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THE MULTIPUNCTURE DEPOT LEPROMIN TEST: I. TECHNIQUE AND ADVANTAGES, II. APPLICATION TO THE STUDY OF BCG-INDUCED LEPROMIN REACTIVITY

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INTRODUCTION

In most areas where leprosy is endemic, the intimacy of village life brings children and adolescents into frequent contact with the disease. In the days of hydnocarpus oil, separation of the children or the patients was the only hope of prevention. The discovery by Fernandez (12) that BCG vaccination converted lepromin negatives to positive, and by Lowe ($^{22, 23, 24}$) that the parent sulfone was nontoxic in smaller doses than had previously been tried, suggested methods of protection and treatment that might be introduced on a wide scale by simple means. These developments stimulated renewed efforts to understand and control the disease, all of which, however, emphasized the need of a pure culture of the bacillus.

The lack of a pure culture has meant that lepromin—as its name implies—has had to be prepared from bacillus-rich lesion tissue of lepromatous patients. In some countries lepromin can be produced only in limited quantities, sufficient for little more than routine use in hospitals —with the prospect, as treatment reduces the number of bacteriologically-positive patients, of becoming insufficient even for that. At the same time, the lepromin test has assumed importance in classification and in the recognition of susceptibles among child and adolescent contacts. The possibility of using a diluted antigen has been investigated by several workers, including Floch (¹⁴), Diniz and Neto (⁹), Schujman (²⁵), Guinto and Wade (¹⁷) and others. The first steps in our own attempts to produce a modified lepromin test (^{2,4,5,6}) included investigations with diluted antigens with and without adjuvants. Adjuvants had previously been used by Fernandez and Mercau (¹³) and especially by Floch (¹⁵).

In Uganda, the lepromatous rate is low and decreasing, and a little

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lepromin must therefore go a long way. This was the primary reason for our work, but we were also sensitive to the fact that persons who are the most strongly reactive often develop indolent ulcers at the sites of injection. This does not add to the popularity of the test, especially if used on the general population. The laymind thinks such an occurrence means weakness, revealing a need for treatment, whereas no response implies safety! It was therefore thought that it would be doubly advantageous if a test could be developed which would economize on antigen and be less likely to mislead or offend public opinion. The multipuncture depot lepromin test (at times hereinafter referred to as "the test") which we first tried in 1955, and have now used on an increasing scale during the last three years, serves both purposes.

MULTIPUNCTURE DEPOT LEPROMIN TEST

Multipuncture injections of a saline suspension of lepromin were found to produce responses in subjects known to be lepromin-positive (^{2,5}). In some patients, when intradermal and multipuncture injections were placed close together to see to what extent they correlated, there was local suppression of one test by the other. If, however, one injection was made for example into the scapular area and the other into the arm, the interference was negligible. The addition of 10 per cent glycerine to the lepromin resulted in stronger multipuncture reactions, an advantage when using dilutions of 1/100. With experience, the reactions could be graded to indicate the degree of positivity that would have been given by the classical intradermal method.

Having observed the adjuvant effect of glycerine, reported about the same time by Floch (¹⁵), we then tried multipuncture injections of lepromin made with the depot medium used by James and Pepys in their trials of intradermal depot tuberculin in sarcoidosis (¹⁹).

Three depot media used successfully are:

(1) Light liquid paraffin, B. P.	8 parts
Anhydrous lanolin	1 part
(2) Light liquid paraffin, B. P.	15 parts
Anhydrous lanolin	1 part
(3) Light liquid paraffin, B. P.	15 parts
Wood alcohol	1 part

The depot lepromin was made by grinding 1 gm. of autoclaved lepromatous tissue in 16 ec. of depot medium and 4 cc. of normal saline. The suspension was easier to make than with saline alone. Filtration was found to be unnecessary. A trace of phenol was added before the product was sealed in ampules. The Heaf multipuncture apparatus with six needles, first introduced for tuberculin testing (Fig. 1), was used throughout the work. It did not appear to matter whether the needles were set to penetrate to a depth of 1 mm. or 2 mm. A small quantity of antigen was dropped from a tuberculin syringe with needle onto the skin surface, the needle plate of the multipuncture apparatus (sterilized by flaming) was then pressed evenly and firmly on the area and the plunger was driven home. The needles, pushed beyond the surface of the plate, made a circle of six punctures in the skin.



