoid than in lepromatous patients (7). When points (*b*) and (*c*) are followed the results may be viewed in terms of the experience reported in the literature, obtained primarily with late readings and unextracted antigens. Finally (*d*) carry over of bacilli from the original inoculum into the test antigen, which is a real possibility with clinical material from this disease, should be ruled out by quantitative data covering the history of the isolate.

To test the foot-pad isolates a suspension of bacilli was prepared from infected foot-pads in the Atlanta laboratory and it was tested at Cebu in lepromatous and tuberculoid patients. Parallel injections of a standard lepromin were given. The reactions to the foot-pad preparation and the lepromin correlated to a high degree.

Materials and Methods

The suspension of acid-fast bacilli from infected foot-pads was prepared from a strain in fourth passage. It was a typical isolate and had originated in September, 1959, from nasal washings of a lepromatous patient from Puerto Rico. The bacillary increase was 5.6×10^2-, 2.0×10^4-, 2.2×10^2-, and 1.1×10^3-fold in the respective 4 passages, or a total of 2.7×10^{12}-fold for all 4 passages. The chance that even 1 bacillus of the original inoculum would have carried over into the skin test dose (1.6×10^6 bacilli) would be extremely small.

The method used for preparation of the suspension was similar to that used for routine harvests of infected foot-pads (1, 2). The mice had been inoculated about 7 months earlier with 5.0×10^3 organisms. The lightly minced foot-pads of 8 mice were placed in the cup of a Mickle tissue disintegator with about 20 3 mm glass beads (kimax solid glass, No. 13500, Kimble Glass Company, Toledo), 4 ml Hanks' balanced salt solution containing 0.05 per cent tween 80 (BSS-tween) added, and vibration carried out for 2 minutes with a measured amplitude of 5 mm. The resulting suspension was then removed to a test tube. The aggregation of skin fibers was accelerated by mixing with bulb and pipette, the aggregates and visible pieces of tissue allowed to settle for 2 minutes, and the supernatant carefully pipetted off (this process called "taking a 2 minute supernatant"). If necessary, a further 2 minute supernatant was taken. The Mickle cup and 2 minute sediments were washed with a single 2 ml portion of BSS-tween, and the resultant supernatant was added to the previous supernatant. An equal volume was then added of 0.25 per cent trypsin (Nutritional Biochemicals Corp., Cleveland, 1:300, in saline buffered to pH 7.6 and containing 0.001 per cent phenol red). The pH of the mixture was adjusted to pH 7.6 with N/100 NaOH, the suspension stirred with bulb and pipette, placed in a 37°C water bath for 5 minutes with occasional agitation by hand, and the suspension again stirred with bulb and pipette. If aggregates of fibers appeared, a 2 minute supernatant was taken. The suspension was then chilled in ice water and centrifuged at 4°C at 3000 RPM for 30 minutes. The supernatant was discarded, and the sediment was resuspended to volume in chilled 0.85 per cent saline buffered to pH 7.3 and containing 0.05 per cent tween 80 (pH 7.3 saline-tween), and the cold centrifugation repeated. The supernatant was discarded, the sediment resuspended to 1 ml in pH 7.3 saline-tween, and thimerosal added to 1:10,000. Each preparation was completed in 1 day, and the final 1 ml stored at 4°C. The yields at several stages during each work-up were determined by counts of acid-fast bacilli. The operation was repeated 16 times (total 128 mice), the final 1 ml portions pooled, a 2 minute supernatant taken, and autoclaving carried out at 15 pounds

for 15 minutes. The total yield for all 16 operations was 70.6×10^7 acid-fast bacteria (AFB) before trypsinization, 70.6×10^7 after trypsinization, 45.3×10^7 before autoclaving, and 22.3×10^7 after autoclaving. The final product had a content of 1.57×10^7 AFB/ml. The standard lepromin (Mabalay) contained 4.18×10^7 AFB/ml, so a portion of it was diluted 1:2.66 to contain 1.57×10^7 AFB/ml. Thus there were 3 products: A, the preparation from foot-pads containing 1.57×10^7 AFB/ml; B, the diluted standard lepromin containing 1.57×10^7 AFB/ml; and C, the undiluted standard lepromin containing 4.18×10^7 AFB/ml. They were marked A, B, and C, and sent to Cebu without further designation.

