THE EFFECT OF PARTIAL PURIFICATION OF LEPROMIN ON THE FERNANDEZ- AND MITSUDA-TYPE RESPONSES IN GUINEA-PIGS[1]

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The mixture of antigens in lepromin preparations makes interpretation of the skin reactions difficult. Our previous studies have shown that both mycobacterial and tissue components are antigenic in guinea-pigs ([6]). It was also shown that autoclaving caused homologous proteins (guinea-pig serum) to become antigenic in the species of origin.

The present study demonstrates that a large proportion of the tissue components in lepromin can be removed by relatively simple fractionations; also that the use of purified suspensions of *Mycobacterium leprae* permits distinction between the types of skin response to the tissue and mycobacterial components of lepromin, at least in sensitized guinea-pigs. The tissue components were found to complicate only the early-phase reactions. The results with these specially-treated bacillary suspensions support the thesis that a positive Mitsuda reaction depends partly upon capacity to destroy the structural integrity of *M. leprae* and thus to release antigens.

MATERIALS AND METHODS

Except for the purification of the bacillus suspension, the materials and procedures used in this experiment have been described in detail ([6]). The previous methods of injection, randomization, and blind-reading provided a satisfactory degree of standardization and reliability.

Eight guinea-pigs were sensitized by monthly intradermal injections of 0.1 cc. of lepromin made by Wade's method ([7]). At the time of the sixth monthly injection, skin reactivities were tested with: (*a*) lepromin (Wade), (*b*) the autoclaved human skin antigen used previously, and (*c*) and (*d*) two purified suspensions of *M. leprae* from autoclaved lepromas.[3] These suspensions were prepared and designated as follows:

Declumped M. leprae.—Five per cent suspensions of autoclaved lepromas were shaken for 3 minutes in the presence of 10 per cent/volume of chloroform to declump the bacilli ([3]). After centrifuging for 2 minutes at 200×G, the supernates were stored while the sediments were treated for 10 minutes at 37°C with the digestion mixtures described below. The two fractions were then combined and centrifuged for 20 minutes at 2000×G to collect the bacilli. The organisms were resuspended in saline containing 0.5 per cent phenol, and their concentration adjusted to agree with the numbers of bacilli in the lepromin used ([3]).

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[1] This work was supported by Grant No. E-1962, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Public Health Service, Bethesda, Maryland.

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Bile-pancreatin M. leprae.—Five per cent suspensions of autoclaved lepromas were incubated at pH 7.8 in the presence of 0.5 per cent bile salts (Bacto ox-gall) and 1 per cent pancreatin (Difco Pangestin 1:75)[4] for 30 minutes at 37°C. Small aliquots of the supernate obtained after centrifuging for 2 minutes at 200×G did not yield precipitable proteins when adjusted to pH 4.6 and boiled for 2 minutes. The sediment was resuspended in the bile-pancreatin preparation and further digested for another 30 minutes. The bacilli were then collected from the pooled digests by centrifugation, and resuspended and standardized in numbers as before.

In both preparations there remained a small amount of insoluble tissue residue, chiefly collagen fibers.

RESULTS

The skin reactions in guinea-pigs attained maximal sizes after the third monthly injection of the Mitsuda-Wade lepromin. The early reaction averaged 7-10 mm. diameter during the first two days, then decreased to a level of about 3 mm. for the next two to three weeks. Since a Fernandez reaction in humans is considered positive if it measures 10 mm., and a Mitsuda reaction usually if it measures 3 mm. (at most 4 mm. ([7])), these degrees of response were similar to those which are arbitrarily classed as positive in man. Bimodal responses, with reduction in size between the Fernandez and Mitsuda phases of the reaction, were not observed in these animals.

Cross reactions of all four antigens were tested at the time of the sixth monthly injection (Fig. 1). The response to the heated skin

[4] This bile-pancreatin solution had been stored for several months in a deep freeze. Subsequent mixtures did not remove the protein as efficiently so that bacilli had to be washed in saline and recentrifuged in order to obtain supernates free of heat-precipitin protein.

