REPRINTED ARTICLE.

STUDIES OF THE LEPROMIN TEST* by DHARMENDRA, M.B., B.S., D.B. and JOHN LOWE, M.D., Leprosy Research Department, School of Tropical Medicine, Calcutta.

Introduction.

About three years ago in the Leprosy Department of the School of Tropical Medicine, Calcutta, a study of the Mitsuda test was planned. The results of this study have been published in Leprosy in India in a series of articles under the general title of 'Studies of the Lepromin Test.' The studies started with an investigation of the classical Mitsuda test; during the course of this study, however, a practically new test has been evolved, and it is realised that further work to be done on the subject is really outside the scope of the general title of the present series. It is therefore felt that the present series which has already run into ten articles may be terminated, and the work done so far may be summarised and reviewed in this concluding article of this series. It is true that some of the lines of work that we had suggested in the opening article of this series have not yet been tackled; these lines, however, can be tackled in a better way with the improved test than with the original Mitsuda test.

The lines of work originally suggested.

In the opening article of this series we (Lowe and Dharmendra, 1940) reviewed the literature on the subject, and discussed the lines of further work necessary to fill in the gaps in our knowledge of the subject. The following lines of work were indicated:

1. Standardisation of methods.
2. A study of the nature of the reaction.
3. A study of the significance of positive and negative reactions.

A perusal of the work reported in the various articles of the present series will show that considerable advance has been made

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along some of the lines indicated, while little has been achieved along the other lines. We shall here review the work done so far.

**Standardisation of methods.**

Widely divergent results of the lepromin test have been reported by different workers in different countries; it is felt that these differences might have been caused partly by the lack of a standard lepromin and of a uniform method of reading results. For an accurate study of this reaction it was therefore desirable that (a) a method of preparing standard lepromin be worked out, and (b) a uniform method of reading results be adopted.

(a) **Preparation of standard lepromin:** At the time of planning this study, no method was available for accurately standardising lepromin. The only precaution taken to ensure some sort of uniformity consisted in keeping a constant proportion between the weight of the lepromatous material used and the saline used to suspend it. Muir (1933) in addition attempted a rough standardisation by making a comparison of bacillary concentration in freshly made material and old material that had given satisfactory results; no actual bacterial count was possible.

During the past two years in the articles of the present series we have suggested three methods for standardisation of the preparations used for the lepromin test. Till a method was available for isolating leprosy bacilli from the nodules, our standard lepromin consisted of a fine suspension of the leprosy bacillus in saline, obtained from the leprous nodules and standardised by making a bacterial count by the Breed's method (Dharmendra, 1941a). Later a method was developed for the complete separation of the bacilli from the leprous nodules (Dharmendra, 1941b), and two standard preparations made from these separated bacilli have been described, first a solution of the protein antigen of the leprosy bacilli standardised by weight of the isolated antigen (Dharmendra, 1941b) and second, a suspension of the partly defatted bacilli standardised by weight of the bacilli (Dharmendra, 1942). The preparations made from the separated bacilli are undoubtedly better than the fine suspension of the leprosy bacilli obtained from the leprous nodules.

Of the two preparations made from the separated bacilli, the solution of the protein antigen has certain advantages over the suspension of the defatted bacilli; the isolated protein is a pure and refined antigen, and with it the late nodular reactions are altogether eliminated, since it produces an early reaction only.

The suspension of the partly defatted bacilli has the advantage over the isolated protein in that it is simpler in preparation.
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It produces a well-marked early reaction and only a slight late reaction; thus by its use the extra labour and special technique involved in isolating the protein is eliminated while many of the advantages of the isolated protein are retained. The suspension of the partly defatted bacilli, standardised by weight, is therefore recommended for routine use. Its preparation has already been described (Dharmendra, 1942). The method consists essentially in obtaining, by the chloroform method, leprosy bacilli free from other elements of the leprous nodules, partly defatting the bacilli by further treatment with chloroform, drying the defatted bacilli, and suspending the dried bacilli in 0.5% carbol-saline, using one milligram of the bacilli to 10 c.c. of the saline. 0.1 c.c. of this suspension is used in the test.

Fernandez and Castro (1941) have recently described a similar method of preparing standard lepromin from bacterial powder. They separate the bacilli from the other elements of the leprous nodules by centrifuging the suspension of the nodules in distilled water at different densities. The yield of the bacilli by this method is, however, much less than by the chloroform method; and weight for weight the chloroform-treated bacilli are more potent than those obtained by the Fernandez method.