TABLE I

Sex and Age Distribution of Patients Tested with Antigens A, B, and C

Age group	Lepromatous			Tuberculoid		
	Male	Female	Total	Male	Female	Total
yrs.						
10–14	—	—	—	2	—	2
15–19	3	—	3	3	4	7
20–29	9	2	11	4	3	7
30–39	11	5	16	4	1	5
40–49	—	—	—	3	—	3
50 over	—	4	4	4	2	6
Total.....	23	11	34	20	10	30

TABLE II

Clinical Status of Patients Tested with Antigens A, B, and C

Lepromatous			Tuberculoid		
Clinical status	No.	Per cent	Clinical status	No.	Per cent
L-1	7	20.6	T-1	7	23.3
L-2	10	29.4	T-2	12	40.0
L-3	17	50.0	T-3	11	36.6
Total.....	34	100.0		30	99.9

The clinical status is given as 1 (slight), 2 (moderately advanced), and 3 (advanced).

The 34 adults with active lepromatous leprosy and 30 patients (28 adults and 2 children over 10 years) with clear-cut tuberculoid lesions were tested simultaneously with the 3 antigens. The two groups were roughly matched for age and sex (Table I), and all were patients at the Eversley Childs Sanitorium, Cebu. The clinical and bacteriological conditions are given in Tables II and III. The tuberculoid patients were, or had been, bacteriologically positive, reactional tuberculoid cases, since positive bacteriology was a condition for admission. Thus they probably tended to be less reactive to lepromin than would have a comparable group of non-reactional tuberculoid cases. The sites of injection were (*a*) left upper forearm, (*b*) left lower forearm, and (*c*) right middle forearm. The dose of 0.1 ml of antigens A, B, or C were in-

jected intradermally into sites *a*, *b*, or *c* at random, as determined previously from a list of random numbers. The reactions were read at 24, 48, and 72 hours for early (Fernandez) reactions of the tuberculin type and at 14, 21, and 28 days for late (Mitsuda) nodular reactions. The recorded measurements were the average of the horizontal and vertical diameters of induration in millimeters.

Although it had been intended that the antigens be treated as unknowns, this was not possible because their gross appearance identified them, antigen *A* being nearly water clear and antigens *B* and *C* definitely turbid. Accordingly the injections were made by Dr. T. Fajardo, Jr., and the reactions were measured by Dr. Guinto without knowledge of the antigen assigned to the site.

TABLE III

Bacteriological Status of Patients Tested with Antigens A, B, and C

Average bacteriological score*	Lepromatous		Tuberculoid	
	No.	Per cent	No.	Per cent
0	—	—	10	33.3
V.S. or +	—	—	16	53.3
++	5	14.7	3	10.0
+++	12	35.3	1	3.3
++++	17	50.0	—	—
Total.....	34	100.0	30	99.9

* Average from smears at all sites tested; V.S. stands for very sparse; + to ++++ for increasing numbers of acid-fast bacteria.

The suspensions of the N isolate of Binford (8, 9) had been prepared and tested at an earlier date. The N preparation was simply the growth in tween-albumin medium, washed thoroughly by centrifugation and resuspension in pH 7.3 saline-tween. The NT preparation originated from infected hamster testes supplied by Dr. Binford. It was purified by a density gradient procedure that was similar to one described for Q fever rickettsiae (10). The gradients were continuous and linear from 3 to 40 per cent sucrose in 1 M KCl with 0.05 per cent tween 80, and 30 minutes at 3000 RPM were required to move the bacillary band to the middle third of the gradient. This band was of a high degree of purity, and it was concentrated by one centrifugation and resuspension in pH 7.3 saline-tween and dialyzed against the same. The final yield 3.38×10^9 AFB from 15.1 gm testes. Both the N and NT antigens were diluted to 5.0×10^7 AFB/ml and autoclaved at 15 pounds for 15 minutes. This concentration had been selected on the basis of comparison to another standard lepromin (Wade), the bacillary content of which was a little greater than the Mabalay lepromin. Application of the density gradient procedure to foot-pad preparations was unsuccessful because of serious losses in acid-fast bacteria.

