THE ANTIGENIC COMPONENTS OF LEPROMIN AS ASSAYED IN GUINEA-PIGS[1]

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Field studies of the epidemiology of leprosy rely principally on clinical diagnosis. It is generally agreed that bacteriologic examination of skin smears should be almost routinely part of the clinical examination, particularly since this determines the "administrative classification" of cases into "open" and "closed." Of the other two procedures used in diagnosis and classification of cases, histologic examination of biopsy specimen is of limited practicability. The lepromin skin test, however, is more practicable, and therefore is being increasingly employed. This test is used to help distinguish between, on the one hand, lepromatous and other tissue-nonreactive ("nonresistant") cases, and on the other hand tuberculoid cases and certain others that are tissue-reactive.

The present studies were undertaken for the purpose of adding to understanding of the immunologic basis of the lepromin reaction. Specific objectives were to compare the relative antigenicity of tissue and bacterial components of the Mitsuda-Wade lepromin, and to determine the degree of cross-response incited by immunization of guinea-pigs with heat-killed suspensions of *Mycobacterium leprae* and *M. tuberculosis* (avirulent bovine, of BCG), both in the presence and the absence of tissue components. On the basis of these results, it is possible to define certain of the major shortcomings in present antigen preparations, and to discuss possibilities for developing preparations of *M. leprae* antigens which may prove useful in epidemiologic studies.

Reviews and conference reports have produced general agreement ([25]) that the Fernandez (early, or 24-48 hour) reaction to lepromin indicates existing local allergy and probably is analogous to the reaction to tuberculin. The Mitsuda (late, or 2-3 week) reaction is thought to be indicative of ability to respond to a deposit of bacillary antigen which serves both as a tissue-sensitizing and a reaction-eliciting (test) dose ([21, 22]). Repeated lepromin testing can induce reactivity in primarily nonreactive healthy volunteers, but there is probably considerable variation in this matter ([24]). A similar response occurs in dogs ([23]). Olmos Castro *et al.* ([20]) reported that 6 Fernandez-negative adult volunteers injected with Wade-Mitsuda lepromin showed an

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average reaction of 3.4 mm. at two days with a gradual rise to 7.5 mm. at twenty-one days. A second Mitsuda test after three weeks produced an average reaction of 15.3 mm. at two days with gradual fall to 10.2 mm. at one week, 11.1 mm. at two weeks and 8.6 mm. at three weeks. The average reactions in 11 cases of tuberculoid leprosy were 20 mm. at two days, 12 mm. at one week, 11 mm. at two weeks, 11 mm. at three weeks and 8.5 mm. at four weeks.

In attempts to separate mycobacteria from tissue in lepromin, chloroform extraction and various physical means have been used. In general, if fragmentation or extraction of the bacilli occurs, the Fernandez reaction is accentuated; and if bacillary integrity is preserved, the Mitsuda reaction is dominant ([4, 14]).

The significance of heated normal tissue components in lepromin has received little attention. De Faria ([3]) reported in a monograph on tests with normal skin extracts prepared according to the Mitsuda-Hayashi techniques; patients with tuberculoid leprosy showed 50 per cent positive reactions with nodules having typical tuberculoid structure histologically. Lepromatous cases showed no reaction. Kooij and Gerritsen ([15]) also reported that normal skin treated in the same way as Mitsuda lepromin, but concentrated 10 times (i.e., tissue 50%), produced small Mitsuda-type responses in approximately 50 per cent of tuberculoid cases, and that similar reactions were not seen in lepromatous cases. Tissue suspensions from relatively bacillus-free tuberculoid lesions have been reported to cause early phase reactions ([17]). In nonleprous patients, Olmos Castro and Arcuri ([19]) found that two injections of lepromin failed to sensitize humans against the tissue components of lepromin-like preparations made from normal skin.[2]

That guinea-pigs can be sensitized to lepromin has been shown ([2, 9]) although negative results have also been reported ([1, 10, 26]). Following BCG inoculation of guinea-pigs, however, positive reactions to lepromin have been produced but with some variation in reports as to whether they were early or late ([2, 11, 18, 27]). Fernandez ([8]) used a lepromin-type preparation prepared from tuberculous lymph nodes of guinea-pigs. In both humans and guinea-pigs there were strong early and late skin responses when there had been previous tuberculin sensitivity, but negative early and weak late responses in the absence of such sensitivity. Melsom ([16]) reported that by injecting large numbers of fresh leprosy bacilli, guinea-pigs could be made tuberculin-positive.

MATERIALS AND METHODS

Guinea-pigs.—Adult animals purchased from two New England farms were maintained on a stock diet of rabbit pellets and cabbage. The backs of these animals were shaved with an electric clipper at least once a week. Weights averaged over 600 gm. in the first series of experiments, and about 300 gm. in the second series.

[2]Since this manuscript was prepared it has been learned that T. F. Davey and S. E. Drewett [*Leprosy Review* **29** (1958) 197-203] also observed that tuberculoid patients showed a positive Mitsuda response, but no Fernandez reaction, to autoclaved human tissue.

Antigens.—All antigens received 0.5 per cent phenol during the final dilution, as a preservative.

Mitsuda-Wade lepromin: A single lot prepared by Dr. H. W. Wade at Culion in 1956, using autoclaved lepromatous nodules that were ground, suspended in saline, filtered through fine nylon fabric (bolting cloth, 20XX), and—when compared with stock lepromin regarding bacillus concentration—diluted to about 3 per cent of original leproma ([25]).

BCG vaccine: Phipps Institute, Lot 588. In the first experiment this was used fresh in a dose of 0.1 cc. intradermally. In the second experiment BCG was killed by autoclaving at 10 lbs. for 10 minutes, and 0.1 cc. of a dilution having a bacillus count equivalent to that of the Wade lepromin ([12]) was injected intradermally.

Tuberculin, PPD: Parke-Davis & Co., Lot No. 032169F, 1 test unit/skin site.

Human tissue antigens: Skin or spleen tissues from autopsies of patients dying with coronary heart disease were treated according to the Wade-Mitsuda method of preparing lepromin. The tissue was autoclaved, ground into a fine paste with mortar and pestle, diluted to 3 per cent, and strained through the nylon bolting cloth used by Wade.

