VOLE BACILLUS ANTIGEN AS A SUBSTITUTE FOR REFINED LEPROMIN[1]

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Lepromin, crude or refined, is widely employed for an intradermal test by leprologists all over the world for the classification of leprosy. The test determines to some extent the state of resistance of the host and the ultimate prognosis. Mitsuda or Wade lepromin is generally employed to elicit late reaction in most of the countries dealing with leprosy, but the refined Dharmendra lepromin producing the early reaction has found favor with the Indian workers. A large bulk of lepromatous tissue is necessary for the preparation of each of these types of lepromin. With the wide-spread use of chemotherapy, active cases of lepromatous leprosy are becoming rare and it is difficult to procure adequate amounts of lepromatous tissue for the preparation of lepromin. Repeated attempts to find a suitable antigen from cultivable mycobacteria have not succeeded in revealing a proper substitute. This paper is an account of successful preparation from the vole bacillus of an antigen that can be used as refined lepromin.

MATERIALS AND METHODS

One strain of *Mycobacterium tuberculosis* var *muris*, was obtained from the National Collection of Type Culture, Colindale, London, for this investigation. This species of mycobacterium was selected in the course of trying different mycobacteria that can be grown easily in culture.

The organism was grown on Loewenstein-Jensen medium at 37°C for 3 weeks. The material for the preparation of lepromin was scraped out in 0.85 per cent saline from a luxuriant 3-week-old growth of the bacillus and autoclaved at 15 lbs. pressure for 30 minutes at 120°C. After centrifugation at 3,000 r.p.m. for 15 minutes the supernatant was removed. The organisms were washed three times in sterile distilled water. The final residue was dried *in vacuo* to be treated with chloroform and ether as in the Dharmendra (1) method of preparation for refined lepromin. Subsequently the material was treated with acetone for 4 days at 37°C. It was then centrifuged and the residue dried *in vacuo*. The final material was suspended in 0.5 per cent carbol-saline in a concentration of 0.1 mgm. per ml. and labeled "VL" antigen.

In another part of the experiment, attempts were made to grow the vole bacillus in Dubos' medium without bovine albumin at 37°C after the 5th passage. A 12-day-old culture of the bacilli after moderate growth in the medium was inactivated at 56°C for 30 minutes, and the suspension of bacilli was centrifuged at 3,000 r.p.m. for 15 minutes. The supernatant was removed and the deposit washed three times with sterile distilled water. The final residue was extracted with chloroform, which was evaporated to dryness. The dried residue was treated with ether and the suspension was centrifuged in the cold for 15 minutes at 3,000 r.p.m. The deposit was dried *in vacuo* and 0.1 mgm. of the powder was suspended in 1 ml. of 0.5 per cent carbol-saline and labeled "V" antigen.

A portion of the powder described above was further treated with acetone for 4 days at 37°C for further purification. The acetone extract was then centrifuged at 3,000

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r.p.m. for 15 minutes. The supernatant was removed and the deposit dried *in vacuo*; 0.1 mgm. of the powder obtained was suspended in 1 ml. of 0.5 per cent carbol-saline and labeled "VA" antigen.

One or another vole antigen was inoculated intradermally into the arm of leprosy patients in doses of 0.1 ml., and standard Dharmendra lepromin was injected into the other arm in most of the cases. The reactions were read after 24 hours to note the early reaction. The area of induration was measured with calipers. An area of induration exceeding the normal thickness of the skin by 1 mm. or more was considered to be the index of a positive reaction, and the reactions were compared with routine Dharmendra lepromin in 681 of the cases. It was also possible to observe late reaction (Mitsuda) at the site of injection ([1]) after 4 weeks in 351 out of the 1,363 patients tested with these antigens. The reaction was examined daily for 5 to 6 days in 250 subjects.

Following the suggestion of J. H. Hanks, lepromin was prepared also from vole bacilli grown in the same way in Dubos' medium without bovine albumin, after killing of the growth by heat at 120°C for 30 minutes in an autoclave. The rest of the procedure was the same as in the case of the antigen marked "V." This lepromin, called "VH" antigen, was tested in the same way on 26 tuberculoid and 14 lepromatous cases of leprosy. Table 1 shows the methods of preparation of the different antigens.

