

RESULTS OF TESTS WITH SERIAL DILUTIONS OF LEPROMIN
IN SEPARATE GROUPS OF NORMAL YOUNG CHILDREN

WITH A COMPARISON OF TWO LEPROMINS AND THE
DHARMENDRA ANTIGEN¹

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Coincidental with increasing emphasis on the use of the lepromin test, there has been a decrease of leproma material suitable for the making of lepromin because of the effects of sulfone treatment. In consequence, the Madrid Congress (¹⁶) recommended that the use of higher than usual dilutions be studied. This is a report of such a study, during which collateral observations were made. It is unique in that it was done with normal young schoolchildren instead of patients, and that none received more than one injection. The results differ widely from those usually reported.

In this connection reference has been made repeatedly to a statement by Pardo-Castello and Tiant (¹⁸) that a 1/3000 dilution will give positive results, but their statement was not based on experimental data. The use of high dilutions was initiated in 1951 by Floch (⁹), who reported that the results with the Mitsuda-Hayashi lepromin diluted to 1/750 could be correlated with those obtained with regular lepromin by increasing the reading one grade (1+).

Partially confirmatory findings have been reported. Diniz and Neto (⁷) used 1/750 and 1/1000 dilutions; but the dose was 0.2 cc., which in effect reduced those dilution values by one-half. It occurred to them that injection of the normal ("control") dose simultaneously with the dilutions in the same individuals might perhaps influence the results of the latter, but they did not pursue that idea to any conclusion. With their double doses and 3 mm. positivity limit the subjects used—mostly BCG-vaccinated preventorium children aged 2-16 years—were 78.7 per cent positive with the 1/20 control, 65.6 per cent with the 1/750 dilution, and 49.3 per cent with the 1/1000 dilution. It was concluded that it is possible to use much-diluted lepromin in routine work, but that, as in tuberculin testing, persons negative to a small dose should be retested with the regular one to pick up the weaker reactors.

Floch (¹⁰) then reported that diminution of positivity does not parallel uniformly the increase of dilution, and that lepromin can be diluted to 1/150 (i.e., 5 times) with no material effect on the intensity of the reaction. Later (^{11, 12}), he reported on the enhancing effect of adding glycerine and paraffin oil, or an extract of normal skin, to the 1/750 dilution to make it behave more like normal lepromin.

Commenting on Floch's work, one of us (²⁴) pointed out that no evidence had been adduced that the full dose given as a "control" might not have influenced the

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degree of reaction to a smaller dose in the same individual, as could be the case if an antibody effect is involved in the Mitsuda reaction.

Schujman (²¹) reported tests with dilutions of both the Mitsuda-Hayashi lepromin (1/100 and 1/750) and the Dharmendra antigen (1/5), the subjects being 40 patients with tuberculoid leprosy varied as regards their known reactivity. The strong reactors gave positive results, although weak with the high dilutions; but of 8 weak reactors only 1 was positive to 1/100, and none to 1/750. Of 4 weak reactors in the test with the diluted Dharmendra antigen, none responded. Schujman concluded that dilute antigens might be of practical use in strong reactors, for a special purpose, but that first tests should always be made with a normal antigen.

Chaussinand (^{2, 3}), from experience with patients, counsels against the use of very high dilutions, or the addition of nonspecific "reinforcing" substances that have no antigenic value. He found that a 1/60 suspension gives practically the same results as the usual full dose, and that a 1/200 dilution is useful for epidemiological investigations although the reactions are weaker.

Basombrio *et al.* (¹) found Floch's reinforced dilute lepromin of promise with respect to economy for testing patients, but unsuitable for testing contacts. In them it gave only 28.5 per cent positives, whereas regular lepromin had given 82.8 per cent in a similar group of contacts.

PRESENT STUDY

The present study was planned primarily to ascertain what results would be obtained with single doses of various dilutions of lepromin in separate groups of healthy young schoolchildren not previously subjected to either lepromin testing or BCG vaccination. The main question was whether, in such normal but immature subjects, any considerable dilution of the antigen could be depended on to give valid results.

Because lepromin is antigenic,² multiple injections were not given any individual, as might be done with a nonantigenic reagent such as tuberculin, lest any reaction elements ("antibodies") engendered by a strong dose might make the results with weak doses stronger. The use of separate groups for the various dilutions unavoidably increased the possibility of chance statistical variations, but the groups were made large enough to minimize such irregularities.

The field work, done in Opon, Mactan Island, Cebu, by the senior author and staff, consisted of two series of tests on schoolchildren, one done in the *poblacion* (town proper) and the other in outlying *barrios* (rural villages). Ages were limited to 6 to 9 years to minimize the effects of aging on the results. In grading the reactions, the recommendations of the WHO expert committee (²⁶) were followed, all late reactions over 4 mm. being regarded as positive.³ Tuberculin (PPD) tests were made, but in connection with other work.

²Since 1937 one of us (²³⁻²⁵) has maintained that the late reaction to lepromin depends upon some immunologic change induced by the test injection itself, a view which has been gaining ground in some circles of late years. To use a term employed by certain students of tuberculosis with respect to the BCG test (²²), this reaction is of the "bacillus-body" type. The injection of BCG, Ustvedt points out, "represents a vaccination, which is something different from a mere test" (referring to the tuberculin test), and no reason is seen to doubt that that idea is applicable to the lepromin reaction.

The designations of the concentrations of lepromin used in this report are based on the stock suspensions, "1/1" meaning the undiluted lepromin. Thus "1/10" means 10 per cent of the lepromin, not of the leproma stock used, and so on (see Table 5); the "1/80" dilution contained only 1.25 per cent of the original lepromin. For approximate correlation with the terms used by previous workers, our "1/10" and "1/40" would be roughly equivalent to Floch's 1/150 and 1/750 dilutions.