BCG plus normal spleen antigen: To approximate the effect of Wade lepromin, an aliquot suspension of spleen antigen prepared as above was mixed with autoclaved BCG in proportions which provide 3 per cent tissue and a bacillus count equivalent to that in Wade's lepromin.

Control serum-protein solution (phenolized serum): Guinea-pig serum was diluted to 3 per cent in 0.5 per cent phenol and autoclaved.

Sensitizations.—At intervals of about one month, 0.1 cc. of each antigen (except viable BCG) was injected in marked areas on the backs of freshly-shaven guinea-pigs. Each inoculation served both as an immunologic stimulus and as a skin-test dose. After sensitization was established, each animal was tested for cross-reactivity by simultaneous injection of all antigens in parallel rows at sites well separated from previous injections. For the injections we used tuberculin syringes, which had passed the WHO-BCG standard leakage tests against 1 kgm. of pressure ([7]), thus ensuring that uniform quantities of material should be injected.

Experiments.—In the first series of experiments, five groups of 6-10 guinea-pigs each were sensitized to the following three antigens: Wade lepromin (2 groups), normal human skin, and living BCG; and a control group was left uninjected until the final cross-testing. In the second experimental series, six groups each composed of 18 guinea-pigs were sensitized to: Wade lepromin, killed BCG, normal human spleen, BCG plus spleen, and phenolized guinea-pig serum; also 1 group was injected with dilute phenol alone.

Randomization was achieved by distributing the guinea-pigs after injection and renumbering for reading purposes. The reader of the reactions never knew which antigen had been used in the animals being read. Further randomization was achieved in the cross-reaction tests by rotating the sites for each antigen at the time of injection.

Reactions were read by the authors, who repeatedly checked against each other to achieve standardization. All reaction sites were read almost every day during the three to six months required for each experimental series. The indurated margins of reactions were palpated by running the finger gently over the skin; pinching or manipulation was avoided since this was found to alter measurements. The average diameter of each reaction was recorded in millimeters. Initial flare reactions which appeared one-half hour after the second and subsequent injections were not recorded.

Daily changes in the average reactions of groups of guinea-pigs sensitized to particular antigens were plotted to produce profiles as shown in the text-figures. Calculations in which the square of the radius was used as an indication of surface area magnified differences somewhat but produced similar curves. Standard errors based on variation in each group were compared in determining the significance of differences observed on the 2nd, 10th, and 20th days.

RESULTS

Patterns of sensitization induced by individual antigens.—Monthly intradermal injections of Wade lepromin induced sensitization in three different experimental groups of guinea-pigs, one of which is shown

TABLE 1.—*Increasing spread of standard deviations of sensitivity reactions to repeated injections of Wade lepromin and human skin antigen (first experimental series with 10 guinea-pigs per group).*

Date of injection	Wade lepromin		Normal human skin antigen	
	Mean of maximum[a] induration in mm.	Standard deviation	Mean of maximum[a] induration in mm.	Standard deviation
July 10	4.3	0.65	---	---
August 7	4.9	0.94	5.7	0.45
August 29	9.5	1.02	8.8	1.5
September 24	11.9	3.05	12.2	1.7
November 23	8	3.9	10.7	4.0
February 21	7.8	2.05	9.3	1.8

[a] Maximum reaction in each animal, whether 24 or 48 hours after injection.

in Text-figure 1-A. In the first experimental series, which was prolonged to include 6 injections, maximal response was elicited by the third monthly injection. The major effect of more prolonged immunization was to increase the standard deviation of responses in the group (Table 1). In the second experimental series, therefore, cross-reactivity was tested at the time of the third monthly injection. It will be noted that when cross tests with all 6 antigens were done simultaneously (Text-fig. 1) the size of the homologous skin reactions was reduced below the previous level when only a single antigen was given.

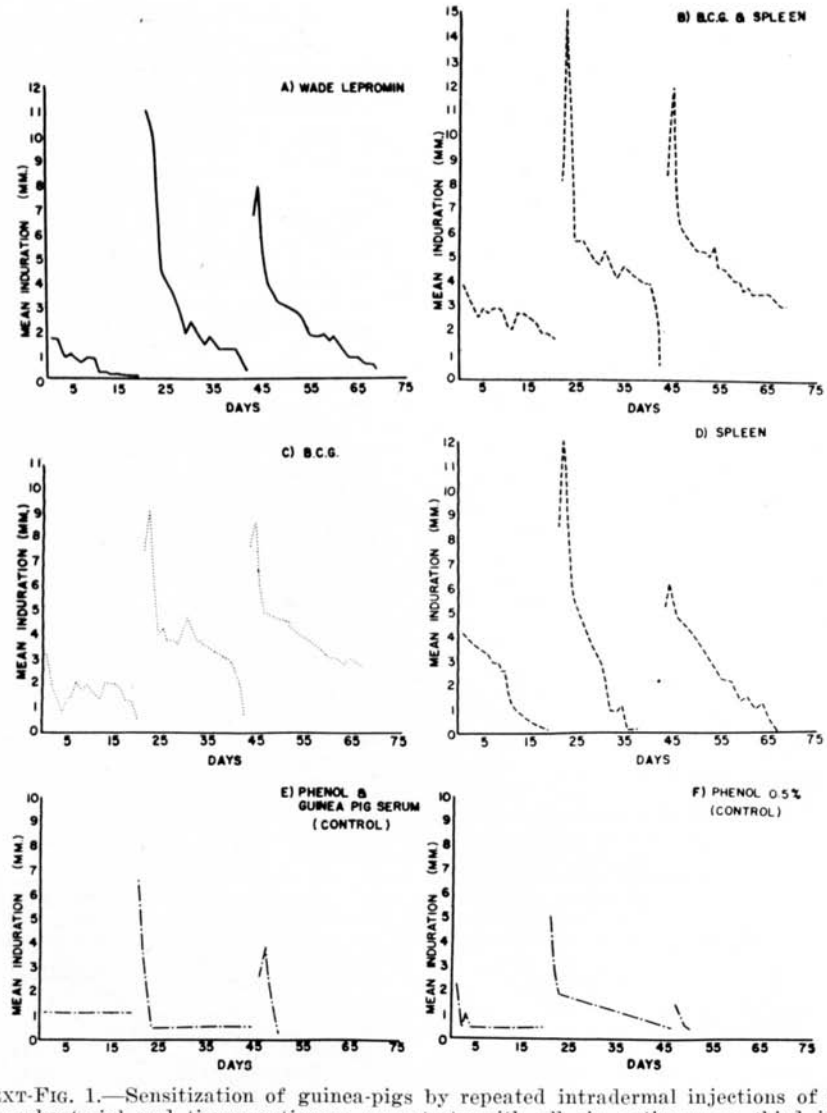
Sensitization of guinea-pigs with autoclaved human tissue antigens followed much the same pattern as with Wade lepromin (Text-fig. 1-D).