RESULTS

None of the 3 antigens gave positive reactions, early or late, in any of the 34 lepromatous patients (Table IV). In marked contrast, among the 30 patients with tuberculoid leprosy definitely positive late (Mitsuda) nodular reactions larger than 4 mm were observed in 20 (66.7 per cent) with antigen A, 21 (70.0

per cent) with antigen B, and in 27 (90.0 per cent) with antigen C. Thus the foot-pad antigen (A) produced Mitsuda reactions that were closely comparable to the standard lepromin of similar bacillary content (B).

The close correlation between antigens A and B is shown in Fig. 1, where the readings in the individual tuberculoid patients at 21 days are plotted. A was perhaps a little weaker than B, and both were weaker than C.

There were differences between A and B in the number of early reactions; probably this is a reflection of the fact that soluble antigens would have been removed in the preparation of A. There were also slight differences in the rate

TABLE IV

Frequency and Size of Early and Late Reactions to Antigens A, B, and C

Antigen	Optimum early reaction (24, 48, or 72 hrs.)						Optimum late reaction (14, 21, or 28 days)					
			Positive						Positive			
	Neg. 0–4.5 mm	Doubtful 5–9.5 mm	+ 10–14.5 mm	++ 15–19.5 mm	+++ 20 mm plus	Per cent pos.	Neg. 0–2.5 mm	Doubtful 3–4 mm	+ 4.5–7 mm	++ 7.5–9.5 mm	+++ 10 mm plus	Per cent pos.
1. Lepromatous, 34 patients												
A	31	3	—	—	—	0	34	—	—	—	—	0
B	34	—	—	—	—	0	34	—	—	—	—	0
C	24	10	—	—	—	0	34	—	—	—	—	0
2. Tuberculoid, 30 patients												
A	19	7	2	2	—	13.3	2	8	15	3	2	66.7
B	16	5	8	1	—	30.0	3	6	15	3	3	70.0
C	9	11	5	3	2	33.3	1	2	18	4	5	90.0

at which the means of the late reactions changed with time, antigen A reaching a peak at 21 days and antigen B at 28 days. Similar differences were seen between different preparations of lepromin.

Reactions to N and NT Suspensions.—The suspensions prepared from the N isolate were tested in leprosy patients also. All had been tested with Mabalay lepromin within the previous 3 months. The late (Mitsuda) reactions were as follows: Of 66 lepromatous patients all had been negative to lepromin; 35 of these were tested with N antigen and 32 (91.4 per cent) were positive; the remaining 31 were tested with NT antigen and 31 (100 per cent) were positive. Of 20 tuberculoid patients 19 (95 per cent) had been positive to lepromin; 10 of these were tested with N antigen and 10 (100 per cent) were positive to N antigen; the remaining 10 were tested with NT antigen and 10