FIRST SERIES

PROCEDURE

These preliminary tests, begun in July 1954, were made with the children of two schools in the Opon *poblacion*. They were taken in lots of five, one being assigned consecutively to each of the five experimental groups. Of the 579 whose reactions were all read, 283 were males and 296 females. The sex distribution in the experiment was fairly uniform. Of the four age years, 6 to 9 inclusive, there were 123, 199, 157 and 100, respectively.

The antigens used were; two lepromins made by different methods, one (W) by Wade (²⁸), the other (M) by E. B. Mabalay (¹³); also, two dilutions, "1/10" and "1/20" of the W. lepromin; and, finally, an authentic lot of the Dharmendra antigen, kindly supplied by him.⁴ The numbers of individuals in each of the five groups are given in Tables 1 and 2.

RESULTS, FIRST SERIES

Early reactions.—Hypersensitivity to lepromin was infrequent in these town children, as shown in Table 1. In full doses the M lepromin caused

TABLE 1.—*Early (Fernandez) reactions in the five groups of the first series; percentages.^a*

Group and antigen	Number tested	Results					Total positive
		Negative	Doubtful	Positive 1+	Positive 2+	Positive 3+	
I. Mabalay 1/1	117	67.5	24.8	7.7	0.0	0.0	7.7
II. Wade 1/1	118	58.5	28.0	10.1	0.9	2.5	13.5
III. Wade 1/10	113	67.2	19.5	11.5	0.9	0.9	13.3
IV. Wade 1/20	119	73.1	21.0	4.2	1.7	0.0	5.9
V. Dharmendra	112	66.9	28.6	2.7	0.9	0.9	4.5

^a Grading: Negative, 0-4 mm.; doubtful, 5-9 mm.; 1+, 10-14 mm.; 2+, 15-19 mm.; 3+, 20 mm. or more.

³Our figures for positivity are consequently lower by the numbers of the doubtful (\pm) readings than they would be if the more usual 3 mm. lower limit had been used. For comparison with results obtained by workers using that limit, simply add the doubtfuls to the positives.

⁴This antigen, received in 1 cc. ampules, was a faintly clouded suspension of which smears showed characteristically rare acid-fast bacilli.

only about one-half as many reactions as the W lepromin (7.7% vs 13.5%), but in view of the small numbers of reactors the difference may have been a chance one.

The Dharmendra antigen gave fewer early reactions than either of the lepromins, only 4.5 per cent. This result was unexpected because that antigen is supposed to give more such reactions than regular lepromin.

The group receiving 1/10 W dilution gave as many early positives as did the one given the full dose, a probably chance result which was not repeated in the second series.

Late reactions.—In this case, as shown in Table 2, the two antigens gave identical results, 75 per cent total positives—a high rate for children so young, although most were only 1+. Had the 3 mm. limit been used, almost all would have been positive. Conspicuously in contrast, only one of the children receiving the Dharmendra antigen showed a reaction over 4 mm., and only 10 (8.9%) of that group would have been positive on the 3 mm. basis.

TABLE 2.—*Late (Mitsuda) reactions in the five groups of the first series; percentages.^a*

Group and antigen	Number tested	Results					
		Negative	Doubtful	Positive 1+	Positive 2+	Positive 3+	Total positive
I. Mabalay 1/1	117	2.6	22.2	69.2	1.7	4.3	75.2
II. Wade 1/1	118	1.7	22.9	63.6	5.9	5.9	75.4
III. Wade 1/10	113	27.4	56.6	15.1	0.0	0.9	16.0
IV. Wade 1/20	119	39.5	49.6	10.1	0.8	0.0	10.9
V. Dharmendra	112	91.1	8.0	0.0	0.9	0.0	0.9 ^b

^a Grading: Negative, less than 3 mm.; doubtful, 3-4 mm.; 1+, more than 4 to 7 mm.; 2+, 8-10 mm.; 3+, more than 10 mm. or any size with ulceration.

^b Considering 3 mm. as 1-plus positive would bring this figure to 8.9 per cent.

With the 1/10 dilution of the W lepromin, the number of positives was reduced drastically, by almost four-fifths, and doubling that dilution caused about one-third further reduction. The decrease did not parallel dilution, for the 1/10 dilution still caused over 20 per cent as many positives as did the full dose, and the 1/20 dilution gave nearly 15 per cent as many.

SECOND SERIES

PROCEDURE

The extended series of tests with dilutions of the W lepromin, done in *barrio* schools, was begun in March 1955, the distribution to the five experimental groups

being done by random sampling. Of the 463 who completed the observations, 204 were males and 259 females. There were 59, 131, 162 and 111 individuals, respectively, of the four different age years. The numbers in the different groups, averaging 92, are shown in Tables 3 and 4. The lepromin concentrations used were 1/1 (control), and 1/10, 1/20, 1/40 and 1/80 dilutions.

These children differed from those of the first series by the facts (1) that they were from a rural environment, (2) that there had been nine months or so of aging since the first series work was begun, and (3) that country children tend to start schooling somewhat later than town children, so that the proportions in the 8- and 9-year groups were larger.

RESULTS, SECOND SERIES

Early reactions.—Fernandez reactions in the full-dose group were, as shown in Table 3, twice as frequent in this series as in the first one with the same lepromin, 26.1 against 13.5 per cent. This difference cannot be explained by a higher frequency of tuberculization, for of the comparable full-dose groups the one in Series 1 was 22 per cent positive to 5 TU, and that in Series 2 was only 25 per cent positive to a double dose, 10 TU.

The numbers reacting to the dilutions were small, but a few did so to the highest dilutions. Analysis shows that this extreme hypersensitivity was closely related to tuberculin positivity.