(b) Reading of results: Above we have described three standard preparations, and have recommended one of them (a suspension of the partly defatted bacilli) for routine use. We shall first describe the criterion of a positive result with this preparation, and then with the other two preparations.

The suspension of the partly defatted bacilli is capable of producing both the early and the late reactions. The positive early reaction is characterised by the appearance, 24-48 hours after the injection, of a definite area of erythema accompanied by an appreciable degree of oedema and thickening of the erythematous area. The thickened erythematous area varies from 10 to 30 mm. in diameter, occasionally more, the average being 15 mm. The late reaction is seen about two weeks after the injections, that is, slightly earlier than the reaction produced by the injection of ordinary lepromin. The late reaction is considerably less marked than that produced by ordinary lepromin; usually it consists of a small nodule from 2 to 4 mm. in diameter; occasionally the nodule is bigger. As a rule, nodule formation is not accompanied by ulceration; rarely however, there may be some ulceration.

The protein antigen isolated from the leprosy bacilli is capable of producing only an early reaction. This early reaction is similar to the one produced by a suspension of defatted bacilli.

The fine suspension of leprous nodules (ordinary lepromin)
capable of producing both the early and the late reaction; the early reaction is less marked and the late reaction is more marked than the similar reactions produced by a suspension of the defatted bacilli. After a study of the late reactions produced by ordinary lepromin in about 500 cases of leprosy of both the types, we (Dharmendra and Lowe, 1942) defined a positive result as follows:

'A progressive infiltration leading to definite nodulation from the second or third week onwards, persisting till at least the fifth or sixth week, often much longer, the nodule in most cases measuring 5 mm. or more in diameter at the end of the fourth week, but occasionally being smaller. Thus the characteristic feature of a positive result is the nature of the reaction (nodular, progressive and persistent) and not its size. The size of the nodule may, however, be used to grade the degree of positive reactions.'

*A study of the nature of the reaction.*

A positive Mitsuda reaction is strongly suggestive of an allergic phenomenon; there are, however, certain features of the test which make the acceptance of this view difficult. Our study of this matter has yielded useful information. Our studies planned to elucidate the nature of the Mitsuda reaction have taken mainly two lines: firstly, the isolation of all the active fractions of the leprosy bacilli, and secondly, the testing of non-contacts. The information obtained by these two methods is summarised below.

*The isolation of active fractions of the leprosy bacilli.* A method was evolved for the separation of bacilli from the other elements of the lepromatous nodules. The separated bacilli are thoroughly ground and the various chemical fractions are isolated from these ground bacilli. A study of the antigenic action of these various preparations has brought out the following points:

1. In the lepromatous nodule, only the bacillary matter is antigenic, producing both the early reaction (24-48 hours) and the late reaction of the Mitsuda type.
2. Of all the chemical fractions (protein, polysaccharides, glycerides, phosphatides and waxes) isolated from the bacilli, only the protein is antigenic.
3. This active fraction (the protein) produces only an early reaction.
4. None of the isolated fractions produces a late reaction of the Mitsuda type.
5. Since the protein fraction is the only antigenic material in the bacilli, and since late reaction is not produced
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by any of the fractions, the classical Mitsuda test is believed to be caused by the protein fraction, which when isolated produces only an early reaction. According to our view the delayed reaction to ordinary lepromin (Mitsuda test) is caused by the slow breaking down of the bacilli contained in it, with the consequent slow liberation of the antigen over a prolonged period.

(6) By providing an explanation for the lateness of the reaction, the above observations have brought the Mitsuda reaction more into line with the allergic skin reactions.

The test in non-contacts: Positive results in non-contacts with the ordinary lepromin have been reported by other workers, and confirmed by us (Jharhendra and Jaihari, 1941). By extracting the isolated leprosy bacilli with different solutions, three active protein fractions were isolated, and it was hoped that one of these fractions might be specific to the leprosy bacillus and give negative results in persons not exposed to leprosy. When tested in non-contacts, however, none of the fractions produced uniformly negative results (Jharhendra and Jaihari, 1943). Nevertheless, with one of the fractions (nucleo-protein isolated by the phosphat-buffer method) the incidence of positive results in the non-contacts was very low (5%). Moreover, there is some other evidence that the nucleo-protein may be the specific fraction of the leprosy bacillus. The lack of specificity may be caused by changes produced in the protein during its preparation by the present method. It may be possible to demonstrate specificity if the protein is isolated in a more natural form.