FIG. 1. Two varieties of the Heaf multipuncture apparatus designed for tuberculin testing, also employed in this study for lepromin testing.

There was an immediate traumatic reaction to the injection which rapidly disappeared, to be followed in those who reacted positively by another response after an interval of days, the period varying in length from one individual to another. Early and late reactions were not conspicuous features, but attention was focused on those which were present at the end of the week. Controlled experiments showed that the depot medium itself had no part in producing the response to the depot lepromin. (If 0.1 cc. of depot medium is injected intradermally, it produces a wheal that may persist for some days; the multipuncture injection does not.)

The Heaf tuberculin-test responses are graded as follows:

- Grade I: Discreet palpable inducation at each puncture.
- Grade II: Fusion of the indurated points to form an edematous ring.
- Grade III: General extension of the induration to form a coin pattern approximately 10 mm. in diameter.
- Grade IV: More extensive induration, with central ulceration.

The reactions of the multipuncture lepromin tests are not as severe. In only a small minority has there been any suggestion of coalescence of the individual indurations. In our original work the reactions were described as:

- Grade I: Four or more palpable discreet papules.
- Grade II: Four or more prominent discreet papules.
- Grade III: Four or more prominent papules with pinpoint ulceration of one or more of them, or all tending to coalesce.

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This method may not appear to be as accurate as measuring an induration, but the margin of error in measuring indurations of less than 5 mm. can be considerable. With experience, estimating the relative size of the papules is not difficult when taken in conjunction with the distance between them (Figs. 2-7). These grades correspond broadly with the international standard used in classifying the Mitsuda reaction. In later work we have found two grades ample for field work, Grades II and III being combined.

The test in lepromatous patients.—Up to this point all tests had been on patients. The results had established the fact that the multipuncture test with depot lepromin was practicable, reliable and economical, and



FIG. 2. A Grade I reaction to the multipuncture depot lepromin test. There are distinct elevations at all 6 of the puncture (deposit) sites, although 2 of them are very small.

FIG. 3. Another Grade I reaction, the individual papillate nodules averaging slightly larger than those in Fig. 1.

FIG. 4. A good Grade II reaction, the papulonodules larger but still distinct except for an apparent trace of fusion between 2 of them.

FIG. 5. A Grade III reaction, with a marked tendency to fusion of the individual elements. (Examining the original photograph with a hand lens, minute pinprick centers are seen over at least 2 of them.)

FIG. 6. Another Grade III reaction, with more fusion of the elements, on 2 of which tiny erosions are evident.

FIG. 7. An ordinary strong Mitsuda reaction, resulting from the usual intradermal injection.

that it caused no inconvenience. It had been suggested by Pepys (private communication) that the lack of response in lepromatous patients might be due to the too-rapid elimination of the antigen from the site of injection. However, the use of the depot medium, which later work showed caused retention of the antigen for a longer time in the tissues, did not provoke a positive response in anyone with this type of leprosy.

ANTIGENS FROM NORMAL, TUBERCULOID AND LEPROMATOUS SKINS

De Faria (¹¹), Floch (¹⁶), Kooij and Gerritsen (^{20,21}), and Davey and Drewett (⁸) have shown that weak responses can be elicited by intradermal injection of saline suspensions of normal skin taken from healthy persons (⁶). It has been argued, however, by Kinnear Brown and associates (³), that the international standard method of calibrating the Mitsuda reaction allows for a nonspecific element, as response of 2 mm. and less are classified as doubtful and those of 3 and 4 mm. as no more than weakly positive. The only question is whether the allowance should be greater. The WHO Expert Committee (²⁷), in its second report (1960), saw no reason to make any adjustment. If, however, a preparation could be made from normal tissue, or any other source, that would produce results really comparable with those of normal lepromin, scarcity of lepromatous tissue would no longer be a problem. We have made several observations in this field.