TABLE 3.—*Early reactions in the five dilutions groups of the second series; percentages.^a*

Group and antigen	Number tested	Results					Total positive
		Negative	Doubtful	Positive 1+	Positive 2+	Positive 3+	
I. Wade 1/1	92	30.4	43.5	13.0	7.7	5.4	26.1
II. Wade 1/10	92	66.3	23.9	7.6	2.2	0.0	9.8
III. Wade 1/20	91	68.1	25.3	5.5	1.1	0.0	6.6
IV. Wade 1/40	96	79.2	16.7	3.1	0.0	0.0	3.1
V. Wade 1/80	92	86.9	8.7	2.2	1.1	1.1	4.4

^a Readings as in Table 1.

Late reactions.—No less than 91.3 per cent of the 92 children in the full-dose group were positive in some degree, as shown in Table 4. Had 3 mm. reactions been classed as 1-plus, every one of this group would have been called positive.

The lack of parallelism between the proportions of Mitsuda positives and the dilutions of the lepromin was striking in this series, as is clear from Table 5. No less than 34.8 per cent of the group receiving the 10

TABLE 4.—*Late reactions in the five dilutions groups of the second series; percentages.^a*

Group and antigen	Number tested	Results					Total positives
		Negative	Doubtful	Positive 1 +	Positive 2 +	Positive 3 +	
I. Wade 1/1	92	0.0	8.7	70.7	10.7	9.9	91.3
II. Wade 1/10	92	15.2	50.0	32.6	0.0	2.2	34.8
III. Wade 1/20	91	22.0	50.5	25.3	2.2	0.0	27.5
IV. Wade 1/40	96	49.0	38.5	12.5	0.0	0.0	12.5
V. Wade 1/80	92	53.3	41.3	5.4	0.0	0.0	5.4

^a Reading as in Table 2.

per cent (1/10) dilution were positive. Even with the 1/80 dose, in actuality not less than a 1/2000 dilution of leproma, 5.4 per cent reacted.

TABLE 5.—*Positive Mitsuda and Fernandez reactions, Series 2; (a) actual percentages and (b) ratios to proportions positive to full (1/1) dose.*

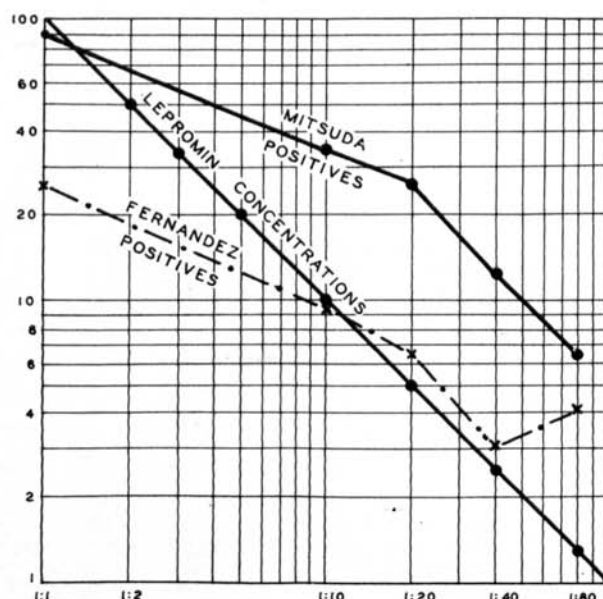
Dilution (and percentage of original lepromin)	Positive reactions, percentages and standard errors		Ratio (per cent) to proportion positive to 1/1	
	Mitsuda	Fernandez	Mitsuda	Fernandez
1/1 (100%)	91.3±2.9	26.1±4.6	100.0	100.0
1/10 (10%)	34.8±5.0	9.6±3.1	38.1	37.5
1/20 (5%)	27.5±4.7	6.6±2.6	30.1	25.3
1/40 (2.5%)	12.5±3.4	3.1±1.8	13.7	11.9
1/80 (1.25%)	5.4±2.4	4.4±2.1	5.9	15.9

The trend of the early reactions being less clearly evident, because of the relatively small proportions of positives, the calculations shown in the second part of Table 5 have been made. This shows percentages figured on the basis of 100 per cent for the number of reactors to the full dose. In these percentages there is a striking parallelism with the comparable percentages for the late reactions—except for the anomalous (obviously chance) rate with the 1/80 dose.

The percentages of Mitsuda and Fernandez reactors, for each strength of lepromin, are plotted in Text-fig. 1. For convenient presentation this figure is drawn on a double logarithmic scale. A straight line for either reaction would mean that the proportions reacting were in direct rela-

tionship to the dilution of lepromin. The figure indicates that this is not the case. The curves for the two reactions show close parallelism, except for the 1:80 reading for the Fernandez reaction.

TEXT-FIG. 1. Showing curves of the Mitsuda and Fernandez reactions, in comparison with that of the concentrations of the lepromin in the various dilutions, drawn on a double logarithmic scale. Neither curve declines in parallel with dilution of the antigen, but the two are essentially in parallel except for that of the Fernandez reaction at the end.



ASSOCIATION OF REACTIONS

Early and late lepromin reactions.—This study affords an excellent example of the frequent capability of young children, at least in the tropics, to develop the Mitsuda reactivity without the pre-existent hypersensitivity represented by the Fernandez reaction, as shown in Table 6. Of the 210 children in the two full-dose W lepromin groups, 4.3 times as many were Mitsuda positive as Fernandez positive—173 (82.4%) *vs* 40 (19.3%). Practically two-thirds of the Mitsuda positives (137, or 65%) were positive for that reaction only; only about one-fifth (36, or 21%) also gave the early reaction. In contrast, almost all (90%) of the 40 Fernandez positives were also Mitsuda positive. Nevertheless, and contrary to the usual expectation, there were a few hypersensitives who failed to give the late reaction.