Conclusion: It is believed that our studies have thrown considerable light on the mechanism of the lepromin reaction. As a result of these studies, the reaction has been brought more into line with the allergic skin reactions. The three features of the test which stood in the way of its being accepted as a test of allergy were as follows: (1) that, clinically, the classical Mitsuda reaction is unlike any other allergic skin reaction; (2) that positive results are seen in persons not exposed to leprosy; and (3) that the patients suffering from the lepromatous (the more serious) type of the disease show negative results.

Our studies have afforded a satisfactory explanation for one of these features, namely, the lateness and the nodular character of the reaction.

There is some hope that as a result of our work a suitable
preparation of the leprosy bacillus may be made available which will not produce positive results in non-contacts; if so, the second anomalous feature will be explained.

Our work has no direct bearing on, and suggests no explanation of, the third anomaly, namely, the negative results in cases of the lepromatous type; however, work with the improved antigen will facilitate the study of this matter. This anomaly undoubtedly needs to be explained; however, its existence should not be considered irreconcilable with the idea of the lepromin reaction being an allergic phenomenon; negative lepromin reaction in lepromatous cases of leprosy may be similar to the negative tuberculin test in advanced cases of tuberculosis, or else it may be caused by some inherent incapacity of the tissues of the lepromatous cases to react allergically to the presence of leprosy bacillus or its products. We may summarise here the evidence for the allergic nature of the test.

1. In appearance, the local changes in the skin produced by the intradermal injection of the isolated antigen are similar to those seen in other allergic skin reactions, for example, the tuberculin test. It is believed that the same antigen is responsible for the nodular reaction seen in the classical Mitsuda test, the lateness and the nodular character of the reaction in this test being caused by the nature of the material injected.

2. In healthy persons living in areas heavily infected with leprosy the incidence of positive results is much higher, and the degree of the reaction is much greater than in healthy persons living in areas where there is little or no leprosy.

3. The response to the injection of active fractions of Myco. leprae is seen not only at the site of the injection, but not infrequently, in leprous lesions away from the site of injection, and also at the site of the previous injections of lepromin. This means that the response is not only local but not infrequently focal also. This is a strong indication of the reaction being allergic.

4. Definitely tuberculoid and definitely lepromatous cases of leprosy are generally believed to be two immunologically distinct groups. The lepromin test gives almost uniformly positive result in one of these groups, that is, in the tuberculoid cases, and almost uniformly negative result in the other group, that is, in the lepromatous cases.

5. The lack of power to react to the antigen of Myco. leprae found in the tissues of the patients suffering from the lepromatous type is a specific one, since the tissues of such patients retain the power to react to other acid-fast bacilli, to some of their products.
and to some irritating substances that have nothing in common with the acid-fast bacilli such as proteoses, weak acids and alkalis, etc.

A study of the significance of a positive or a negative reaction.

No study has yet been made of the significance of a positive or a negative result in contacts. The study needs to be carefully planned and will involve repeated observations over a long period. A start has been made of the study of the prognostic value of the test in cases of leprosy. All cases attending for treatment at the Leprosy Department of the School of Tropical Medicine, Calcutta, are being tested as a routine, and in time we hope to collect valuable data on the prognostic value of the test. A study of this nature necessarily involves observations over a long period.

At this stage we are not in a position to report a detailed study and to accurately assess the prognostic value of the test in cases of leprosy. However, an analysis of the results of the test in a large number of cases of leprosy, and subsequent developments in some of the cases during the limited period of about three years, have brought out certain points bearing on this subject.

Our findings, in general, support the view generally held that, in cases of leprosy of all types, the lepromin test is of some value in prognosis, a positive result indicating a favourable prognosis.

Observations in the neural cases: There is some evidence to show that lepromin-negative neural cases are more likely to become lepromatous than the lepromin-positive cases. Only one of the large number of neural cases tested by us has so far become lepromatous since testing; this was a simple neural case with a negative lepromin reaction. We have also some evidence to show that the neural cases with marked reaction to lepromin are more likely to subside. Even apart from actual subsidence, a positive test indicates a better prognosis; for example, bacteriologically positive cases are more likely to become bacteriologically negative if the result of the lepromin test is positive.

