IMMUNOLOGICAL STUDIES ON LEPROSY: SEPARATION AND EVALUATION OF THE ANTIGENS OF *MYCOBACTERIUM LEPRAE*

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STUDIES of delayed-type hypersensitivity in animals sensitised with various mycobacteria, cell walls, protoplasm, old tuberculin, PPD, and other protein and polypeptide fractions from mycobacterial cells have been reported extensively (Kanai, Youmans and Youmans, 1960; Larson *et al.*, 1961 and 1966, Magnusson, 1961 and 1962, Magnusson, Engback and Bentzon, 1961; Baer and Chaparas, 1966; Larson, Baker and Baker, 1968; Beam, Stottmeier and Kubica, 1969; Birnbaum and Affronti, 1970; Baker, Hill and Larson, 1972; Chaparas and Hedrick, 1973). These studies have been directed primarily towards an evaluation of skin reactivity to fractions derived from BCG and other mycobacteria. The few studies on *Mycobacterium leprae* have been concerned mainly with lepromin-like preparations of organisms obtained from human lepromata or from *M. leprae* infections of mice. The latter have been reported by Shepard and Guinto (1968) and by Draper, Rees and Waters (1968). Only one study of the immunological characterisation of antigens derived from *M. leprae* has been reported thus far (Abe *et al.*, 1972).

In the studies reported here, efforts have been made to separate antigens from a cell extract prepared from human tissue-derived *M. leprae*, by chromatographic methods, and to test these antigens for their ability to elicit skin reactivity in the guinea pig.

MATERIALS AND METHODS

Mycobacterial strains. The strains used in this study were: *M. leprae*, the acid-fast bacilli derived from the tissues of armadillos infected with *M. leprae* (Kirchheimer and Storrs, 1971), *M. lepraemurium*, *M. johnei*, *M. avium* 5, *M. intracellulare*, and the Bacillus Calmette Guérin (BCG). The first four organisms listed were separated from infected tissues; the remainder were grown *in vitro*, with the exception of BCG, which was used directly from a vaccine preparation.

Preparation of cell extracts (CE). The method for preparing the CE of *M. leprae* has been described (Navalkar, 1971). CEs from other tissue-derived strains were prepared in the same manner. The other mycobacterial strains, with the exception of BCG, were first grown *in vitro*, and the cells obtained by filtration on a Büchner funnel were processed to obtain the CEs. The methods for growing the organisms and for preparing the CEs were those described previously (Navalkar, Dalvi and Patel, 1975). No CE was prepared from BCG; these organisms were used as whole cells.

Chromatographic procedures. Gel-filtration chromatography of *M. leprae* CE was performed on DEAE-cellulose. The column (25 × 450 mm) was equilibrated with 0.02M Tris-HCl buffer (pH 8.0), charged with 2 ml of the CE, and elution begun with the same buffer.

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TABLE I

Results of skin-tests in guinea pigs sensitised with Mycobacterium leprae and other mycobacteria

Skin-test antigens	Size (mm) of skin reactions, at 48 h, in animals sensitised with					
	<i>M. leprae</i> , CE	CE from acid-fast bacilli from armadillo	<i>M. lepraemurium</i> , CE	<i>M. avium</i> 5, CE	<i>M. johnei</i> , CE	BCG
<i>M. leprae</i> , CE	20 × 20	20 × 20	10 × 10	15 × 15	8 × 10	8 × 10
CE from acid-fast bacilli from armadillo	20 × 18	25 × 20	10 × 10	18 × 18	...	8 × 10
<i>M. lepraemurium</i> , CE	15 × 20	10 × 10	20 × 20	12 × 12	...	6 × 8
<i>M. avium</i> 5, CE	10 × 10	12 × 15	8 × 10	25 × 25	...	10 × 10
<i>M. johnei</i> , CE	8 × 10	6 × 8	10 × 12	15 × 15	20 × 16	8 × 8
PPD	5 × 5	—	—	—	8 × 10	20 × 18
Lepromin, Dharmendra	6 × 5	7 × 6	—	—	6 × 6	6 × 8
Lepromin, WHO	12 × 12	10 × 12	4 × 5	6 × 8	...	5 × 5
Fraction A of <i>M. leprae</i>	15 × 10	20 × 18	8 × 10	8 × 6	5 × 5	5 × 5
Fraction B of <i>M. leprae</i>	15 × 15	15 × 15	8 × 10	6 × 5	—	—
Fraction C of <i>M. leprae</i>	20 × 18	10 × 12	—	—	—	8 × 8

CE = cell extract; figures in **bold type** indicate homologous reactions; ... = not done; — = no visible reaction.

As the elution progressed, the molarity of the buffer was increased to 0.2M, keeping the pH constant. The eluate was collected in 3- or 4-ml fractions, and the adsorption of each fraction at 260 and 280 nm was determined with a Beckman D Spectrophotometer. The eluates were pooled according to the protein peaks and concentrated by pervaporation at room temperature to one-tenth of their original volume. Each concentrate was screened for the presence of antigens by comparative immunodiffusion analysis, use being made of the already established antigen-antibody system (Navalkar, 1971). To obtain sufficient quantities of each fraction, a series of such separations and immunodiffusion tests was carried out. Each immunodiffusion plate was stained for protein with amido black, and for polysaccharide with Schiff reagent (Crowle, 1961).