The early reactions in general were so few that the data in Table 6 are not discussed further except to note that with the full-dose lepromins the Fernandez negatives gave almost as many Mitsuda reactions as did the Fernandez positives, and among the barrio children quite as many. The situation here, with these young normal children, is of course very different from the usual findings in tuberculoid leprosy patients, or perhaps even from what would be obtained in healthy adults.

Fernandez and tuberculin reactions.—Correlation with the tuberculin reaction (positive, as tested, in about 23% of the total group) is noted only with respect to the Fernandez positives in the groups tested with

TABLE 6.—Association between Fernandez and Mitsuda reactions.

Series and group	Fernandez positive		Fernandez negative		Mitsuda positive		Mitsuda negative	
	No.	Per cent Mitsuda positive	No.	Per cent Mitsuda positive	No.	Per cent Fernandez positive	No.	Per cent Fernandez positive
<i>First Series (poblacion children)</i>								
I. Mabalay 1/1	9	88.9	108	74.1	88	9.1	29	3.4
II. Wade 1/1	16	87.5	102	73.5	89	15.7	29	6.9
III. Wade 1/10	15	60.0	98	9.2	18	50.0	95	6.3
IV. Wade 1/20	7	0.0	112	11.6	13	0.0	106	6.6
<i>Second Series (barrio children)</i>								
I. Wade 1/1	24	91.7	68	91.2	84	26.2	8	25.0
II. Wade 1/10	9	88.9	83	28.9	32	25.0	60	1.7
III. Wade 1/20	6	50.0	85	25.9	25	12.0	66	4.5
IV. Wade 1/40	3	0.0	93	12.9	12	0.0	84	3.6
V. Wade 1/20	4	25.0	88	4.5	5	20.0	87	3.4

the W lepromin and its dilutions. In the full dose groups the 40 early reactors were 62.5 per cent positive to tuberculin, against 14.7 per cent of the 170 Fernandez negatives; and the proportions were essentially the same with the 1/10 dilution groups, 66.7 per cent of 24 against 14.9 per cent of 181.

However, there was an interesting difference in tuberculin rates in the relatively few subjects who showed hypersensitivity with the higher dilutions. The 13 positives to the 1/20 dose were 84.7 per cent tuberculin positive, and all of the 7 Fernandez reactors to the higher dilutions also reacted to tuberculin.

COMPARISON OF RESULTS IN SERIES 1 AND 2

Here we have observations with the W lepromin that are not a part of the original study plan, but that arise from the fact that—by force of circumstances—there were differences between the two lots of children tested. These were, a slight one in age and a more definite one in environment.

Regarding the Mitsuda reaction in the full-dose tests, the difference between 75 per cent positives in the town children (Series 1) and 91 per cent in the country children (Series 2) is a significant one in the statistical sense. In random sampling a difference as great or greater would be expected only about once in 400 trials.

The differences with the dilutions used in both series were greater. With the 1/10 dilution, the percentage reacting in Series 2 was more than twice that in Series 1; and with the 1/20 dilution the increase was even greater.

With respect to the Fernandez reactions induced by the dilutions, no such differences between the two series are seen. What differences there are may be ascribed to chance. The situation is different with the full-dose results in that the *poblacion* children gave only 13.5 per cent early reactions, whereas the *barrio* children gave 26.1 per cent positives.

In a search for an explanation of the differences between the two series, special consideration has been given, with negative results, to the two age differences mentioned, i.e., the higher proportion of 8- and 9-year-old children in the second series children, and the nine months or so of aging in that series.

Mitsuda positivity in such children increases with age, and up to a certain point rapidly. For example, in another study made on Mactan Island, with extremely young children (⁸), it was found that only 4.9 per cent of 81 aged 6-11 months reacted positively, but 17.9 per cent of 145 aged 1 year and 36.5 per cent of 170 aged 2 years.⁵ In an earlier study of the general population of Cordova municipality, also on Mactan Island, based on 1,851 tests of supposed noncontacts (¹⁴), the percentages of Mitsuda positives by 5-year groups increased as follows: 0-4 years, 15.7; 5-9, 38.2; 10-14, 60.3; 15-19, 80.3; and 20 years and over, 96.4. The degree of reactivity also increased, so that the percentages of stronger reactions (2+ and 3+ together) ranged, respectively, 0.6, 3.0, 7.6, 14.3, and 49.6. These increases with aging are all the more conspicuous because the lepromin used in that study was a relatively weak one.

An analysis of the data of the present study with respect to age, summarized in Table 7, has shown that of the full-dose group of Series 1 the children who were 6-7 years of age were 71.8 per cent positive, against 80.9 per cent of those 8-9 years old. Since this difference, which represents the average "spontaneous" increase in two years, is statistically not significant (and in Series 2 there was no difference whatever) any increase of reactivity in the Series 2 lot due to the added 9 months of age could not have been sufficient to explain the differences in results observed.

As for the disproportion of older children, the 8-9 years lot constituted 44.9 per cent of the full-dose group in Series 1, and 59.0 per cent in Series 2. However, adjusting the percentages of the tables to the age distribution of a standard population composed of 1,042 children in the

⁵Of the 396 total, maximum age 35 months, 23.2 per cent were Mitsuda positive, but only 2.3 per cent reacted to 5 TU of tuberculin. Exposure to leprosy can also be ruled out as a factor in the production of the lepromin positivity in those children.

two series divided into age groups of 6-7 years (512) and 8-9 years (530), resulted in percentage changes which were of no significance. It would seem that there must be some other explanation than the age factor for the observed difference between the two lots of full-dose children.