Observations in the lepromatous cases: Some workers have studied the results of the test in the lepromatous cases in relation to the subsidence of the disease and have brought out the following points: (1) of active lepromatous cases, those with a positive lepromin test are more likely to improve. (2) When lepromin-negative cases show subsidence of the disease, the lepromin test sometimes becomes positive with the subsidence. (3) A subsided lepromatous case that is lepromin-positive is less likely to relapse than a similar lepromin negative case.
Our work lends support to the first point: of the active lepromatous cases, those with a positive lepromin test are more likely to improve. Of the 141 lepromatous cases tested by us during the months from June 1940 to March 1941, 12 have since become bacteriologically negative and inactive. Seven of these belong to the large lepromin-negative group of 127 cases, and 5 to the small group of 14 cases with a weak positive lepromin reaction.

Our experience with the test in the subsided lepromatous cases does not support the second point, namely, that with the subsidence of the disease in lepromin-negative cases the lepromin test tends to become positive. In none of the subsided cases has a negative lepromin reaction changed into a positive reaction; in some cases with a weak positive reaction the reaction has even become weaker.

None of our subsided lepromatous cases has so far had a relapse, and thus our present work gives no information on the third point, namely, that a subsided lepromin-positive lepromatous case is less likely to relapse than a similar lepromin-negative case. Our general experience of the test, however, would lead us to expect this.

Observations in cases of doubtful classification: In cases of doubtful classification a strongly positive lepromin reaction practically rules out the possibility of the case being lepromatous.

Conclusion: There is, therefore, a considerable indication that, in cases of leprosy of all types, the lepromin test is of definite value in prognosis.

The possibility of using lepromin injections for immunizing against leprosy.

A study has not yet been made of the possibility of turning a lepromin-negative healthy person into a lepromin-positive, by means of injections of lepromin. If this were possible, the question whether such a change was followed by increased immunity could then be investigated.

We have, however, attempted without success to change a weak positive or negative reaction seen in lepromatous cases and also in a few neural cases into a positive reaction by means of repeated testing (Dharmendra, Lowe and Mulherji, 1944a). Eighty-nine such cases, 27 neural and 62 lepromatous, were tested repeatedly. Monthly tests for a period up to 18 months failed to enhance the reaction to lepromin in the large majority of the cases tested. In only 10 of the 89 was slight increase observed, in 2 of the 62 lepromatous and 8 of the 27 neural cases. Thus the experience of some other workers in this matter has not been confirmed.
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The practical value of the work reported in this series.

Above we have indicated the progress made along the various lines of work suggested by us in the opening article of this series. We may now summarise the practical advantages that have accrued as a result of our work.

1. The test has been simplified both for patients and workers. In the classical Mitsuda reaction the readings have to be made weekly for four weeks or longer; with the defatted bacilli or the isolated protein antigen, the early (24-48 hours) reaction is quite clear-cut and no late readings are needed. The classical Mitsuda reaction usually results in the production of large nodules which may ulcerate; the defatted bacilli produce only a slight late reaction, the nodules usually measuring 2 to 4 mm., rarely ulcerating; the isolated protein antigen produces no late reaction whatsover.

2. A refined antigen which is capable of accurate standardisation has been made available for the test. The antigen used for the Mitsuda reaction was very crude; it consisted essentially of a boiled suspension of leprous nodules from which gross tissue particles had been eliminated. The bacterial powder separated from the leprous nodules by the chloroform method is practically free from any tissue and the suspension prepared from this can be standardised by weight of the bacilli; the protein fraction of the bacilli is a still further refined and a chemically pure product and its solution can be very accurately standardised.

3. The work has thrown a considerable light on the mechanisms of the reaction. By providing a satisfactory explanation for the lateness and the nodular character of the lepromin test (the classical Mitsuda reaction), and by demonstrating that a solution of the antigen responsible for this reaction produces only an early reaction of the tuberculin type, our work has brought the lepromin test more into line with other allergic skin reactions. Moreover, results so far obtained with the different protein fractions of the bacilli are very encouraging, and it is possible that further work on these lines might yield a fraction specific to the leprosy bacilli. In that case a diagnostic allergic skin reaction for leprosy infection would be evolved.

4. It is believed that as a result of our present work, further immunological work in leprosy has been facilitated; a refined and pure antigen which can be accurately standardised has been made available, and the investigations have been rendered easier both for patients and workers.
Some other points regarding the Mitsuda test arising out of the present work.