Twenty-five leprosy patients, strongly positive to depot lepromin or with strong Mitsuda reactions, were tested with a 1/20 depot preparation of bacteriologically-negative tissue from an active tuberculoid lesion. The reactions in 17 were negative; 7 gave Grade I responses, and 1 a Grade II reaction. Nineteen of the 25 patients were also tested with an antigen made from skin of a healthy person. None of them reacted.

Although the effects of BCG vaccination will be discussed later, it may be said here that the 10 patients of this group of 19 who were tuberculin negative were given BCG by intradermal injection, but no changes occurred at the depot sites.

Thirty-seven healthy school children were then tested simultaneously with the three following special antigens made up 1/20 with depot medium: (1) normal skin from a healthy person, (2) active tuberculoid tissue (bacteriologically negative), and (3) bacteriologically-negative tissue from the skin of a resolving lepromatous patient; and they were also tested with (d) normal lepromin. The results were as follows:

Antigen	reacting		
Normal skin	0		
Tuberculoid leprosy tissue	0		
Bacteriologically-negative lepromatous tissue	3 (8%)		
Normal lepromin	28 (76%)		

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The three children who were positive to the bacteriologicallynegative lepromatous tissue antigen were very strongly positive (Grade III) to normal lepromin.

To finish with this group, it may be said that 36 of them were vaccinated with BCG. This caused no change in any of the depots containing the antigens other than lepromin. The 8 lepromin-negatives vaccinated (one of them was absent)showed conversion at the lepromin sites. Furthermore, 22 of the 28 who were positive to the first lepromin test showed an increased response.

Nineteen other children who were lepromin positive were tested with similar antigens made up with isotonic saline. None reacted to normal skin or tuberculoid tissue, while 5 reacted very feebly to the bacteriologically-negative tissue from the lepromatous patient. BCG vaccination did not produce any change in any of the depot sites.

These results show that response to lepromin given by the multipuncture method is independent of any tissue component. The weak responses by some patients to antigen from tuberculoid tissue contrasts with the lack of response by any of the healthy lepromin-positive schoolchildren. As the same preparation was used in all the tests, there may be something in the individual response that is stimulated or exaggerated by the type of disease. The few weak responses to bacteriologically-negative lepromatous tissues were probably due to undetected bacilli present in the antigen. The absence of change in the depot sites after BCG vaccination, other than in those in which normal lepromin had been deposited, supports the belief that the bacilli are the essential element in lepromin.

LEPROMIN CONVERSION AFTER BCG VACCINATION

Two antigenic factors are involved in the usual studies of the effect of BCG vaccination on lepromin reactivity. First is the lepromin used to detect the nonreactors, and then the BCG vaccine used for the purpose of inducing lepromin reactivity.

Wade (²⁶) has suggested that the test dose of lepromin helps to condition the individual to react, it being a "microvaccination" as the BCG skin test has been called (Ustvedt). Ignacio, Palafox and José (¹⁸) induced lepromin reactivity by repeated lepromin injections, results which have since been confirmed by Bechelli (¹). Doull, Guinto and Mabalay (¹⁰) concluded that 11.5 per cent of the lepromin positives after BCG vaccination were due to "natural" (i. e., unidentified) causes, 7.2 per cent to the initial lepromin test dose, and 33.4 per cent to the actual vaccination.

Some of the investigations that are summarized here were prompted by Wade in a private communication. Children in comparable groups were tested with lepromin, tuberculin or both. The lepromin was a 1/20 depot preparation (except where stated otherwise), and was administered by the multipuncture method. Tuberculin PPD was

given as a Heaf test or as a Mantoux test, in the latter case the dose being 5 TU. Children with less than 10 mm. Mantoux or Grade II Heaf were vaccinated with BCG, the assumption being that small and intermediate responses are not generally due to infection with the tubercle bacillus. The expression "tuberculin positive" and "tuberculin negative" are related to these standards. The earlier observation in this field included the following. (4, 5)