Whereas with living BCG vaccine a single injection produced strong sensitivity (Text-fig. 5), it was necessary with heat-killed BCG to repeat the injection to get maximal response (Text-fig. 1-C). Primary injections of heat-killed BCG produced bimodal skin responses whether during sensitization (Text-fig. 1-C) or in final cross testing of guinea-pigs sensitized to other antigens (Text-fig. 4C). As sensitivity increased after the first injection of BCG, skin reactions did not show bimodal curves with a dip on the 5th day.

Reactions to combined BCG plus human spleen were consistently larger than to BCG alone or to the spleen alone (Text-fig. 1-B).

To provide a parallel for the presumption that heated human tissue in lepromin may have antigenic effects in humans, a control group of animals was injected with autoclaved guinea-pig serum. Responses of over 6 mm. occurred, with clearing in four days. When 0.5 per cent phenol alone was injected somewhat smaller reactions occurred, that preparation presumably being more irritating than those in which phenol was combined with proteins.

Comparison of cross reactions to heterologous antigens in guinea-pigs sensitized with one preparation.—Data from cross-reactivity test will be presented in two ways. The first analysis compares cross reactions of all 6 antigens in each group of guinea-pigs sensitized to a particular antigen (Text-figs. 2 and 3). The second analysis compares



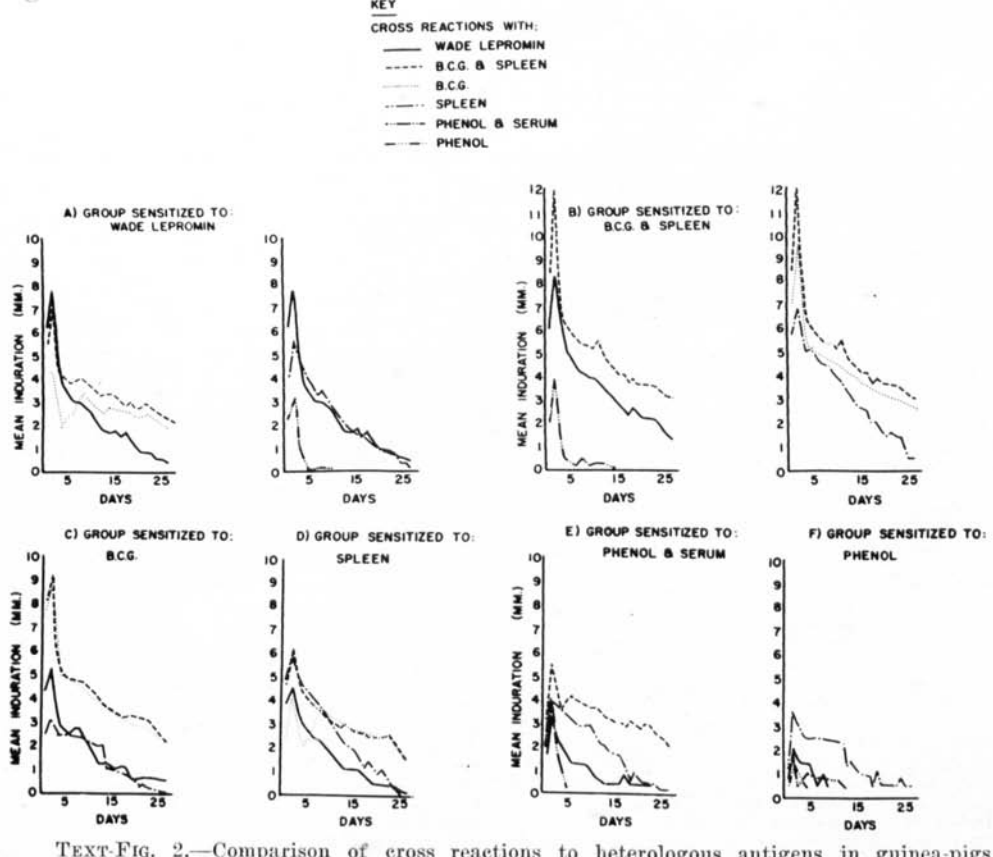
TEXT-FIG. 1.—Sensitization of guinea-pigs by repeated intradermal injections of homologous mycobacterial and tissue antigens; cross-tests with all six antigens on third injection reduced the size of homologous reactions. (Each line shows the average reactions of 18 guinea-pigs in each experimental group.)

the responses elicited by each test antigen in the 6 groups of guinea-pigs sensitized to various antigens (Text-fig. 4). The first analysis shows cross over between test antigens and the second cross over in immunizing capacity.

Essentially similar cross reactions were observed in both first and second experimental series. In the group sensitized to Wade lepromin (Text-fig. 2-A) the early-phase reaction to BCG plus spleen and to spleen was almost as great as that to Wade lepromin. The late-phase reactions to BCG and BCG plus spleen were considerably larger than

that to Wade lepromin, even though the numbers of acid-fast bacilli in each preparation had been adjusted to equivalence. In order to visualize better the relative contributions of tissue and mycobacterial antigens, graphs were constructed (Text-fig. 3) in which the response to the human spleen antigen was used as a baseline. When differences from the responses to the spleen antigen were plotted, the reaction to Wade lepromin was demonstrated to be strong during the early-phase response; subsequently the curve followed the response to spleen alone. It will be noted that BCG plus spleen showed both early- and late-phase responses, while the BCG reaction was primarily late-phase.