In addition to the progress made along the various lines of work suggested by us in the opening article of this series, certain other points regarding the Mitsuda test have arisen out of the work.

The results in cases of the different types of leprosy: An analysis of the results of the test with ordinary lepromin in over 600 cases of leprosy of all types (Dharmendra and Lowe, 1942) brought out the following points:

1. Of the lepromatous cases, 90% gave negative results, 10% weak positive, and none strong positive. The positive results were commoner in cases which showed clinical and/or histological abnormality but were not confined to them.

2. Of the 'doubtful' cases, 40% gave positive results, four-fifths of these being weak positive. A correlation of the results with the histological findings in these cases showed that the positive results were seen chiefly in cases which were either definitely tuberculoid or else showed a tuberculoid element in histology. No 'doubtful' cases which on histological examination showed only lepromatous changes gave more than a weak lepromin reaction, and even such reactions were very few.

3. In cases classified as 'neuro-anæsthetic' the incidence and the degree of positive results were high.

4. In the 'neuro-macular' cases there was a high incidence of positive results, the incidence and degree of positive reaction increasing from 'simple' through 'tuberculoid not major' to 'major tuberculoid.' Of 'simple' cases, 20% gave negative results, 34% weak positive, and 44% moderate or strong positive; whereas of the 'major tuberculoid' cases none gave negative results, 16% weak positive, and 84% moderate or strong positive. In cases classified as 'tuberculoid not major,' the figures were intermediate.

Correlation of the results of the lepromin test with bacteriological findings in the neuromacular cases: We (Dharmendra and Lowe, 1942) found that the incidence and degree of positive results
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in all the sub-types ('simple,' 'tuberculoid' and 'major tuberculoid') were higher in the bacteriologically-negative cases than in the bacteriologically-positive ones. These weaker reactions have been shown not to be caused by the presence of the bacilli; both the findings are probably caused by a third factor.

Correlation of the results of the lepromin test with the clinical activity of the neuro-vascular lesion: We (Dharmendra, Lowe and Mukherji, 1942b) have also found that the incidence and degree of positive results are higher in clinically inactive cases than in clinically active cases of the same sub-type. In individual cases, however, the reaction does not become stronger with the subsidence of activity. These findings have been interpreted as indicating that those cases which show the stronger reaction to lepromin are more likely to subside.

Variations in the degree of reaction to lepromin with variations in the time of the year when this test is done: It appears that the degree of reaction to lepromin in many patients showing the neural type of leprosy is influenced, apart from any other factor, by the season of the year. We (Dharmendra, Lowe and Mukherji, 1942b) have reported that the same lot of lepromin used in the same patients showing no change in the clinical condition, gives a stronger reaction in summer and weaker reaction in winter.

The early reaction to lepromin: Till recently the phenomenon of early reaction to lepromin had attracted little attention. Most workers had considered the early reaction, sometimes seen in the first three days after injections of lepromin, to be of little significance. Fernandez (1940), however, made a special study of the early reaction and reported that it was always present in cases giving marked late reaction and that an early reaction had the same significance as the late reaction.

We (Lowe and Dharmendra, 1941) have confirmed the report of Fernandez that, after the intradermal injection of lepromin, an early erythematous reaction of tuberculin type is seen in most cases of leprosy that give the classical late reaction. We compared the early and late reaction in a series of 300 cases with the ordinary lepromin and found a high degree of agreement. We also confirmed the report of Fernandez that the early reaction is caused by a soluble antigen.

Having confirmed the significance of early reaction to lepromin, we extended our work to find out the mechanism of this early reaction; and we believe that our studies have thrown considerable
light on the mechanism of not only the early but also of the classical Mitsuda reaction.

By making a comparative study of early and late reaction to lepromin and to filtrate from that preparation, Fernandez concluded "that early and late reactions are probably brought about by different substances or toxins of the Hansen's bacillus." Our work disproves the presence of two different antigens, one responsible for the early reaction and the other for the late. We have shown that both the early and the late reaction can be explained on the basis of the protein antigen that we have isolated from the Hansen's bacillus. Moreover, our work provides no indication of the existence of a separate antigen producing a late reaction (Dharmendra, 1941b); it is the slow liberation of the antigen from the breaking down of the bacilli contained in the lepromin, that is responsible for the late reaction.

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