Ten uninfected children, in contact with their mothers who were patients, were each tested with depot lepromin in the concentrations of 1/20 and 1/100. One child reacted positively to both tests, whereas the other 9 were negative to both 5 weeks after testing. The 9, being tuberculin-negative, were now vaccinated with BCG. At the same time they were retested with 1/20 depot lepromin. Four weeks later the *second* lepromin test showed a positive reaction in every child. The sites of the original lepromin tests showed no change for another two weeks, but then 7 became positive to both concentrations; in the other 2 children there was a visible alteration at the test sites. Thus, in 7 children the antigen at the sites of the first injections was retained in the skin for 11 weeks, long enough to act as an indicator. It would probably have acted similarly in the other two children had the interval been shorter. The fact that the *first* lepromin tests were the *last* to become positive, suggests that the conversion was not due to their action in producing sensitization, but to the BCG vaccination.

A number of healthy school children, negative to both tuberculin and depot lepromin, were vaccinated with BCG three weeks after they had been tested with lepromin (⁵). Of those who were tested with the 1/20 depot lepromin, 40 children out of 45 converted; 1 did not, 3 were indefinite, and 1 could not be read because he developed extensive scabies. Of those who were tested with 1/100 depot lepromin, 15 out of 16 converted; 1 did not. Of 11 other children who were tested with both lepromin dilutions at the same time and returned for examination, 9 showed conversion at both test sites, while 1 did not convert.

Including the 9 children referred to earlier, where the interval was 5 weeks before vaccination, at least 71 out of 83 children were shown by the original depot tests to have converted. The results with the 1/100 antigen were not greatly different from those with the stronger preparation. The conversion rate was similar to that obtained by other workers using intradermal lepromin tests with 1/20 saline antigen before and after the BCG vaccination.

This work was extended on a sufficiently large scale to give dependable results with respect, first, to the influence of tuberculin testing on lepromin reactivity, and, second, the effect of BCG vaccination. The results are summarized in Table 1.

With reference to the first part of the table, the difference between Groups A and B is 3 per cent, less than the standard error (SE) which is 3.83. The difference between Groups B and C is 4 per cent, again less

TABLE 1.—Positivity rates	with	depot	lepromin	with	or	without	tuberculin	testing,
		or B	CG vaccin	ation.				

Group	Number of	Lepromin positive	
	children	Number	Per cent
A. One lepromin test only	472	264	56
B. Heaf tuberculin and depot lepromin			
simultaneously	255	150	59
C. Heaf tuberculin test 5 weeks earlier	124	68	55
Totals	851	482	57
D. Mantoux tested 6 months earlier, with BCG vaccination of the 116 tuberculin negatives	179	154	86
E. Heaf-tested 5 weeks earlier, with BCG vaccination of the 118 tuberculin negatives	148	128	87
Totals	327	282	86

than the S. E. which is 5.43. These differences are therefore not significant.

Referring to the second part of the table, the difference between the percentages of lepromin positives in the vaccinated and the unvaccinated groups are 7 and 10 times the S. E. This difference, therefore, is highly significant.

The children in Groups D and E were retested with tuberculin, but 11 of the 327 did not return for reading. The results, given in Table 2, are grouped to show the number of lepromin positives among the naturally-occurring tuberculin positives, which are the controls; and the number of lepromin positives among those who became tuberculin positive after BCG vaccination.

Of the 82 children in the control group (naturally tuberculin positive), 77 per cent reacted positively to lepromin, whereas of the 231 children who had become tuberculin positive after BCG vaccination, 92 per cent were lepromin positive. The difference of 15 between these

TABLE 2.—Com	parison of	lepromin and	tuberculin	results.
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	Lepromin results							
Tuberculin test used	In natural tuberculin positives		In converted tuberculin positives		In persistent negatives		Total	
	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.		
Mantoux	40	13	105	9	0	2	169	
Heaf	23	6	107	10	0	1	147	
Total	63	19	212	19	0	3	316	
		32	2:	31	100	3		

percentages is three times the S. E., which is significant. Only 3 subjects were persistently tuberculin negative (i. e., did not respond to the BCG vaccination), and the failure of them to react to lepromin is noteworthy.