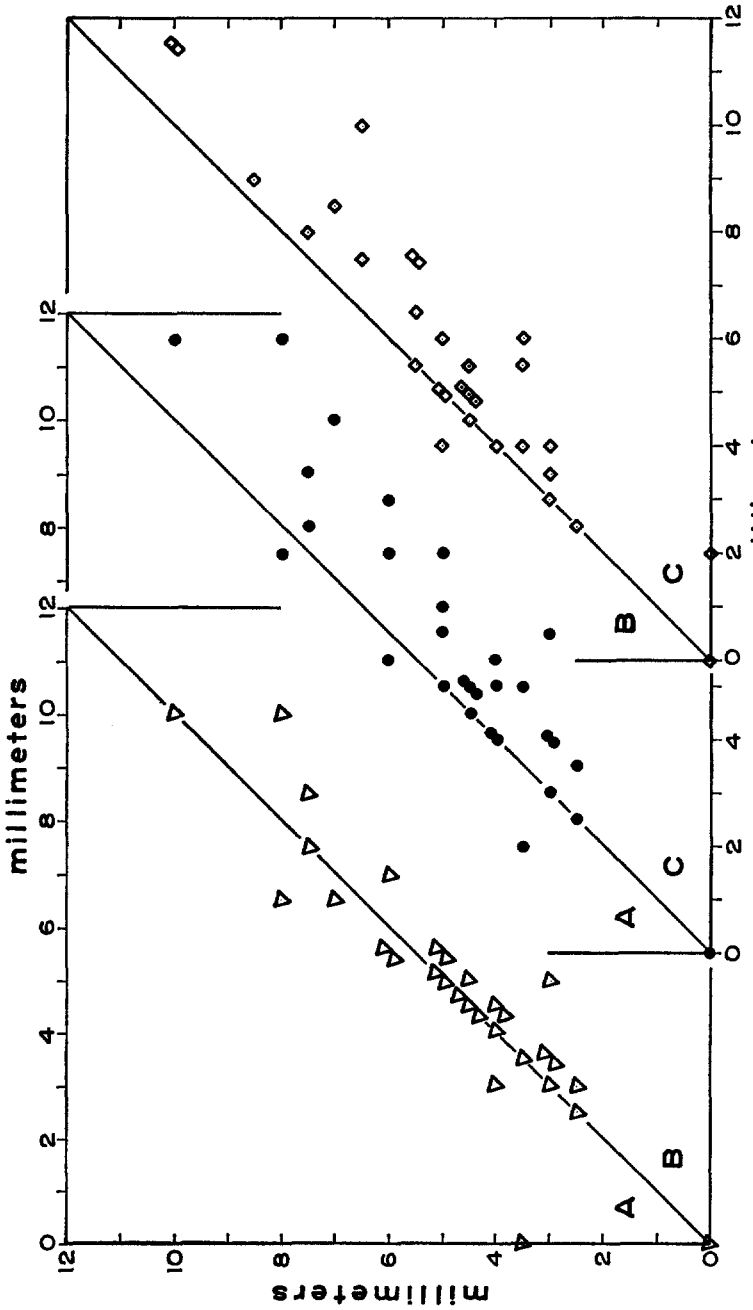

FIG. 1. Skin test readings (Mitsuda, 21 days) in the tuberculoid patients. Correlations between antigens A and B, A and C, and B and C.

(100 per cent) were positive. Thus there were no important differences in the reactivity of lepromatous and tuberculoid patients to suspensions of the N isolate. The early (Fernandez) reactions to the N antigens were also about the same in the two types of patients.

TABLE V

Late (Mitsuda) Skin Reactions to Various Bacillary Antigens in Leprosy Patients

1. Lepromin (contains *M. leprae*): Negative in lepromatous, positive in most tuberculoid patients.
2. Foot-pad isolates: Same as lepromin, (this paper).
3. N isolate in hamster testes: Positive in 91 to 100 per cent of lepromatous and tuberculoid patients (this paper).
4. *M. tuberculosis:* Positive in estimated* 88 per cent of lepromatous patients (Guinto, 11).
5. *M. avium:* Positive in estimated* 83 per cent of lepromatous patients (Guinto, 11).
6. *M. leprae-murium:* No correlation with lepromin (Hayashi, 3; Muir, 12; Sato and Sato, 13; Ohtawara and Kamamura, 14).
7. *M. phlei:* No correlation with lepromin (Hayashi, 3).

8–12. Purported isolates of *M. leprae* by Clegg, Needham, McCoy, Duval, and Kedrowsky: No correlation with lepromin (Hayashi, 3).

13–18. BCG, *M. fortuitum, M. rhodocrous, M. marinum, M. phlei, M. smegmatis:* No correlation with lepromin in 48 leprosy patients (16 lepromatous, 30 tuberculoid, and 2 atypical) (McFadzean, 15).