Although there was little or no difference related to age in the full-dose groups, there is interest in the differences of the results in the two

TABLE 7.—*Influence of age on Mitsuda positivity in Series 1 and 2, serial lepromin dilutions.^a*

Lepromin concentration	Ages 6-7 years		Ages 8-9 years	
	No. tested	Mitsuda positive per cent	No. tested	Mitsuda positive per cent
(a) <i>First series</i> (tested July 1954)				
1/1	71	71.8	47	80.9
1/10	57	10.5	56	21.4
1/20	65	4.6	54	18.7
(b) <i>Second series</i> (tested March 1955)				
1/1	34	91.2	58	91.4
1/10	37	27.0	55	40.0
1/20	38	15.8	53	35.8
1/40	41	4.9	55	18.2
1/80	40	5.0	51	5.9

^a Ages of both series as of June 1954 according to the school records. Those of the second series children are therefore increased by the approximately nine months interval between the testing of the two series.

age groups with the 1/10 and 1/20 dilutions. In all instances the 8-9 year subgroups gave higher reaction rates than the 6-7 year children, more conspicuously so with the 1/20 dilution.

Also noteworthy is the fact that in both age groups the Series 2 subgroups showed more reactivity than the corresponding Series 1 subgroups. It cannot be said that the differences with the 1/1 dose are at all significant, or that those with the dilutions are of high significance. For example, a difference as great as or greater than that between 21.4 and 40.0 of the 8-9 years subgroups with the 1/10 dilution would be expected in random sampling about one in 27 times; and the difference between 18.4 and 35.8 with the 1/20 dilution about once in 20 times. Nevertheless, the differences are consistently in the same direction. The sugges-

tion is strong that some conditioning factor had been operative to make the barrio children more responsive to small doses than the poblacion children.

DISCUSSION

In view of the youth of the subjects of this study, the high frequencies of late reactions with the full doses of lepromin are striking. There is no reason whatever to ascribe the results to contact with leprosy, and the results of the tuberculin testing done confirmed previous experience⁽¹³⁾ that there is not a high rate of latent tuberculosis in Mactan children.

Nor can these results be ascribed to any peculiarity of the lepromin. The two lots used in Series 1 were of equal Mitsuda antigenicity, despite the differences of manufacture. They did differ, however, in their ability to elicit the early or hypersensitivity reaction, which suggests that autoclaving (W lepromin) may perhaps be more effective than boiling (M lepromin) in making available the elements upon which that kind of reaction depends.

That Dharmendra's antigen should be so extremely ineffective was quite unexpected. It is supposed to give more early reactions than ordinary lepromin, but among our young subjects—for some reason which escapes us—it caused even fewer than the 1/20 lepromin dilution. As for the late reactions it is equally remarkable, despite the fact that this antigen admittedly⁽⁶⁾ gives fewer and weaker late reactions than regular lepromin in leprosy patients, that even on the 3 mm. basis of positivity only 9 per cent of the reactions could have been recorded as positive.⁶ The reason may be that in the chloroform-ether serial treatment of the bacilli in the preparation of this antigen there is extracted so much of the bacillary lipid content—including the acid-fast waxy element, which plays an important role in the antigenicity of mycobacteria, at least with the tubercle bacillus (4, 19, 20, 27). The findings would seem to favor the view of the nature of the Mitsuda reaction which has been expressed. It would seem that the extraction has a fair higher effect in tests with immunologically immature subjects than with adults.

Regarding the primary question of this study, i.e., whether or not dilutions sufficiently high to be materially economical of leproma material are suitable for routine field testing—the findings show clearly that they are not. The obvious reason is that the subjects tested were very different from any used before, in youth and immaturity for one thing, and for another thing in lack of previous lepromin testing, or BCG vaccination, or overt leprosy infection.

Furthermore, no individual had more than one test injection. However, our data offer no direct evidence regarding the possibility that a full ("control") dose in the same individual might heighten the response

⁶So far as we are aware nobody, not even Dharmendra himself (personal communication), has made definitive tests with his antigen in young, healthy children.

to a fractional dose, for it was not practicable to set up for each dilution a control group receiving both doses.

Whatever may have been the factors responsible, our results differ widely from those of the previous investigators who worked with subjects, patients or preventorium children, who previously had been subjected to various interventions affecting their immunologic status. Only one known report has data of similar significance, namely, that of Basombrio and associates who found that a Floch preparation might perhaps be useful with actual patients, but was entirely unsuitable for testing contacts.

It may be that a 1/50 or 1/60 dilution of a good leproma pool could be used regularly (Floch, Chaussinand), but that it would be unsafe to use a much greater dilution for practical field work is indicated by the sharp drops in positive results (from 75 to 16% in Series 1, and from 91 to 35% in Series 2) obtained with our "1/10" dilution. In this our findings are in disagreement with those of Floch and Chaussinand in patients, and with the latter's opinion that a 1/200 dilution could be used for "epidemiologic investigations" with due regard for weaker reactions. Even for testing patients, one would not deliberately choose a preparation known to give a material proportion of false negatives, necessitating full-dose retests of all nonreactors and preventing comparisons of the degrees of reactivity of those responding in the two tests.

Other considerations are secondary in the present connection, however intriguing. Regarding correlation of the two reactions, in the full-dose groups there was the usual close correlation in one direction only, i.e., of the late reaction with the early one, 90 per cent of the Fernandez positives being also Mitsuda positive. As regards the reverse relationship, no less than 78 per cent of the Fernandez negatives gave late reactions. Here is further evidence, if such evidence were still needed, against the view that the Mitsuda reactivity depends on pre-existing hypersensitivity, or "sensitization"—that, in effect, the late reaction is merely an extension of the early one due to slow break-down of the bacilli (⁵). On the other hand, a few cases showed that Mitsuda positivity is not obligatory in the presence of hypersensitivity, emphasizing the fact that the two reactions are different phenomena.