Among 615 other comparable children there were 400 natural tuberculin positives, of whom 289 were lepromin positive (i. e., 72 %). If the two series are taken together, 352 children out of 482 natural tuberculin positives were lepromin positive (i. e., 73 %), while 212 out of 231 who became tuberculin positive after BCG vaccination were lepromin positive (i. e., 92 %). The difference of 19 between the percentages is seven times the S. E. of the 2.7. If the tuberculin positives are separated according to the tuberculin test used, there is little difference.

BCG vaccination and lepromin reactivity.—The normal lepromin reactor rates increased from 29 per cent at ages 5-6 to 78 per cent at ages 15-16. After BCG vaccination the rate rose to 86 per cent. The highest natural lepromin reactor rate encountered was 60 per cent, the positives including 36 per cent with a Grade I response and 24 per cent who were Grade II or stronger. After BCG vaccination, a comparable group of children—i.e., with the same age composition—had 90 per cent lepromin positives, of whom 32 per cent were Grade I and 58 per cent Grade II or stronger. The vaccination increased significantly both the total reactor rate and the strengths of the individual responses. This is in line with the observations of Convit (7).

Use of a diluted antigen.—A group of 615 children, 65 per cent of whom reacted positively to tuberculin, had a lepromin reactor rate of 59 per cent. The 274 children in a comparable group with a tuberculin rate of 66 per cent gave a lepromin rate of 45 per cent to a 1:100 depot lepromin. The stronger antigen was more efficient in provoking a reaction in those with the capacity to respond in any degree. The diluted antigen distinguished between those who would react satisfactorily to normal lepromin and those whose response would be negative or inadequate.

DISCUSSION

We have described a modification of the usual lepromin test. The results at three weeks have correlated well with those of the regular Mitsuda test. The antigen is easier to prepare than the saline suspension and it spreads more easily on the skin. The test is simple to apply, and it is highly economical of the antigenic material. Using a tuberculin syringe with a platinum-iridium needle (size S. W. G. 17) as a dropper, so that the smallest drop—but not the needle—touched the skin, we found it possible to get 25 multipuncture tests from 0.1 cc. of a 1/100 preparation. One gram of autoclaved tissue will therefore provide sufficient depot lepromin of this strength for more than 25,000 multipuncture tests, or 5,000 in a strength of 1/20 25,000. The classical method will provide sufficient only for 1,000 in the weaker concentration, and 200 in the stronger. The economy is still more pronounced when the depot preparation is used as an indicator to register the effect of BCG vaccination, because the number of necessary tests is then halved. Ulceration does not occur, and the test occasions no inconvenience whatever. It is therefore less likely to offend or mislead public opinion. With practice it is possible to grade responses to compare with those based on the international standard.

The oily medium retards absorption, creating a depot which lasts long enough to indicate lepromin conversion after BCG vaccination. Prolonged retention of the antigen in the tissue did not itself induce a response, but depot injections given five weeks before BCG vaccination eventually indicated lepromin conversion; although, significantly, later than the depot injection made at the time of vaccination. It follows, therefore, that the BCG vaccination alone was responsible for the change.

Although weak reactions to the intradermal injections of normal tissue have been obtained, the multipuncture test using a depot preparation of normal skin did not provoke responses in either patients or healthy subjects. The test may therefore be considered more delicate and specific than the Mitsuda test, since the response must be due solely to the bacillary content of the antigen. The feeble reactions by a few lepromin-positive patients to the depot antigen from tuberculoid tissue contrasts with lack of response by any lepromin-positive healthy subject. This may have been a coincidence; on the other hand, it could imply a component in the host's reaction that is influenced by the type of the disease. As no change was produced by BCG vaccination in any depot site other than those containing the bacillary antigen, it follows again that the bacillus content is the essential element in lepromin, and that preparations without bacilli cannot be substituted.

Tuberculin testing did not influence the response to lepromin, whether done at the same time or earlier. The change after BCG vaccination was beyond any dispute, and was prompt. Simultaneous testing of the general population is therefore practicable.

The lepromin reactor rate among the artificially-stimulated tuberculin positives was significantly greater than that among the naturallyoccurring tuberculin positives. The direction of this variation supports the use of BCG vaccination as a practical measure to produce lepromin conversion.