Statistical analysis (Table 2) of the 0.8 mm. difference between maximum reactions on the second day to Wade lepromin and to BCG plus spleen did not show significance because of the relatively large standard error of difference of 0.5. The 1.0 mm. difference between reactions to these two preparations on the 10th day is, however, highly significant, with a standard error of difference of 0.03. On the 20th day the difference was 1.9 mm. and the standard error of difference again 0.03.



TEXT-FIG. 2.—Comparison of cross reactions to heterologous antigens in guinea-pigs sensitized with one preparation. (Each line shows the average reactions of 18 guinea-pigs in each experimental group.)

TABLE 2.—*Cross-testing with six antigens in groups of guinea-pigs sensitized by two previous injections of each antigen.*[a]

Previous sensitization of group	Days after injection	Test antigens					
		Lepromin[a]	BCG & spleen[a]	BCG[a]	Spleen[a]	Phenol-serum[a]	Phenol[a]
Wade lepromin	2	7.8 (0.6)	6.9 (0.6)	4.2 0.6)	5.6 (0.4)	3.2 (0.9)	2.6 (0.3)
	10	2.7 (0.2)	3.7 (0.2)	2.9 (0.2)	2.9 (0.3)	0.1 (0.1)	
	20	0.9 (0.2)	2.8 (0.2)	2.4 (0.1)	0.8 (0.3)		
BCG + spleen	2	8.8 (0.8)	11.9 (1.8)	9.5 (1.4)	6.8 (0.9)	3.8 (1.9)	2.4 (0.6)
	10	4.0 (0.3)	4.5 (0.5)	4.5 (0.2)	3.9 (0.1)	0.3 (0.3)	0.1 (0.2)
	20	2.2 (0.2)	3.6 (0.3)	3.0 (0.2)	1.5 (0.2)		
BCG	2	5.1 (0.6)	9.2 (1.2)	8.7 (0.8)	3.0 (0.4)	1.2 (0.2)	1.9 (0.2)
	10	2.2 (0.1)	4.4 (0.2)	4.1 (0.3)	2.3 (0.2)		
	20	0.6 (0.2)	3.2 (0.2)	2.9 (0.2)	0.3 (0.2)		
Spleen	2	4.5 (0.4)	6.6 (0.5)	4.1 (0.6)	6.1 (0.5)	2.0 (0.3)	2.1 (0.3)
	10	1.9 (0.2)	3.4 (0.4)	3.4 (0.5)	3.2 (0.2)		
	20	0.5 (0.2)	2.5 (0.2)	2.4 (0.1)	1.0 (0.1)		
Phenol-serum	2	3.2 (0.3)	5.3 (0.8)	3.9 (0.2)	3.8 (0.4)	3.8 (1.2)	1.8 (0.4)
	10	1.1 (0.3)	3.6 (0.3)	2.9 (0.4)	2.9 (0.5)		0.2 (0.2)
	20	0.4 (0.2)	2.3 (0.1)	2.0 (0.2)	2.3 (0.3)		
Phenol	2	2.0 (0.5)	2.8 (0.3)	3.0 (0.6)	3.8 (0.5)	2.0 (0.4)	1.5 (0.5)
	10	0.5	1.5 (0.6)	0.7 (0.5)	2.5 (0.2)		
	20	....	0.8 (0.5)	1.0 (0.1)	0.8 (0.5)		

[a] Mean skin reactions in mm. for each group of 18 animals in second experimental series, within parentheses standard errors for group.

In the group of guinea-pigs sensitized to BCG plus spleen (Text-fig. 2-B), further clarification of the relative contribution of tissue and mycobacterial antigens is possible because we had purposefully constituted the combined antigen from separate known components. Significant differences between reactions to BCG alone and BCG plus spleen, therefore, can be attributed to the tissue components. In graphs constructed with the response to spleen alone as a baseline, responses to the three preparations, Wade lepromin, BCG and BCG plus spleen (Text-fig. 3-B), showed secondary rises which are essentially parallel. BCG plus spleen produced the largest reaction; BCG was next, and Wade lepromin the smallest.

In guinea-pigs sensitized to heated BCG alone (Text-fig. 2-C), strictly mycobacterial sensitization produced strong and almost parallel responses to BCG and to BCG plus spleen. Only during the first three days was the response to Wade lepromin significantly greater than that to spleen.

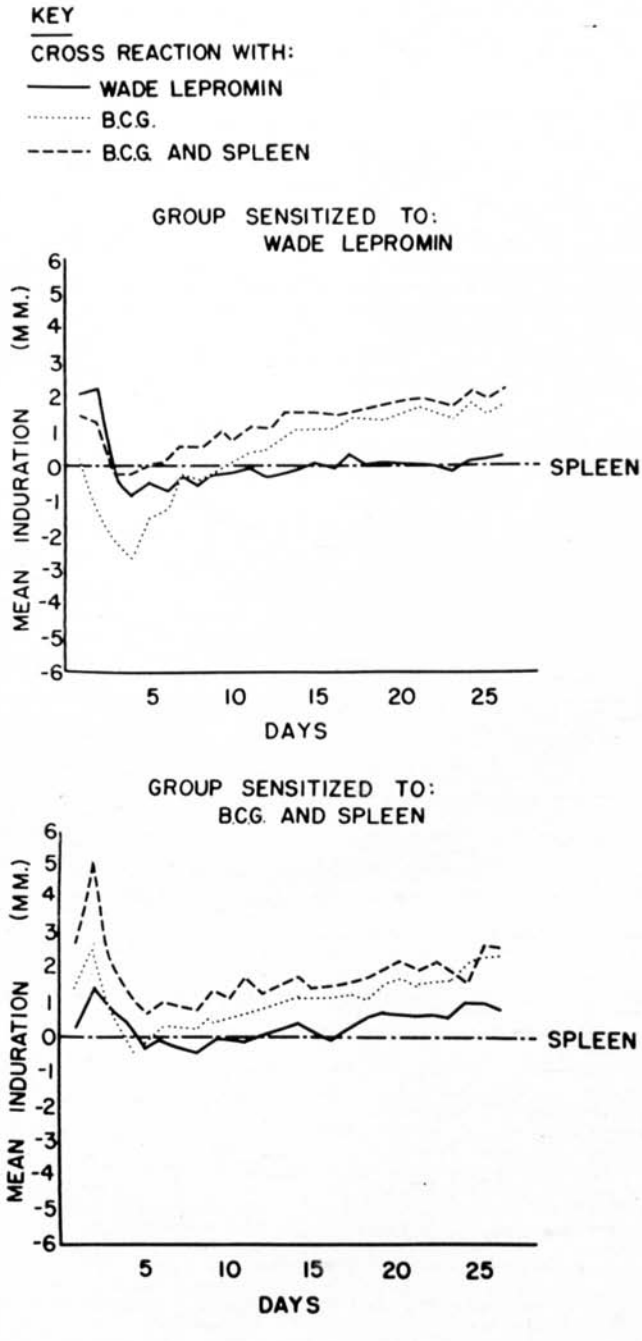
In the group sensitized to spleen alone (Text-fig. 2-D), reactions to spleen and to BCG plus spleen were parallel for eight days. Thereafter, the curve for spleen continued to decline while BCG and BCG plus spleen proceeded at higher levels. There was a relatively poorer response to Wade lepromin.