Several points of interest are seen in the results with fractional doses, apart from the well-established lack of parallelism between degrees of dilution and rates of the late reaction. For one thing, the early-reaction rates showed a similar lack of parallelism. In view of the general understanding of the nature of the reaction, this is a point for further investigation.

With the dilutions there was increase of the relative frequency of Mitsuda negativity in Fernandez positive cases. Of 11 early reactors to the 1/20 dilution, only 3 also gave the late one, and only 1 of the 7 who gave the early reaction to the smaller doses. Evidently, very small amounts

of the soluble antigen element of the lepromin were capable of setting off the early reaction in some of the hypersensitive individuals, whereas the correspondingly reduced numbers of bacilli present were usually incapable of inducing the late reaction.

There was seen with the dilutions an age difference that did not appear in the full-dose groups. In the latter the 6-7 year and 8-9 year subjects gave about the same Mitsuda rates, but in the dilutions groups the older subjects in both series showed the higher positivity rates. Thus the two years of aging has an effect that was demonstrated only with weak doses of lepromin.

The higher dilutions brought out an even closer correlation with tuberculin positivity in the few individuals who gave positive Fernandez reactions with them than did the full-dose tests. In a population of children who were about 23 per cent tuberculin positive as tested, the 41 individuals who were Fernandez positive with the 1/1 or 1/10 concentrations were 64 per cent tuberculin positive, while the 20 who gave early reactions to the higher dilutions were 90 per cent positive. With very small doses of lepromin, tuberculin hypersensitivity seems to have an especially marked influence in determining Fernandez positivity, perhaps a causative one.

Finally, the dilutions brought out increasingly greater differences of Mitsuda reactivity between the two series, as has been said. Thus the barrio children not only gave higher Mitsuda rates with the full doses, but taken as a whole they were materially stronger reactors as measured by the ability of larger proportions of them to respond positively to the smaller doses—a fact not evidenced by the strength-of-reactions readings in the full-dose groups.

There remains an intriguing question: Why were the children of Series 2 more reactive than those of Series 1? The difference of Mitsuda reactivity to the full dose is statistically significant, and as noted the differences in the dilutions groups were more marked. Although nothing definite can be said of a comparison of the early reaction rates in the two series, because the reactors were so few, note is nevertheless made of the fact that the relatively high rate with the full dose in Series 2 seems not to have held up proportionately with smaller doses. Under the circumstances this observation cannot be regarded as more than suggestive, but it is reminiscent of the difference between the results with high and low doses of tuberculin (e.g., 100 and 10 or 5 TU) which, at least in the southeastern United States, is more prevalent in rural than urban areas⁽¹⁵⁾.

Considering all of the operative factors in connection with the observed series differences, the only remaining one that seem possibly pertinent is that of environment. Series 1 was composed of town children (not actually urban), Series 2 of truly rural children—most of the former accustomed to wearing shoes, the latter predominantly barefooted even when attending school, and in general living nearer to the soil.

It seems possible that the hypothetical factor involved (i.e., the "natural causes" of lepromin reactivity⁽⁸⁾) may be the nature of the one which is responsible for the "naturally-acquired, nonspecific" reactivity to large doses of tuberculin studied especially by Palmer and associates, first in the United States and later internationally under WHO auspices^(15, 27). This nonspecific element is supposed to be influenced by geographic conditions, and to be more prominent in rural than in urban areas. It has been found to be highly prevalent in the Philippines. Beyond this speculative suggestion we cannot go, except to say that the matter should be properly investigated in suitable regions, preferably comparing city (truly urban) and rural children. If there be such a nonspecific factor influencing reactivity to lepromin, the fact should be established.

SUMMARY AND CONCLUSIONS

To ascertain whether or not high dilutions of lepromin would be practical for the field testing of normal populations, two series of tests were made with healthy schoolchildren aged 6-9 years. The first series comprised five groups averaging 116 children each, all of Opon *poblacion* (town), the second one five groups averaging 92 each, all of outlying *barrios* (rural villages). None had been lepromin tested before or BCG vaccinated, and none was given more than one injection of lepromin. In the first series two lepromins made by different methods (M, by Mabalay, and W, by Wade) were compared with each other and with the Dharmendra antigen, and two dilutions (1/10 and 1/20) of the stock W lepromin were also used. The tests in the second series were with five concentrations of the W lepromin: 1/1 (i.e., undiluted), 1/10, 1/20, 1/40 and 1/80.

With respect to the late reaction, the two lepromins in Series 1 gave the same results, 75 per cent positives. The M stock, however, elicited fewer early reactions than the W stock, suggesting that boiling may be less effective than autoclaving in freeing the soluble antigenic elements involved in that reaction. In Series 2 the full-dose W lepromin gave rise to more positive reactions of both kinds than it had in Series 1, and the dilutions gave increasingly large differences with respect to the late reaction. The Dharmendra antigen proved ineffective in eliciting either type of response in these very young and immature subjects, this result with the late reaction probably due to the extraction of the lipids during manufacture.

As for the effectiveness of the dilutions in eliciting the Mitsuda reaction, there was a great decrease of positives to the 1/10 (i.e., 10%) dilution, about 80 per cent in Series 1 and about 60 per cent in Series 2. Beyond that the curve dropped more rapidly. It follows that a dilution as high as 1/10 would be entirely unsuitable for routine testing in field work.

⁷Analogous nonspecific reactions to histoplasmin and coccidioidin have been reported, and also to penicillin in persons never exposed to the drug.

The data on correlations of the early and late reactions to full doses show, as usual, that early reactors usually give the late reactions also (but not obligatorily, because there were 10% exceptions), whereas over 80 per cent of the Fernandez negatives gave the Mitsuda reaction, a high degree of dissociation. As for the results with the dilutions in Series 2, there was a striking parallelism between the two reactions. A suggestion is seen that tuberculin positivity had a progressively large influence on the Fernandez reaction to high dilutions.