It probably means also that in this series the standard of tuberculin positivity was fixed too low, although is was higher than that used elsewhere to determine the level at which BCG vaccination should be introduced to stimulate tuberculin sensitivity. Thus Mantoux infiltrations greater than 10 mm., and Heaf reactions stronger than Grade I, include a number in which the sensitivity is due to agents other than the

tubercele bacillus. The corollary follows, reached by an approach quite different from that used by tuberculosis workers, that the genuinely tuberculin-sensitive person may be detected only by reference to standards based on local investigations. These observations are supported by the fact that the tuberculin reactors exceeded those who were positive to lepromin, the reverse of what was expected.

Apparent sensitivity to tuberculin or lepromin may therefore include elements due respectively to infection with tubercle bacilli, lepra bacilli, and unidentified but antigenically related bacilli. Any combination may be present. The tubercle bacillus gives the most powerful but possibly the less frequent stimulus. The increase in the lepromin reactor rate and in the strengths of the reactions after BCG vaccination suggests that those who had benefited had had no previous contact with the tubercle bacilli, or that whatever contact there had been had for some reason been inadequate. The increase in the lepromin reactor rate from 29 per cent at ages 5-6 to 86 per cent within one month after BCG vaccination—that is, to a level not achieved naturally by age 16, which was 78 per cent—justifies the use of BCG to promote lepromin positivity.

Doubt has been thrown on the part played by BCG in producing lepromin conversion because lepromin injected intradermally in larger doses than were used in this work can itself sensitize, although to a less extent. Our investigations were so planned, however, that the test injections could not interfere. The results confirmed the conclusions formed when using a single depot test to indicate the effect of BCG vaccination.

In the anxiety to establish the respective roles played by the injection of lepromin and BCG vaccination, the importance of the sequence lepromin test—vaccination—lepromin test is sometimes overlooked. The synergic or adjuvant actions of the lepromin and the BCG may perhaps produce more lepromin conversions than either could alone. The subject needs to be explored more fully, because a combined technique may provide the answer to the problem of persons who at present remain persistently lepromin negative. The value of BCG vaccination is not lessened because lepromin itself helps to sensitize. The raising of the threshold at which vaccination is employed may also cause an increase in the number of lepromin conversions.

SUMMARY

1. The technique and advantages of the multipuncture depot lepromin test are described. The reaction produced is due to the bacillary content of the antigen; the presence of tissue elements does not affect the result.

2. A dilution of 1/100 provokes fewer positive reactions, but is adequate to separate those who would react satisfactorily to normal antigen and those whose response would be negative or marginal.

3. A single test may be used to indicate the effect of BCG vaccination.

4. The proportion of lepromin positives among the artificiallystimulated tuberculin positives was greater than among the naturallyoccurring tuberculin positives. The reasons for this are discussed. The test dose of lepromin did not have any part in the production of lepromin sensitivity.

5. In the mechanism of the lepromin test—BCG vaccination lepromin test sequence, the lepromin injected may help to create the reactivity that develops. This should not obscure the importance either of the BCG vaccination or of the whole sequence.

RESUMEN

1. Se describen la técnica y las ventajas de la prueba de la lepromina con depósito por multipunción. La reaccion producida-se debe al contenido bacilar del antígeno sin que la presencia de elementos histológicos afecte el resultado.

2. Una dilución de 1/100 provoca menos reacciones positivas, pero resulta adecuada para separar a los que reaccionarían satisfactoriamente al antígeno normal y a aquéllos cuya respuesta sería negativa o limítrofe.

3. Puede usarse una sola prueba para indicar el efecto de la vacunación con BCG.

4. La proporción de positivas a la lepromina entre las positivas a la tuberculina excitadas artificialmente fué mayor que entre las positivas excitadas naturalmente a la tuberculina. Discútense las razones de esto. La dosis de prueba de la lepromina no desempeñó el menor papel en la producción de sensibilidad a la lepromina.

5. En el mecanismo de prueba de la lepromina—vacunación BCG—resultado de la prueba de la lepromina, la lepromina inyectada puede ayudar a crear la reactividad que se forma. Esto no debe eclipsar la importancia ya de la vacunatión con BCG o de todo el resultado.

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