The control group sensitized with phenolized guinea-pig serum (Text-fig. 2-E) showed some cross reaction with each test antigen. Phenol alone produced no cross sensitization; the moderate reaction to human spleen is probably not important, because it is no more than that observed following primary injections of spleen.

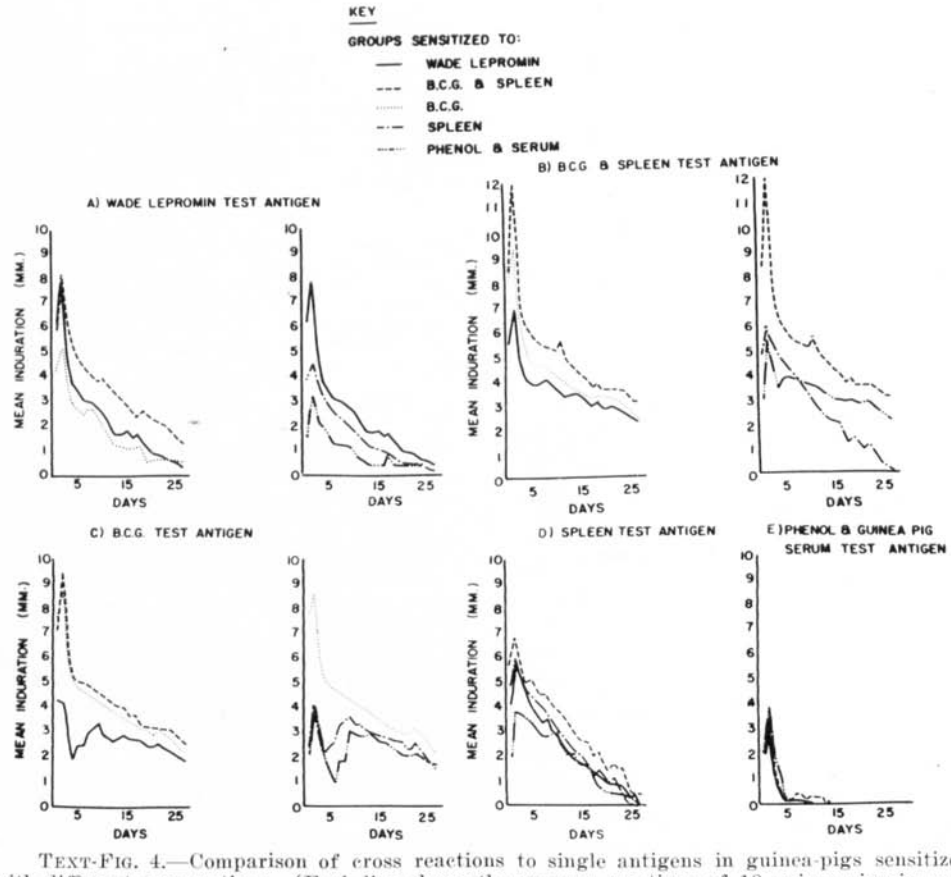
Comparison of cross reactions to single antigens in guinea-pigs

sensitized with various preparations.—The data from the second experimental series are plotted to compare responses resulting from differences in sensitization (Text-fig. 4).

Wade lepromin (Text-fig. 4-A) produced equal early responses in the two groups sensitized to combined tissue and mycobacterial antigen.



TEXT-FIG. 3.—Comparison of cross reactions to heterologous antigens in guinea-pigs sensitized with one preparation. Patterns of response to mycobacterial components shown by plotting against reactions to spleen as a baseline. (Each line shows the average reactions of 18 guinea-pigs in each experimental group.)



TEXT-FIG. 4.—Comparison of cross reactions to single antigens in guinea-pigs sensitized with different preparations. (Each line shows the average reactions of 18 guinea-pigs in each experimental group.)

Late response to lepromin, however, was higher in the group sensitized to BCG plus spleen than in the group sensitized to lepromin itself. It appears then that, although lepromin produced as good early-phase sensitization as BCG plus spleen, the late-phase sensitization was significantly inferior. The response to Wade lepromin in guinea-pigs sensitized to BCG alone and to spleen alone was not significantly different.

The BCG-plus-spleen test antigen (Text-fig. 4-B) produced a sharp early-phase response in guinea-pigs sensitized with any mycobacterial antigen, with the response in BCG-alone groups falling midway between the BCG-plus-spleen and Wade lepromin groups. Guinea-pigs sensitized to spleen alone did not maintain as high a late response to BCG-plus-spleen antigen as did the control animals sensitized to phenolized guinea-pig serum.