Analysis of the age factor has shown no significant difference between the younger (6-7 years) and older (8-9 years) children to the full dose. There was, however, an apparent influence of that factor in the reactions to the higher dilutions.

As for the differences in positivity rates between the two lots of children, the fact that the rural groups gave the larger figures suggests that they may have been influenced by some environmental factor which affected their immunological background, one that is more prevalent in the countryside than in the town, possibly but not necessarily analogous to the one studied in recent years in connection with the so-called nonspecific reactivity to tuberculin.

RESUMEN Y CONCLUSIONES

A fin de averiguar si las diluciones altas de lepromina resultarían o no prácticas para la comprobación en campaña de poblaciones normales, se ejecutaron dos series de pruebas en escolares sanos de 6-9 años de edad. La primera serie comprendía cinco grupos que promediaban 116 niños cada uno, todos de la población de Opon; la segunda, cinco grupos promediando 92 cada uno, todos procedentes de los barrios campestres. Ninguno había sido comprobado con lepromina o vacunado con BCG anteriormente y ninguno recibió más de una inyección de lepromina. En la primera serie, se comprobaron mutuamente y con el antígeno de Dharmendra dos leprominas preparadas con distintos métodos (M, por Mabalay, y W, por Wade), usándose además dos diluciones (1/10 y 1/20) de la lepromina stock W. En la segunda serie, se hicieron las pruebas con cinco concentraciones de la lepromina W: 1/1 (es decir, sin diluir), 1/10, 1/20, 1/40 y 1/80.

Con respecto a la reacción tardía, las dos leprominas dieron los mismos resultados en la Serie 1: 75 por ciento de positivas. Sin embargo, la M stock provocó menos reacciones tempranas que la W stock, señalando así que la ebullición tal vez sea menos eficaz que el tratamiento al autoclave para poner en libertad los elementos antigénicos solubles que intervienen en esa reacción. En la Serie 2, la lepromina W a dosis completa produjo más reacciones positivas de ambos géneros que en la Serie 1, y las diluciones acusaron diferencias cada vez mayores con respecto a la reacción tardía. El antígeno de Dharmendra se mostró ineficaz en lo relativo a provocar una u otra clase de reacción en estos sujetos tan jóvenes e inmaduros, debiéndose probablemente este resultado en la reacción tardía a la extracción de los lípidos durante la fabricación.

En cuanto a la eficacia de las diluciones para provocar la reacción de Mitsuda, hubo una gran disminución de las positivas a la de 1/10 (o sea, 10%), aproximadamente de 80 por ciento en la Serie 1 y de 60 por ciento en la 2. A partir de ahí, la curva descendió más rápidamente. Despréndese de esto que una dilución hasta

de 1/10 resultaría absolutamente inapropiada para la comprobación corriente en campaña.

Los datos sobre correlaciones de las reacciones tempranas y tardías a las dosis completas revelan, como de costumbre, que los reactores tempranos suelen manifestar también las reacciones tardías, (pero no forzosamente, pues hubo 10% de excepciones), en tanto que más de 80 por ciento de los Fernandez negativos mostraron la reacción de Mitsuda (disociación de alto tenor). En lo tocante a los resultados obtenidos con las diluciones en la Serie 2, hubo un notable paralelismo entre las dos reacciones. Nótese una indicación de que la positividad a la tuberculina ejerció un efecto progresivamente grande sobre la reacción de Fernandez a diluciones altas.

El análisis del factor edad no reveló mayor diferencia entre los niños más pequeños (6-7 años) y los mayores (8-9 años) a la dosis completa. Hubo, no obstante, un influjo aparente de dicho factor en las reacciones a las diluciones más altas.

Con respecto a las diferencias en las tasas de positividad entre los dos lotes de niños, el hecho de que los grupos rurales acusaran las cifras más altas sugiere que tal vez fueran afectadas por algún factor del ambiente que modificó su posición inmunológica, siendo el mismo más prevaleciente en el campo que en el centro urbano, y posible pero no forzosamente análogo al estudiado en los últimos años en relación con la llamada reactividad anespecífica a la tuberculina.

ADDENDUM. Since this article was prepared, our attention has been called to a recent report on the effects in leprosy patients of moderate dilutions of lepromin, by Yanagisawa and associates [*La Lepro* **25** (1956) Special Number, pp. 53-58]. In different groups of patients a full dose was injected in one arm and a single dilution (1/2, 1/4 or 1/8 of the original stock) in the other arm. Referring only to the late reactions to the Mitsuda antigen, the numbers of positive reactors who gave lesser reactions (mostly slightly less) to the dilutions than to their own control full doses were: with 1/2, 84 of 191 (43%); with 1/4, 140 of 185 (76%); with 1/8, 116 of 144 (81%). It was concluded that, to lessen wastage of lepromin and to avoid side effects, the 1/8 dilution of the Mitsuda antigen could be used but that new criteria for reading of the reactions would have to be established experimentally. According to Yanagisawa (personal communication), it would be impossible in Japan to extend such studies to either schoolchildren or adults in the normal population.

Overlooked is the fact that Azulay and Convit [*THE JOURNAL* **15** (1947) 264-266] used simultaneously both integral lepromin and a 1/10 dilution of the same stock in testing 73 syphilis patients in Cleveland, Ohio. The full dose (1/1) elicited 74 per cent positive reactions, the 1/10 dilution 33 per cent. The authors thought that "A direct relationship between the proportion showing a positive reaction and the concentration of the bacilli in the antigen was thus verified."

