ANTIGENIC ANALYSIS OF LEPROMIN BY AGAR-DIFFUSION[1]

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INTRODUCTION

The identification and the establishing of relationships among the various antigens associated with the mycobacteria have been the subject of innumerable investigations. The classical serologic technqiues of complement-fixation, agglutination, and precipitation have been employed in this endeavor by many investigators. Information has also been provided by sensitizing both normal and tanned erythrocytes with bacillary components of mycobacteria and subsequently testing these antigen preparations with appropriate antisera (1, 5, 6).

Thus far the application of these techniques to the serologic study of leprosy has not been highly rewarding, due to the difficulties encountered in obtaining suitable antigenic material (3). Despite this, Olmos Castro and Bonatti (7) have devised a microflocculation test which depends on chloroform extracts of lepromatous tissue as the antigen. Although others may have undertaken similar investigations, there is little evidence of this in the literature.

A relatively new method for studying antigenic relationships takes advantage of the independent diffusion and precipitation of antigens and antibodies in gels (8). This technique has proved suitable for examining mixed serologic systems, i.e., systems composed of multiple antigens, or antibodies, or both. Recently several investigators (2, 9,10) have applied this procedure for the analysis of mycobacterial antigens.

The present report describes the results of preliminary experiments designed to determine the practicability of employing the agar diffusion technique in the study of antigens present in lepromin, and to relate these antigens if possible to those found in old tuberculin.

MATERIALS AND METHODS

Antigens.—The lepromin employed in this study was of the Hayashi-Mitsuda type, made of pooled lepromas and preserved with 0.5 per cent phenol, prepared by Dr. E. Mabalay of the Cebu Skin Dispensary, Cebu City, Philippines.

Old tuberculin (OT), 4 X International Standard, was generously supplied by Dr. W. S. Hammond, Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.

Sera.—Sera were obtained from the following sources. Philippines: (a) Two samples of pooled sera from adult patients with lepromatous and with tuberculoid leprosy, and (*b*) 44 sera from normal, nontuberculous school children, 6-9 years of

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age, living on Mactan Island, Cebu (an area in which leprosy is endemic), obtained through the cooperation of Drs. James A. Doull and Ricardo S. Guinto of the Leonard Wood Memorial.[2] All of these children were tuberculin negative (PPD, 10TU); 38 of them gave positive late reactions to lepromin. Their sera were obtained prior to the lepromin testing. Columbus: (*c*) Forty-four sera from nontuberculous children, aged 6-9 years, from Children's Hospital; From the Ohio Tuberculosis Hospital, (*d*) 35 sera of patients with tuberculosis, and (*e*) paired sera from 5 members of the hospital personnel taken before and after BCG immunization.

Agar-diffusion.—The various techniques of agar-diffusion analysis used in this laboratory have been reported elsewhere (2, 10). Bacto-agar medium, 0.8 per cent, is employed as the diffusion matrix. The agar is prepared in phosphate-buffered saline (pH 7.4) with sufficient glycine to effect a 1.0M concentration. Six cc. of this agar is poured into a flat-bottomed Petri dish and permitted to solidify.

Filter-paper discs are placed 7 mm. apart on the agar surface, and the test serum and antigen are introduced onto opposing discs by means of dropper pipettes to the point of saturation. The plates are incubated in the cold (4°C) and observed at three-day intervals for lines of precipitation by viewing in oblique lighting. Care is exercised to minimize temperature change during the viewing, since this leads to distortion.

Frequently it is necessary to concentrate serum samples in order to obtain high antibody levels. This is accomplished by pretreating the serum disc with the test serum and drying *in vacuo*. This procedure is repeated and, subsequent to placing the disc on the agar surface, the serum is applied for the third time. Thus there results a three-fold concentration.

EXPERIMENTAL

Reactivity of lepromin in agar with leprosy sera.—Aliquots of the sera from lepromatous and tuberculoid leprosy cases were concentrated on paper discs by evaporation and tested, by means of agar diffusion, with varying concentrations of lepromin (undiluted, and 1:15, 1:30 and 1:60 dilutions). Each of the serum discs was placed in the center of an agar plate, and the antigen-bearing discs were arranged radially from the central disc.

A typical reaction is represented by the photograph (Fig. 1). Inasmuch as it was ascertained from this experiment that undiluted lepromin gave stronger reactions than did the dilutions, that preparation was employed in all subsequent studies. Difficulty in obtaining photographs with sufficient contrast to permit satisfactory reproduction has necessstated depicting results of other agar diffusion reactions diagrammatically (Text-figs. 1 and 2). With lepromin as the antigen, the lepromatous serum demonstrated two lines of precipitation, whereas only one line was observed with the tuberculoid serum (Text-fig. 1).