Previous exposure to BCG changes the shape of the reaction profile to BCG (Text-fig. 4-C). Guinea-pigs receiving BCG for the first time showed the characteristic bimodal response, but the middle dip did not

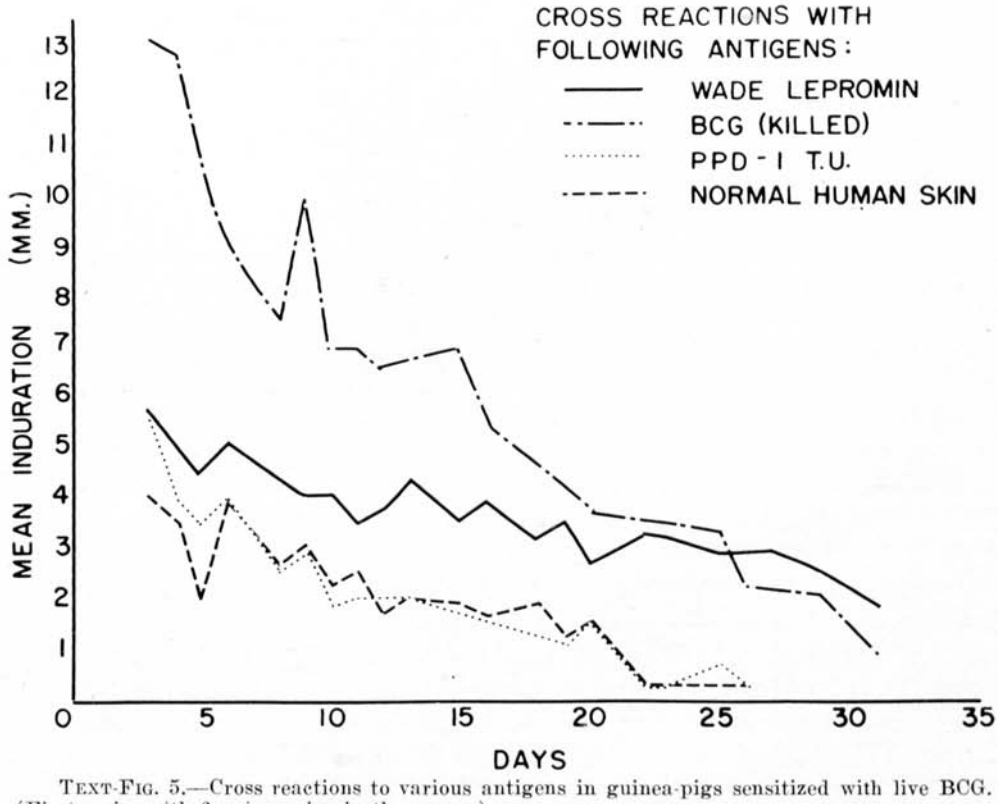
occur if animals had previously received BCG or BCG plus spleen. Since a typical bimodal BCG curve occurred in animals which had been sensitized by Wade lepromin, it seems that leprosy bacilli have little effect in modifying response to tubercle bacilli.

The response to the human spleen antigen (Text-fig. 4-D) in groups sensitized to all preparations was nonspecific, starting high and declining steadily. This is probably related to the heterogeneity of antigenic components in the test antigen.

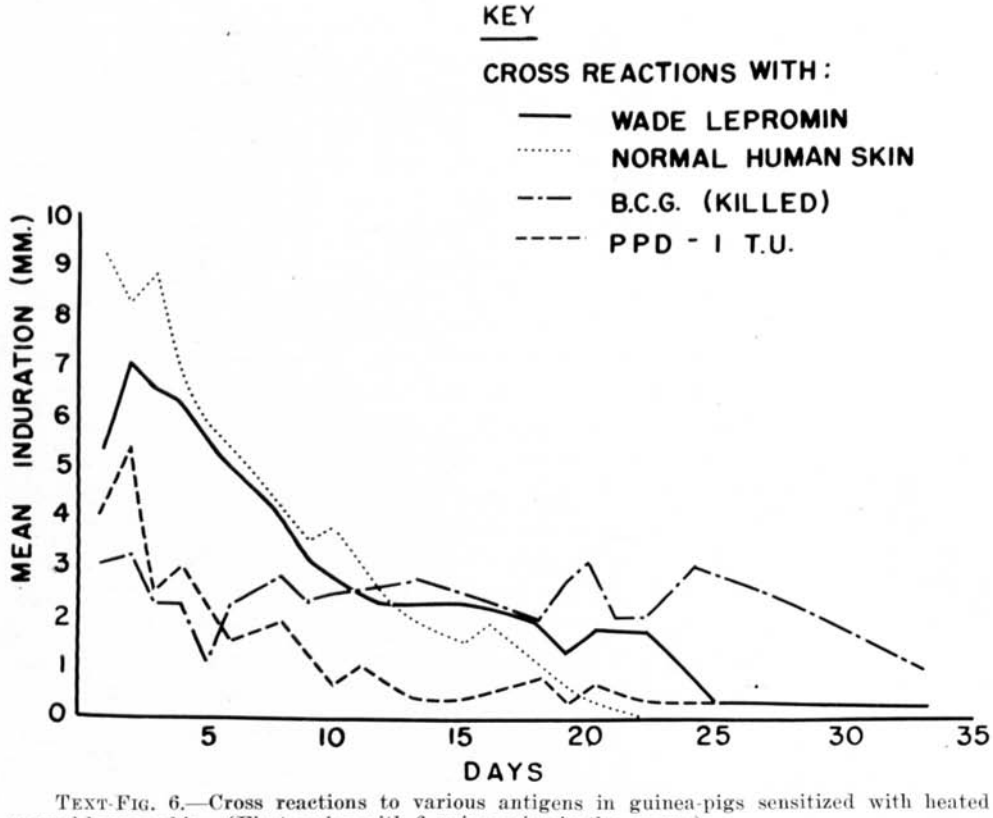
The response to phenolized serum as a test agent (Text-fig. 4-E and Table 2) was nonspecific and of relatively brief duration. The second-day response (Table 2) to phenol-serum in groups of guinea-pigs sensitized to lepromin, BCG plus spleen, and homologous serum, all were larger than those in the remaining three groups.