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REFERENCES

1. BASOMBRIO, G., JONQUIERES, E. D. L. and SANCHEZ CABALLERO, H. J. Test de Mitsuda con leprominos de Floch. *Leprológia* (Buenos Aires) **1** (1956) 151-155.
2. CHAUSSINAND, R. Standardisation de la lépromine. *Internat. J. Leprosy* **24** (1956) 284-291.
3. CHAUSSINAND, R. A propos de l'utilisation d'antigène dilué pour l'épreuve à la lépromine. *Bol. Serv. Saúde Públ. (Lisboa)* **3** (1956) 179.
4. CHOUCROUN, N. The role of the lipo-carbohydrate of the tubercle bacillus in hypersensitivity, acid-fastness, and acquired resistance. *Tuberculology (Denver)* **11** (1949) 25-30.
5. DHARMENDRA and LOWE, J. The immunological skin tests in leprosy. Part II. The isolated protein antigen in relation to the classical Mitsuda reaction and the early reaction to lepromin. *Indian J. Med. Res.* **30** (1942) 9-15.
6. DHARMENDRA, MUKERJEE, N. and KHOSHOO, P. M. A comparative study of three antigens for the lepromin test. *Internat. J. Leprosy* **22** (1954) 311-321.
7. DINIZ, O. and NETO, H. A. Results of the use of the dilute Mitsuda antigen. *Internat. J. Leprosy* **22** (1954) 144-146.
8. DOULL, J. A., GUINTO, R. S. and MABALAY, M. C. Effect of BCG vaccination, lepromin testing, and natural causes in inducing reactivity to lepromin and to tuberculin. *Internat. J. Leprosy* **25** (1957) 13-37.
9. FLOCH, H. Sur la vaccination par le BCG en prophylaxie antilépreuse. Etude de la para-immunité et de la para-allergie entre lèpre et tuberculose. *Publ. No. 249, Inst. Pasteur de Guyane*, December 1951.
10. FLOCH, H. Discussion sur les résultats obtenus dans la réaction de Mitsuda à l'aide d'antigènes dilués. *Internat. J. Leprosy* **22** (1954) 139-143.
11. FLOCH, H. Enhancement of positivity of the Mitsuda reaction obtained with diluted antigen. *Internat. J. Leprosy* **23** (1955) 295-300.
12. FLOCH, H. Sur la réaction de Mitsuda intradermo-réaction à l'aide d'extrait phénique de peau normal. *Internat. J. Leprosy* **24** (1956) 292-296.
13. GUINTO, R. S., DOULL, J. A. and MABALAY, E. B. Tuberculinization and reactivity to lepromin; association between lepromin and tuberculin reactions in schoolchildren in Cordova and Opon, Cebu, Philippines. *Internat. J. Leprosy* **23** (1955) 32-47.
14. GUINTO, R. S., DOULL, J. A. and MABALAY, E. B. A note on the lepromin reaction in males and females of the general population of Cordova, Mactan Island, Cebu, Philippines. *Internat. J. Leprosy* **23** (1955) 131-134.
15. HAYASHI, F. Mitsuda's skin reaction in leprosy. *Internat. J. Leprosy* **1** (1933) 31-38.
16. [MADRID CONGRESS] Technical resolutions. *Immunology of leprosy. Mem. VI Congr. Internac. Leprol., 1953; Madrid, 1954*, pp. 96-104; *also Internat. J. Leprosy* **21** (1953) 527-535.
17. PALMER, C. E., FEREBEE, S. H. and PETERSEN, O. S. Studies of pulmonary findings and antigen sensitivity among student nurses. VI. Geographic differences in sensitivity to tuberculin as evidence of nonspecific allergy. *Publ. Hlth. Rep.* **65** (1950) 1111-1131.
18. PARDO-CASTELLO, V. and TIAN, F. R. Leprosy; the correlation of its clinical, pathologic, immunologic and bacteriologic aspects. *J. American Med. Assoc.* **121** (1943) 1264-1268.

19. RAFFEL, S. Chemical factors involved in the induction of infectious allergy. *Experientia* **6** (1950) 410-419; *abstract in* *Internat. J. Leprosy* **20** (1952) 170-171.
20. RUSSE, H. P. and BARCLAY, W. R. The effect of isoniazid on lipids of the tubercle bacillus. *American Rev. Tuberc. & Pulmon. Dis.* **72** (1955) 713-717.
21. SCHUJMAN, S. The use of dilute antigens in the lepromin tests. *Internat. J. Leprosy* **23** (1955) 291-294.
22. USTVEDT, H. J. The BCG-test. WHO/Exp. Vac. TBC/6. 1953 (mimeographed).
23. WADE, H. W. Problems of allergy in leprosy. *Festschrift Bernhard Nocht Hamburg: Augustin*, 1937, pp. 652-655.
24. WADE, H. W. The immunology problem. *Internat. J. Leprosy* **6** (1938) 95-101 (editorial).
25. WADE, H. W. The Mitsuda phenomenon. *Internat. J. Leprosy* **9** (1941) 101-104 (editorial).
26. WADE, H. W. Lepromin testing with diluted antigen. *Internat. J. Leprosy* **22** (1954) 218-220 (editorial).
27. WHITE, R. G., COONS, A. H. and CONNOLLY, J. M. Studies on antibody production. IV. The role of a wax fraction of *Mycobacterium tuberculosis* in adjuvant emulsions on the production of antibody to egg albumin. *J. Exper. Med.* **102** (1955) 83-104.
28. [WORLD HEALTH ORGANIZATION] Expert Committee on Leprosy; first report. *World Hlth. Org. Tech. Rep. Ser. No. 71*, 1953, pp. 28.
29. [WHO TUBERCULOSIS RESEARCH OFFICE] Further studies of geographic variation in naturally acquired tuberculin sensitivity. *Bull. World Hlth. Org.* **12** (1955) 63-83.