TABLE 1.—*Method of preparation of different antigens.*

Antigen	Culture medium	Heat treatment used	Chloroform-ether extraction	Acetone extraction
VL	Loewenstein-Jensen	120°C for 15 min.	Yes	Yes
V	Modified Dubos	56°C for 30 min.	Yes	No
VA	Modified Dubos	56°C for 30 min.	Yes	Yes
VH	Modified Dubos	120° for 30 min.	Yes	No

RESULTS

The results of the intradermal tests with these different antigens, read at 24 hours, are summarized in Table 2.

The reading of results after 2 to 7 days revealed that the induration gradually started to disappear in some cases after 4-5 days and persisted in 10 cases up to about 4 weeks, leading to formation of a nodule. Erythema, however, subsided after 48 hours.

The results of testing with Dharmendra antigen and the "V" antigen in opposite arms of the same patient are presented in Table 3. It will be seen that the results are closely similar.

The "lepromin" prepared after autoclaving the vole bacillus ("VH") induced reaction in 6 out of 14 lepromatous cases of leprosy (42.9%) and in 16 out of 26 tuberculoid cases (61.5%).

DISCUSSION

The interest in the lepromin reaction created by Mitsuda in 1916 was further enhanced by the observations of Lowe and Dharmendra ([6,7]) and Rotberg ([9]). It has not yet been possible to elucidate the nature and significance of this reaction because of inability to grow

TABLE 2.—*Results of intradermal tests after 24 hours with different antigens.*

Antigen	Typical tuberculoid			Typical lepromatous		
	Total no. of cases	Positive reaction	Negative reaction	Total no. of cases	Positive reaction	Negative reaction
VL	293	246 (84.0%)	47 (16.0%)	111	35 (32.0%)	76 (68.0%)
Dharmendra	681	580 (85.1%)	101 (14.8%)	266	30 (11.2%)	236 (88.7%)
V	1,013	842 (83.1%)	171 (16.9%)	350	33 (9.4%)	317 (90.6%)
VA	78	57 (73.0%)	21 (27.0%)	18	1 (5.5%)	17 (94.5%)

TABLE 3.—*Comparative results of Dharmendra's antigen and "V" antigen in the same patient.*

Antigens	Typical tuberculoid			Typical lepromatous		
	Total no. of cases	Positive reaction	Negative reaction	Total no. of cases	Positive reaction	Negative reaction
Dharmendra	681	580 (85.1%)	101 (14.8%)	266	30 (11.2%)	236 (88.7%)
"V" antigen	681	572 (83.9%)	109 (16.0%)	266	22 (8.2%)	244 (91.7%)

M. leprae in artificial culture media and find a suitable experimental model in the absence of a susceptible animal. In spite of the efforts of Fernández [3], Dharmendra [1], and others, preparation of this skin testing antigen still has to be made from leprosy bacilli not completely free from human tissues. Tuberculin-like antigens from other cultivable mycobacteria were not found to behave like the crude or refined lepromin used at present. Many healthy persons and tuberculous subjects react to both lepromin and tuberculin, while a florid case of lepromatous leprosy can react strikingly to tuberculin but not to lepromin, and there is no parallelism between tuberculin and lepromin reactivity in cases of tuberculoid leprosy [4].

Because of the lack of a pure lepromin preparation, it has not yet been possible to standardize an antigen that could be used as an important epidemiologic tool in different countries employing uniform technic. The lepromin test has assumed an important role in the study of leprosy cases with respect to classification and prognosis, and in the examination of contacts with respect to probable resistance to infection [10].