Live BCG as a sensitizing agent.—In the first experimental series, live BCG vaccine was used in a single 0.1 cc. intradermal injection to immunize a group of guinea-pigs. Text-figure 5 shows that the subsequent response to killed BCG was highly specific. Wade lepromin maintained a high level response through the late phase more effectively than it did in guinea-pigs immunized with killed BCG (Text-fig. 2-C).

Normal human skin as a sensitizing agent.—In the first experimen-



TEXT-FIG. 5.—Cross reactions to various antigens in guinea-pigs sensitized with live BCG. (First series with 6 guinea-pigs in the group.)



TEXT-FIG. 6.—Cross reactions to various antigens in guinea-pigs sensitized with heated normal human skin. (First series with 6 guinea-pigs in the group.)

tal series, normal human skin antigen was used (Text-fig. 6). It appears to be a more effective sensitizing antigen than the human spleen preparation (Text-fig. 2-D), but the contours of the curves are essentially the same.

DISCUSSION

The data presented make possible a clearer evaluation of the significance of the lepromin reactions both in terms of the properties of heated antigens and of the types of immune response.

The first point requiring emphasis is that autoclaved proteins are not devoid of antigenicity in the species of origin (Text-fig. 1-E). Autoclaved homologous serum was a weak test antigen (Text-fig. 4-E). However, when used as a sensitizing agent, it enhanced the responses to other antigens (Text-fig. 2-E). The large standard error of the homologous reactions in Table 2 is indicative of the considerable variation between individual guinea-pigs in susceptibility to autosensitization. In that animal, the antigenicity of the heated human-tissue components of lepromin is as great as that of mycobacterial components. These observations are consistent with findings in many immunologic studies on heated proteins, namely, that heat denaturation decreases

but does not obliterate antigenicity. Such denaturations meanwhile produce protein derivatives which are antigenic in the species of origin. This subject has already been reviewed by Hanks and Wallace ([13]), who point out that mycobacterial proteins also may be expected to show decreased antigenicity and broadened specificities as a result of heating. Since heated mycobacteria are readily permeated by tissue components, they concluded that preparation of *M. leprae* skin-test reagents would be improved by separating the bacilli fresh from unheated leproma tissue and then inactivating the bacilli by some method which would cause minimal modification of their protein configurations.

The usefulness of the present Mitsuda-Hayashi-Wade lepromin might first be considered in terms of the purpose for which it is to be employed. It has often been stated that the major specificity of the Mitsuda reaction is its negativity in lepromatous cases. If interest is primarily in diagnostic classification or prognosis, the presence of heated tissue components may cause no difficulty in lepromatous patients, since they usually are unable to react to either tissue or mycobacterial components. In tuberculoid patients, antigens which assay response to *M. leprae* alone would, at least theoretically, be preferable. Although frequency of a capacity to react to heated human antigens seems not to have been studied in a general population, it would be surprising if tuberculoid leprosy were the only chronic infection which gives rise to autoimmunizations. Until this question is investigated, or surveys are conducted with purified bacillary preparations, skin reactivity to heated human proteins must be considered among the undetermined causes of reactions to lepromin individuals without recognized prior mycobacterial infection. Field studies by Doull and colleagues ([5]) demonstrated that in children less than three years old who were not exposed to leprosy, 12 per cent spontaneously developed reactivity to lepromin during ninety days of observation. They also presented evidence ([6]) indicating that only 16 per cent of such spontaneous Mitsuda conversions could be attributed to exposure to *M. tuberculosis* and related organisms.

For epidemiologic purposes a distinction must be made between the two questions which presumably can be tested by recording the Fernandez phase and the Mitsuda phase[3] of skin reactions. First, has there been prior mycobacterial sensitization? Second, what is the capacity to respond to *M. leprae?*

In assaying the immunologic status of individuals, there appear to be three types of allergic response which need to be recognized.

[3]The results of the present experiments are consistent with Wade's views ([21, 22]) of the mechanism of the late or Mitsuda-type response to integral or bacillary antigen. Intact mycobacteria in an intradermal depot release antigen which produces or enhances local sensitization. As this sensitization rises, the amount of antigen remaining in the local tissues to serve as a test dose is decreasing. The period when local allergy and released tests antigen are both high enough to react is the time when the late Mitsuda reaction will be observed.

TYPE 1: A positive Fernandez reaction indicates pre-existing allergy, and suggests a significant prior exposure to mycobacteria or closely related antigens.

TYPE 2: A positive Mitsuda reaction following a negative Fernandez reaction suggests that there has been no significant prior exposure to appropriate mycobacterial antigens, but that there is an ability to develop allergy rapidly.

TYPE 3: A positive Mitsuda reaction following a positive Fernandez reaction probably is due in part to the "booster" effect of the injected antigen.

In testing for capacity to respond to *M. leprae*, an inherent difficulty arises from uncertainty in distinguishing between individuals in Types 2 and 3. If a Mitsuda response is preceded by a positive Fernandez reaction, some credit must be given to prior sensitization and not solely to new and rapid response. If it is not preceded by a Fernandez reaction, a positive Mitsuda response probably indicates an inherently strong ability to react. Unfortunately, heat-killed *M. leprae* has not been shown in humans, or here in guinea-pigs, to be a potent antigen. It may, therefore, be doubted whether surveys of late response to integral antigens will afford an adequate test of innate capacity for immune response. Without previous deliberate and uniform exposure of all persons to this mycobacterial antigen, it can never be known whether failures to achieve a Mitsuda-type response are due to low capacity to generate response or to lack of appropriate prior exposure.

Observations following primary injections of a more potent antigen (killed BCG) into guinea-pigs illustrate this point. Six of 86 guinea-pigs (11%) given killed BCG failed to develop the characteristic late response following a primary injection of lepromin. These animals, however, were able to make positive response to the second and subsequent injections of antigens.