Identity tests.—Identity tests were performed using these two test sera and a hyperimmune anti-BCG rabbit serum. Separate discs were saturated with each of the sera and placed 7 mm. from a centrally-located lepromin-saturated disc. After several days' incubation in the cold, a common continuous precipitate (or line of identity) was observed between

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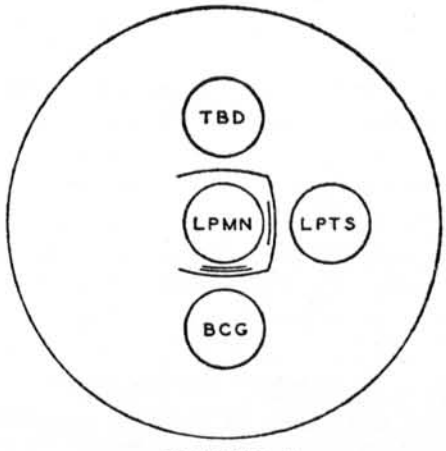
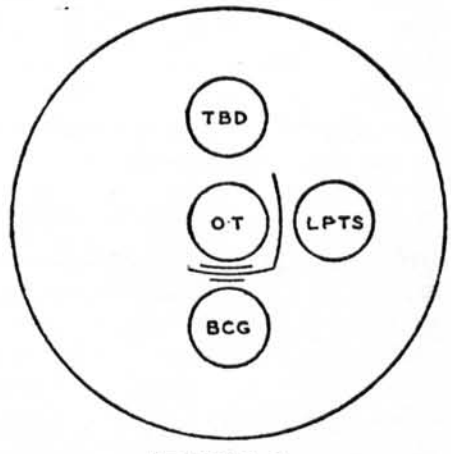
each serum disc and the antigen disc, indicating that an antigen contained in the lepromin reacted with an antibody common to each of the test sera (Text-fig. 1). Lines of nonidentity also were noted with the lepromatous and the anti-BCG serum.



TEXT-FIG. 1.



TEXT-FIG. 2.

TEXT-FIGS. 1 and 2. 1 (left): Agar diffusion showing a continuous common line of identity between lepromin (central disc) and the three test sera, tuberculoid and lepromatous leprosy, and rabbit anti-BCG. 2 (right): With tuberculin (OT, central disc) there is a line of identity between the antigen and only the lepromatous and anti-BCG sera. The tuberculoid serum failed to react. In both diagrams the discontinuous lines indicate reactions of nonidentity.

The same procedure was repeated using OT as the antigen. Preliminary experiments showed that a 1:30 dilution reacted optimally in this test system. A line of common identity was observed between the lepromatous and the anti-BCG sera, as well as nonidentity lines. However, the tuberculin failed to react with the tuberculoid serum (Text-fig. 2).

"Blocking" tests.—Futher evidence of the presence of common antigens in old tuberculin and lepromin was obtained by "blocking" tests. These are performed in much the same manner as are the more classical inhibition tests; i.e., the test serum and the suspected "inhibiting antigen" are mixed and incubated. This mixture is then tested in the presence of the specific antigen. The absence of a reaction indicates that the suspected antigen and the specific antigen are identical. Precisely the same principle is employed with the "blocking" test in agar.

When equal volumes of the OT dilution and each of the leprosy sera were first mixed in test tubes, incubated for 30 minutes at 37°C, and then examined by agar diffusion using lepromin as antigen, no reactions were observed. In order to preclude the possibility that the observed inhibition by tuberculin was due to a nonspecific reaction, the tuberculin was mixed with normal human serum in varying proportions and tested with lepromatous serum. No inhibition of reactions could be detected.

It is interesting to note that, although the tuberculin failed to react with tuberculoid leprosy serum in the agar diffusion test, it blocked the reaction between that serum and lepromin.

It appears that lepromin is less effective as a blocking agent than tuberculin. When 1 volume of the lepromatous serum was mixed with 1, 2, or 3 volumes of lepromin and the incubated mixtures tested, as above, against the tuberculin, only part of the reaction was inhibited. Since mixing the serum with the antigen effected a dilution of the serum, corresponding saline-diluted controls were included in the experiment, and in no instance was the reaction altered.