The results reported here indicate that antigen prepared by chloroform and ether extraction of the vole bacillus grown in modified Dubos' medium may be able to serve the purpose satisfactorily and replace the lepromin used at present. Antigen "V" was used as a lepromin-testing antigen on a large number of leprosy patients of different types since "VL" and "VA" antigens were weak after acetone extraction and autoclaving seems to have a similar effect on "VH" antigen. About 83 per cent of 1,013 cases of tuberculoid leprosy and only 9.4 per cent of 350 lepromatous cases have reacted to this antigen ("V") on intradermal inoculation, as compared to 85.1 per cent and 11.2 per cent re-

spectively with Dharmendra's refined lepromin. Antigen purified further by acetone extraction ("VA") was found to be rather weak; it induced local reaction in only 73.0 per cent of tuberculoid cases. The antigen ("VH") prepared after killing the organism by heat at 120°C for 30 minutes made the antigen nonspecific; this effect probably was due to denaturation of the protein, as 6 out of 14 lepromatous cases reacted to this preparation.

The antigen ("VL") prepared from vole bacilli grown in Loewenstein-Jensen medium was found not to be of differentiating value, as it caused reaction in 32.0 per cent of 111 lepromatous cases and 84.0 per cent of 293 tuberculoid cases. The fact that the organism was autoclaved and the antigen extracted further with acetone, might explain the difference.

The technic of this test, particularly the standard of reading the reaction, needs some comment. The standard laid down by the Madrid Congress ([8]) as well as by the Expert Committee of WHO ([10]) was not followed by Dharmendra, who graded the results on the size of erythema ([2]). The thickness of normal skin varies a great deal when measured with a caliper; for this investigation, therefore, the essential criterion for a positive reaction was considered to be induration exceeding the normal thickness of the skin by at least 1 mm. The zone of erythema was at least 10 mm. in diameter in all the positive cases. The results could be read more easily after 48 hours when induration in almost all the cases was more pronounced, but in an outpatient department it is more convenient to read the results after 24 hours, when the patients are more likely to return. The late Mitsuda reaction was observed to develop in 10 out of 350 cases. It was not surprising that the late reaction was not seen in many cases, as the antigen was used in a refined state and was immediately available to excite tissue reaction.

If these results with the technic given are confirmed by other workers in different countries, further details on this refined antigen free from human tissue can be worked out and an international standard prepared. The want of such a standard is really great ([5]). The antigen described, which can be prepared in any amount at almost negligible cost, may be a welcome substitute for the crude or refined lepromin used today.

SUMMARY

Vole-antigen prepared by chloroform and ether extraction of vole bacilli grown in Dubos' medium without bovine albumin and killed by heating at 56°C for a half hour, was used successfully as a substitute for lepromin in testing 1,363 cases of leprosy. It was compared with the classical Dharmendra lepromin in 947 cases. The Fernández or early reaction was elicited in 83.1 per cent of tuberculoid cases and 9.4 per cent of lepromatous cases. The antigen prepared by killing the organism by heat at 120°C for a half hour, or when extracted further with acetone, was not found to be satisfactory.

RESUMEN

El antígeno del ratón campestre (vole) preparado por la extracción con cloroformo y éter de bacilos del ratón campestre cultivados en el medio de Dubos sin albúmina bovina y muertos a 56°C durante media hora, fué usado exitosamente como un substituto de la lepromina en el examen de 1,363 casos de lepra. Fué comparado con la lepromina Dharmendra clásica en 947 casos. La reactión temprana, o reacción de Fernández, fué incitada en el 83.1% de los casos tuberculoides y 9.4% de casos lepromatosos. El antígeno preparado matando el organismo a 120°C durante media hora, o extraído mas adelante con acetona, no fué encontrado satisfactorio.

RÉSUMÉ

De l'antigène-campagnol (vole-antigen) préparé par extraction au chloroforme et à l'éther de bacilles du campagnol-développés sur milieu de Dubos sans albumine bovine et tués par chauffage à 56°C pendant une demi-heure a été utilisé avec succès comme substitut de la lépromine pour tester 1363 cas de lèpre. Cet antigène a été comparé avec la lépromine classique de Dharmendra dans 947 cas. Chez 83.1% des cas tuberculoïdes et 9.4% des cas lépromateux l'injection a entraîné une réaction précoce, dite de Fernández, positive. L'antigène n'a pas été considéré comme satisfaisant lorsque les microorganismes avaient été tués par chauffage à 120°C pendant une demi-heure, ou quand ils avaient subi une extraction supplémentaire par l'acétone.

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