Epidemiologic studies to test for the incidence of prior infection, it seems to us, depend on the eventual development of a leprolin or purified *M. leprae* extract and on testing for its specificity in producing Fernandez-type reactions. In guinea-pigs, as in humans, the antigens of BCG produce early-phase cross reactions to *M. leprae*. The cross-sensitization produced by sensitization with heated leprosy bacilli seems to be weaker, although Melsom ([16]) showed that unheated leprosy bacilli can cause guinea-pigs to become tuberculin positive. Intensive study will be needed to learn if sensitization by tubercle bacilli and by *M. leprae* can be distinguished by comparing the size of reactions produced after simultaneous injections of tuberculin and a corresponding leprolin.

1. Skin sensitization was produced in groups of guinea-pigs by a single injection of living BCG or by monthly intradermal injections of one of the following autoclaved antigens: Wade-Mitsuda lepromin,

normal human skin, normal human spleen, normal guinea-pig serum, BCG, and BCG plus normal human spleen.

2. The only antigen which regularly produced a distinct bimodal response with both early- and late-phase reactions was heated BCG. Repeated injections of BCG or combination of heated BCG with heated tissue antigens, eliminated the dip between the two phases. Previous sensitization with Wade lepromin did not alter the diphasic BCG curve, which indicates that lepromin produced little cross-sensitization against BCG.

3. In guinea-pigs sensitized to lepromin, strong early-phase cross reactivity occurred after skin testing with heated human tissue antigens. Late-phase cross reactions in these animals to preparations containing heated BCG were even larger than those to lepromin itself.

4. Animals sensitized to a BCG-plus-spleen preparation constituted to resemble lepromin showed both early- and late-phase mycobacterial cross-sensitization to lepromin. These cross reactions were smaller than when the two BCG preparations were used as test antigens, but larger than homologous late-phase reactions to lepromin in guinea-pigs sensitized to lepromin. This indicates that BCG is more effective than *M. leprae* as both an immunizing and a test antigen.

5. Guinea-pigs sensitized to heated BCG showed demonstrable, but weak, cross reactions with Wade lepromin only during the early-phase response.

6. Guinea-pigs sensitized to heated human spleen showed early-phase response to other antigens containing human tissue.

7. Heated guinea-pig serum produced moderate cross-sensitization to each of the other antigens. It also showed distinct early-phase cross reactions when tested in guinea-pigs sensitized to each of the preparations containing tissue components.

CONCLUSIONS

1. Autoclaving human tissue does not destroy its antigenicity, and heated proteins are antigenic in the species of origin.

2. Sensitization with Mitsuda-Wade lepromin causes enhanced response to both tissue and mycobacterial components. The degree of immune response is less than that induced by autoclaved BCG, or by autoclaved BCG plus tissue components.

3. Separation of *M. leprae* from tissue components, therefore, seems an essential step in the preparation of skin-test reagents for epidemiologic investigations. It should then be possible to determine the relative usefulness of soluble antigens and intact bacilli for detecting in the individual: (*a*) prior sensitization, or (*b*) capacity for enhancement of immune response.

RESUMEN

1. Se produjo sensibilización cutánea en grupos de cobayos con una sola inyección de BCG vivos o con inyecciones intradérmicas mensuales de uno de los siguientes

antígenos esterilizados al autoclave: lepromina de Wade-Mitsuda, piel humana normal, bazo humano normal, suero normal de cobayo, BCG y BCG más bazo humano normal.

2. El único antígeno que produjo con regularidad una neta respuesta bimodal con reacciones de fase tanto incipiente como tardía fué el BCG calentado. Las inyecciones repetidas de BCG o la combinación del BCG calentado con antígenos histológicos calentados eliminaron el declive entre las dos fases. La previa sensibilización con la lepromina de Wade no alteró la curva bifásica del BCG, lo cual indica que la lepromina produjo poca sensibilización cruzada contra el BCG.

3. En los cobayos sensibilizados a la lepromina, se presentó intensa reactividad cruzada en la fase incipiente después de la comprobación cutánea con antígenos de tejidos humanos calentados. En estoa animales, las reacciones cruzadas de fase tardía a las preparaciones que contanían BCG calentados fueron aun mayores que las observadas a là lepromina misma.

4. Los animales sensibilizados a una preparación de BCG-más-bazo, compuesta de modo que semejara lepromina, revelaron sensibilización cruzada micobacteriana a la lepromina de fase tanto incipiente cuanto tardía. Estas reacciones cruzadas fueron más pequeñas que cuando se usaban las dos preparaciones de BCG como antígenos de ensayo, pero mayores que las reacciones homólogas de fase tardía a la lepromina en los cobayos sensibilizados a la última. Esto indica que el BCG es más eficaz que el *M. leprae* como antígeno, ya inmunizante o de ensayo.

5. Los cobayos sensibilizados al BCG calentado revelaron reacciones cruzadas observables, pero débiles, con la lepromina de Wade únicamente durante la respuesta de fase incipiente.

6. Los cobayos sensibilizados al bazo humano calentado revelaron respuesta de fase incipiente a otros antígenos que contenían tejido humano.

7. El suero calentado de cobayo produjo moderada sensibilización cruzada a cada uno de los demás antígenos. Reveló además netas reacciones cruzadas de fase incipiente al ser ensayado en cobayos sensibilizados a cada una de las preparaciones que contenían componentes de tejidos.

CONCLUSIONES

1. El tratamiento de tejido humano al autoclave no destruye su antigenicidad y las proteínas calentadas son antigénicas en la especie de origen.

2. La sensibilización con la lepromina de Mitsuda-Wade produce una respuesta acrecentada a los componentes tanto histológicos como micobacterianos. La intensidad de la inmunirreacción es menor que la de la provocada por el BCG esterilizado al autoclave o por el BCG esterilizado al autoclave más componentes histológicos.

3. La separación del *M. leprae* de los componentes histológicos parece, pues, un tiempo indispensable en la preparación de reactivos de cutirreacción destinados a investigaciones epidemiológicas. Resultaría, pues, posible determinar la relativa utilidad de los antígenos solubles y los bacilos intactos para descubrir en el individuo: (*a*) sensibilización anterior o (*b*) capacidad para intensificar la inmunirreacción.

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