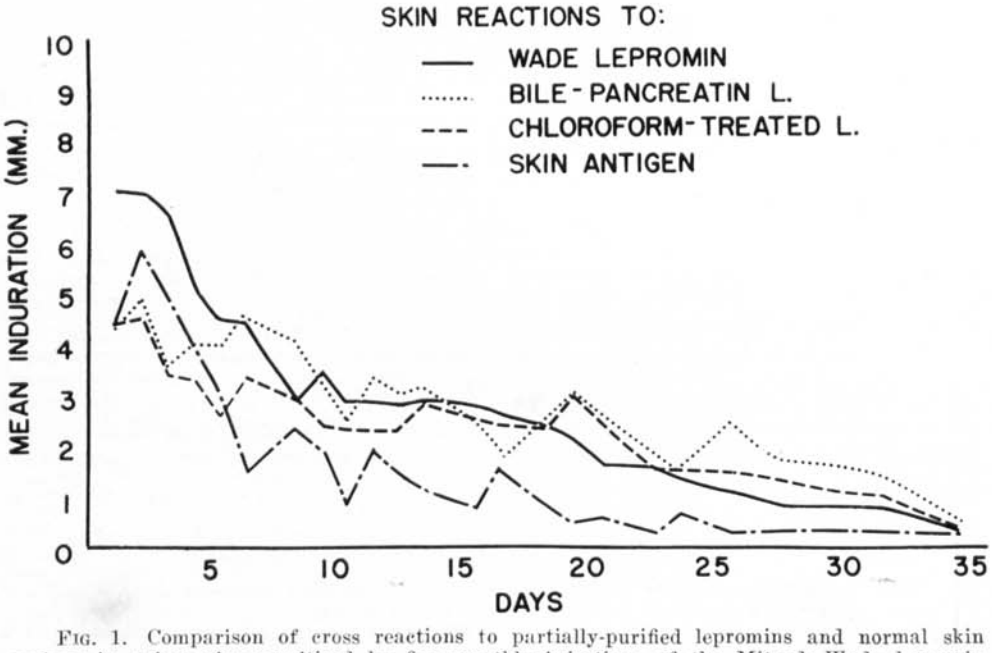

FIG. 1. Comparison of cross reactions to partially-purified lepromins and normal skin antigen in guinea-pigs sensitized by five monthly injections of the Mitsuda-Wade lepromin (average reactions of 8 guinea-pigs).

antigen was greatest on the second and third days, but then fell to about 1 mm. in 10 days, paralleling the early response to the lepromin although at a lower level. This suggests that a portion of the early response to lepromin is to tissue components, and that the balance is to mycobacterial proteins.

During the first few days, responses to the two purified suspensions of *M. leprae* were smaller than those to skin antigen and to lepromin. From the 7th to the 18th days the average size of skin reactions to the bacillus suspensions corresponded to those induced by the lepromin. Thereafter, the average reactions to purified bacilli persisted at slightly higher levels.

DISCUSSION

The results of this study indicate that in guinea-pigs sensitized with lepromin the early phase of skin sensitivity is due both to tissue components and bacillary fractions, while the more persistent (or Mitsuda) phase of reaction is due solely to the mycobacteria. Davey and Drewett ([1]), however, have reported that in patients with tuberculoid leprosy normal skin antigen did not produce Fernandez reactions, but did produce moderate Mitsuda reactions which were equivalent to those produced by a suspension of leprosy bacilli removed from tissues by grinding in chloroform. Even larger reactions were produced by the standard Mitsuda-Wade lepromin.

The chloroform-ether method for preparing Dharmendra's antigen causes marked diminution of the Mitsuda phase of skin reactions ([2, 5]). It must be emphasized, therefore, that the two procedures here employed for purification of the bacillus suspension were designed to maintain the integrity of the bacilli, and that the late-phase skin reactions to these purified preparations persisted somewhat better than those to the lepromin. Treatment with chloroform equivalent to 10 per cent of the volume of lepromin had been shown to declump the bacilli without measurable decrease in their numbers and without appreciable loss of acid-fastness ([3]).

This method was chosen to produce declumped bacilli which could be purified by centrifugal methods. However, it was found that brief treatment of the low-speed sediments with bile and pancreatin permitted inclusion of more bacilli in the final collections. Purification of bacilli by means of bile and pancreatin was an adaptation of unpublished methods (J.H.H.) for producing purified suspensions of active *M. leprae murium* from fresh tissue homogenates after a single high-speed centrifugation. If heat-denatured proteins are not bound in complex structures such as bacterial cells, tryptic degradation proceeds rapidly. This was, therefore, a logical means of obtaining a selective action against antigenic components of tissue origin. The results with this antigen also served as a control on the possibility that declumping in dilute chloro-

form might cause an important loss in capacity to elicit the Mitsuda phase of the lepromin response.

It seems probable that only structurally-bound proteins are retained in bacilli which have been rendered permeable by heat and chloroform ([4]) and then subjected to enzyme digestion and washing. The persistent Mitsuda reactions induced by such a preparation add new support to the prevailing view that Mitsuda positivity indicates a capacity to destroy the structural integrity of *M. leprae*.

SUMMARY AND CONCLUSIONS

Purification of intact *M. leprae* from autoclaved lepromas was achieved by a single high-speed centrifugation after either of two preparatory steps: declumping in 10 per cent chloroform or digestion with bile and pancreatin.

In guinea-pigs sensitized by five monthly injections of Mitsuda-Wade lepromin, human tissue antigens appear to contribute only to the early-phase (2-3 day) cutaneous reactions to lepromin.

The purified bacillus suspensions elicited the persistent (Mitsuda) phase of response in the same manner as did the lepromin used. The antigens which incite the Mitsuda reaction are retained in the whole bacilli even after they are made permeable by heat and chloroform, and in spite of enzyme digestion and washing. The late-phase response requires, therefore, a capacity to localize and destroy bacilli at a significant rate.

RESUMEN Y CONCLUSIONES

Se obtuvo la purificación de los *M. leprae* intactos procedentes de lepromas tratados al autoclave con una sola centrifugación a alta velocidad después de uno de los tiempos preliminares: desaglutinación en cloroformo al 10 por ciento o digestión con bilis o pancreatina.

En cobayos sensibilizados con cinco inyecciones mensuales de lepromina de Mitsuda-Wade, los antígenos de tejido humano no hicieron más que exagerar la fase incipiente (2-3 días) de las cutirreacciones a la lepromina.

Las supensiones bacilares purificadas provocaron la fase persistente (Mitsuda) de reacción del mismo modo que la lepromina usada. Los antígenos que incitan la reacción de Mitsuda se retienen en los bacilos íntegros aun después de permeabilizarlos con el calor y el cloroformo, y a pesar de la digestión con enzima y del lavado. La respuesta de fase tardía requiere, por lo tanto, la capacidad para localizar y destruir los bacilos en una forma apreciable.

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