Comparison of sera of children of Cebu and of Columbus.—In view of these results, it seemed advisable to determine if sera from normal young children residing in an endemic leprosy area might contain antibodies with specifities directed toward antigens present in either lepromin, tuberculin, or both. Accordingly, sera from 44 school children of Cebu, Philippines, and an equal number of sera from Children's Hospital in Columbus, were concentrated and examined by agar diffusion. Both tuberculin and lepromin were employed as antigens. Of the 44 sera from Cebu, 43 (97.7%) reacted positively with lepromin, whereas only 7 (15.9%) positive reactions with lepromin were observed among the 44 sera of the Ohio children. All of these sera failed to react with old tuberculin (Table 1).

TABLE 1.—*Reactivity of test sera as determined by agar diffusion, with old tuberculin and lepromin used as antigens.*

Sera tested	Agar reactivity		No. of lepromin-reactive sera blocked by OT
	Old tuberculin	Lepromin	
Philippine children, 44	0 (0.0%)	43 (97.7%)	43
Columbus children, 44	0 (0.0%)	7 (15.9%)	0
Tuberculous adults (Columbus), 35	4 (11.4%)	8 (22.8%)	0
Hospital personnel, 5-pairs (before and after BCG)	0 (0.0%)	0 (0.0%)	0
Lepromatous leprosy (pooled)	Positive	Positive	(Blocked)
Tuberculoid leprosy (pooled)	Negative	Positive	(Blocked)

Each lepromin-positive serum was mixed with an equal volume of 1:30 old tuberculin, concentrated, and tested for reactivity with lepromin. The tuberculin "blocked" all of the Philippine sera from reacting, while no inhibition was observed with the locally-procured sera. It will be recalled that these Philippine sera were from blood samples drawn prior to lepromin testing and, therefore, the antibody demonstrations cannot be attributed to the antigenicity of lepromin, i.e., the bacilli contained in the test agent.

Additional evidence for the specificity of the above reaction was obtained when 35 sera from known cases of tuberculosis were tested for

lepromin reactivity. Eight of these sera reacted positively. However, these reactions were not blocked by old tuberculin in any instance, contrary to the results observed with the Philippine sera.

Paired sera from five members of a hospital staff, obtained before and after BCG vaccination, were tested against lepromin and OT in the same manner, and they failed to react.

DICUSSION

Two antibodies possessing a specificity for the same antigen will form a continuous line of precipitation when measured simultaneously by agar diffusion. This continuous line is referred to as a "line of identity" (see text-figures). Failure of the lines to meet is indicative of antibodies of different specificities (4).

Since a common line of identity was observed between the tuberculoid leprosy and lepromatous leprosy sera with lepromin as antigen, it can be concluded that these sera contained an antibody in common. Additional lines of precipitation indicated that there is more than one antigen present in lepromin.

On repeating the procedure using old tuberculin as the antigen, it was noted that the lepromatous serum also contained antibodies specific for antigens in tuberculin. However, no evidence of such antibodies could be detected with the tuberculoid serum by this direct method of testing.

The results of "blocking" tests showed that antigens in old tuberculin would block both of the leprosy sera from reacting with lepromin. On the other hand, lepromin was incapable of completely blocking lepromatous serum from reacting with tuberculin. These findings suggest, therefore, that there is at last one antigen, as detected by agar-diffusion, which is common to both lepromin and old tuberculin.

The failure of old tuberculin to react directly with the tuberculoid leprosy serum, while effectively blocking the same serum from reacting with lepromin, is at present not understood. Two of several possible explanations are offered: either a soluble complex formed between OT and the tuberculin serum, or a nonspecific binding occurred. In this regard it should be recalled that normal serum did not inhibit reactions.

Practically all of the sera of the Philippine children (43 of 44) were serologically active by the diffusion technique employed, and demonstrated characteristics peculiar to each of the leprosy sera tested. Inasmuch as (*a*) these sera reacted with lepromin, and (*b*) the reaction could be blocked by old tuberculin, they resembled both the lepromatous and tuberculoid leprosy sera. In their failure to react directly with the old tuberculin, on the other hand, they resembled the tuberculoid leprosy serum. In any event, an antibody was demonstrated in these sera that was specific for a lepromin antigen.

Although small percentages of sera from the nontuberculous Columbus children and from the tuberculous adults were also found to react with an antigenic element in lepromin, this element was not the same as the

one responsible for the reactions with the sera of the Philippine children or of leprous adults, since the reactions were not blocked by tuberculin. Furthermore, sera from BCG-vaccinated hospital personnel failed to react.

It would seem then that the lepromin that was used contains an antigen capable of reacting with sera from Philippine children, but which does not cross-react with sera from tuberculosis patients or other individuals (normal children or BCG-vaccinated adults) residing in Columbus, Ohio, a region where leprosy is practically nonexistent.