* Estimated on the assumption that patients that reacted to tuberculins for these organisms would have had positive Mitsuda reactions. In reality only those who were tuberculin-negative were tested with bacillary antigens and 65 and 46 per cent, respectively, responded with Mitsuda reactions.

DISCUSSION

The specificity of the lepromin reaction is given by the results in Table V in which are gathered results from this paper and other sources. The reactions of none of the 16 other mycobacterial cultures correlated with those of lepromin. Thus the failure of the lepromatous patient to react to lepromin is specific, and it is not part of a general lack of reactivity such as the general failure to manifest tuberculin-type hypersensitivity to all antigens by patients with sarcoidosis, Hodgkin's disease, and advanced malignant states (16). McFadzean's results (15) also attest to the specificity of the Mitsuda reactions to other mycobacteria, since he failed to find correlation between antigens of other mycobacteria, except between BCG and *Mycobacterium marinum*, species already known to be antigenically related.

The relationship of the lepromin reactions to more widely known immunologic skin tests is indicated by the timing. The early (Fernandez) reaction seems to be a tuberculin-type reaction in every respect. ("Tuberculin-type" is here used synonomously with "delayed-type" of the general immunologic literature

in order to avoid confusion with the late, Mitsuda, reaction to lepromin.) The late (Mitsuda) reaction suggests the graft rejection phenomenon in its slowness; there is enough time for a new immunologic response to form against the introduced antigen itself. The lateness of the Mitsuda reaction may also be a function of the time at which protoplasmic antigen is released from intact bacilli; *M. leprae* (17) in common with other slowly growing mycobacteria (18) has an antigen-free outer surface, in that it is not stainable with fluorescent antibody until it is broken open. The classical criterion of tuberculin-type sensitivity, its transferability with white cells, has not been tried with lepromin reactivity.

The lepromin reaction has been viewed in leprology as an indicator of resistance to the disease (6). The lepromin negativity of the lepromatous patient can also be looked on as a form of immunologic tolerance (19), since it represents a specific inability to respond immunologically to antigen present in the tissues in large amounts (20). There is, of course, no conflict in these two views. This is perhaps best brought out by an analogy in generalized vaccinia. Kempe (21) reports that in vaccinia necrosum, a progressive form that is always fatal when untreated, there is usually complete lack of antibody formation to vaccinia virus, even though there can be normal antibody response to other antigens. There is also lack of tuberculin-type sensitivity to heat-killed vaccinia virus. He describes a case that continued to progress in spite of massive administration of antibody. Recovery occurred, however, when immune white cells were administered.

SUMMARY

Lepromin, a product containing *Mycobacterium leprae* from patients' tissues, fails to elicit skin reactions in lepromatous patients; this non-reactivity was utilized as a means of identifying the mycobacterium isolated in mouse foot-pads from leprosy patients. To do this a suspension of acid-fast bacilli prepared from mouse foot-pads infected with a typical isolate was compared to human lepromin. The isolate was in fourth passage, and the multiplication since first isolation had diluted the original inoculum to insignificant levels. Three preparations were tested: A, A foot-pad preparation containing 1.6×10^7 organisms/ml, B, lepromin diluted to the same bacillary content, and C, undiluted lepromin containing 4.2×10^7 organisms/ml.

The reactions were compared in 34 lepromatous and 30 tuberculoid patients. All 34 lepromatous patients were negative in both early (Fernandez) and late (Mitsuda) reactions to all 3 antigens. In the tuberculoid patients the late reactions were positive in 66.7, 70.0 and 90.0 per cent to antigens A, B, and C, respectively. The size of the reactions to A and B were closely correlated in the individual patients.

Experience in leprosy patients with suspensions prepared from 16 other

mycobacterial cultures is reported or reviewed. None of them produced reactions that correlated with those of lepromin. Thus the lack of reactivity to lepromin of lepromatous patients appears to be a specific phenomenon.

The results provide evidence that the foot-pad isolates and *M. leprae* are immunologically identical.

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