Despite the obvious limitations placed on the experiments here reported by the failure to examine more sera, the merit of utilizing agar-diffusion techniques for investigating the immunologic aspects of leprosy seems apparent. Not only might such procedures provide methods for studying the antigenic components of variously prepared lepromins, but also they may be of value in ascertaining or establishing the serologic response of patients and in investigating the epidemiology of this disease with particular reference to the relationship between serum antibodies and skin reactivity.

SUMMARY

1. A method has been described for investigating antigenic characteristics of lepromin.
2. By the agar diffusion technique, old tuberculin and lepromin have been shown to possess antigens in common as well as distinctive antigenic components.
3. Sera from Filipino children, residing in an endemic leprosy area, reacted with lepromin with a specificity not observed with normal and tuberculous sera obtained from patients in Columbus, Ohio.

RESUMEN

Usando como antiígenos una lepromina de Hayashi-Mitsuda y una tuberculina antigua (TA), se han aplicado la serorreacción de difusión en agar y una prueba obstaculizadora que aporta información complementaria a varios sueros procedentes de Cebú, en las Filipinas (consideradas como "zone endémica" para lepra), y de Columbus, Ohio (donde no existe lepra).

La lepromina (sin diluir) produjo una reacción de "identidad común" con dos sueros combinados de leprosos, lepromatoso y tuberculoide, y también con un suero cunicular anti-BCG. La AT (dilución al 1:30) no produjo reacción con el suero de lepra tuberculoidea, aunque los otros dos sueros acusaron reacciones positivas.

La TA agregada como elemento obstaculizador a los sueros leprosos resultó eficaz, impidiendo en ambos la reacción a la lepromina en la prueba de difusión. Por otro lado, la lepromina no sirvió más que parcialmente para bloquear el suero lepromatoso con respecto al antígeno de TA.

En la segunda fase de este estudio, el antígeno de lepromina acusó resultados positivos con 43 de 44 (98%) sueros de escolares filipinos no tuberculosos de 6 a 9 años de edad; sólo con 7 de 44 (16%) sueros de niños no tuberculosos de 6 a 9 años de un hospital de Ohio; 8 de 35 (23%) sueros de adultos tuberculosos de otro hospital de Ohio; y con ninguno de los sueros de 5 adultos del personal del hospital que habían sido vacunados con BCG. En cambio, el antígeno de TA no produjo reaccines de difusión más que en el grupo tuberculoso y sólo en 4 (11%) de los miembros de éste.

Recálcase el hecho de que la TA produjo obstrucción, con respecto a las pruebas de difusión con la lepromina como antígeno, en todos los 43 sueros de niños filipinos que habían reaccionado con la lepromina en la prueba inicial (lo mismo que hizo con los sueros combinados de casos de lepra), mientras que no ejerció tal efecto obstructor sobre los sueros reactores a la lepromina, procedentes de niños y adultos tuberculosos de Ohio.

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Description of Plate

Plate (15)

Fig. 1. Typical agar diffusion reaction showing a line of precipitation between old tuberculin (central disc) and lepromatous serum (right). Compare Text-figs. 1 and 2.

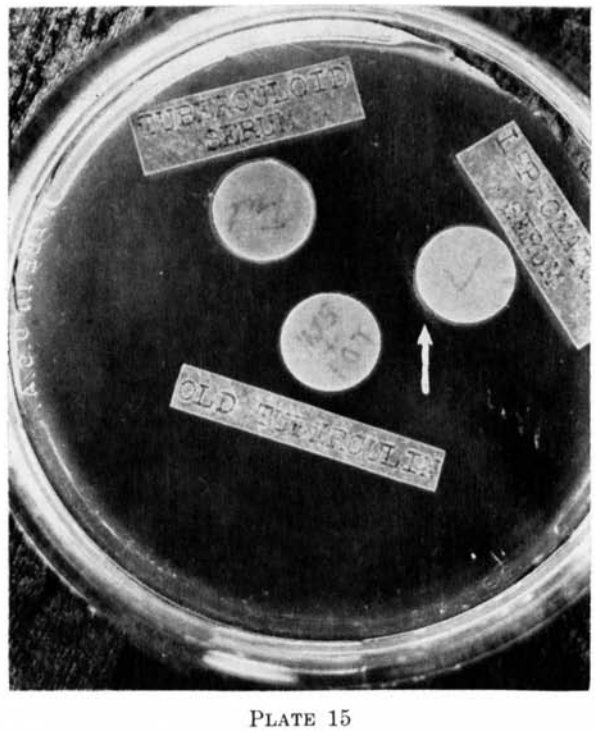

PLATE 15