Skin reactivity. Female Hartley-strain guinea-pigs were used. Before sensitisation, the animals were skin-tested with PPD and lepromin. Groups of three animals, weighing 300–350 g were sensitised, one group with each of the CEs. Two 1-ml injections of a mixture of the antigen with an equal volume of Freund's incomplete adjuvant were administered subcutaneously over the sternum, with an interval of 3 weeks between injections. The BCG-sensitised group received only one injection of the standard vaccine-dose. Six weeks after the first injection, the animals were skin-tested with 0.1 ml of one of the following antigens administered intradermally: the homologous CE, PPD, Dharmendra and WHO standard lepromins, and the antigen-active fractions. The extent of erythema, oedema and induration was measured in two directions and recorded at 6, 24 and 48 h.

TABLE II

Results of passive cutaneous anaphylaxis (PCA) studies in guinea-pigs

Challenged (by cardiac puncture) with	Size (mm) of PCA reactions at the site of intradermal injection of serum from animals sensitised with						
	<i>M. leprae</i> , CE	CE from acid- fast bacilli from armadillo	<i>M. lepraemurium</i> , CE	<i>M. johnei</i> CE	<i>M. avium</i> 5, CE	<i>M. intracellulare</i> , CE	BCG
<i>M. leprae</i> , CE	12 × 12	16 × 12	—	—	—	—	—
CE from acid-fast bacilli armadillo	16 × 12	25 × 20	8 × 8	—	—	15 × 15	—
PPD	—	10 × 10	6 × 6	—	—	15 × 15	20 × 20
Lepromin, Dharmendra	—	20 × 25	—	—	—	—	8 × 10
Lepromin, WHO	—	—	—	—	—	—	—
Fraction A of <i>M. leprae</i>	10 × 12	10 × 8	5 × 5	—	—	—	5 × 5
Fraction B of <i>M. leprae</i>	12 × 10	—	5 × 5	—	—	—	—
Fraction C of <i>M. leprae</i>	25 × 25	—	—	—	—	—	—

CE = cell extract; — = no visible reaction.

Passive cutaneous anaphylaxis (PCA) reactivity. Hyperimmune sera, obtained from the sensitised animals just described, were titrated in young guinea-pigs to determine the dilution most suitable for eliciting good PCA reactions. Then, 0.1 ml of the optimum dilution of each serum was injected intradermally into a shaven area of skin of a Hartley-strain guinea-pig (weight 200–250 g); 18 h later, 0.5 ml of each antigen mixed with 1 % Evans' blue dye was administered to the animals by cardiac puncture. The extent of blue coloration appearing within 15 min. of challenge at the site of each intradermal injection was measured in two directions and recorded. This was further confirmed by dissecting the local site of reaction and determining the extent of dye fixation in the subcutaneous tissue.

RESULTS

Skin reactivity

Table I shows the sizes of the skin reactions observed in guinea pigs sensitised with various mycobacteria and skin-tested with the various antigens. Each result represents an average of three experiments, with three animals per group in each experiment. The control animals exhibited no reactions or only weak reactions to all of the skin-testing materials. In the test animals, the homologous CEs elicited stronger reactions than did the heterologous CEs. Of particular interest are the reactions elicited by the various antigen-positive fractions. It is apparent that two fractions—A and B—produced skin reactions in most of the animals sensitised with other mycobacteria, whereas one fraction—C—produced reactions only in the *M. leprae*-sensitised and, to a lesser degree, in the BCG-sensitised guinea-pigs. Apparently fractions A and B each contained more than one antigen, whereas fraction C contained a single antigen. An extract of normal human tissue did not elicit a reaction in these animals. The reactions to PPD and to Dharmendra lepromin in the animals sensitised with *M. leprae* were rather weak, when compared with the reaction elicited by WHO lepromin.

PCA reactivity

Table II shows the sizes of the PCA reactions in guinea pigs given intradermal injections of hyperimmune serum from sensitised animals and challenged into the heart with various antigens. Animals challenged with *M. leprae* CE gave positive reactions at the homologous site and at the site at which serum from the animals sensitised with the armadillo-derived acid-fast bacilli had been injected. Once again, the specificity of fraction C—the fraction containing the single antigen—is apparent. When given into the heart this fraction reacted only with the serum from *M. leprae*-sensitised animals. Neither PPD nor the two lepromin preparations, when injected by this route, produced reactions at the sites of injection of serum from *M. leprae*-sensitised guinea-pigs. In the animals injected with PPD, there were positive reactions at sites at which the serum from animals sensitised with *M. intracellulare*, BCG and the armadillo-derived *M. leprae* had been injected, and in the animals given Dharmendra lepromin, only the latter two sites showed positive reactions. The WHO lepromin did not elicit any positive reactions.

DISCUSSION

Many investigators have employed chromatographic methods to obtain single antigens from mycobacterial preparations (Chaparas and Baer, 1964; Kniker and La Borde, 1964; Norlin, 1965; Abe *et al.*, 1972). Although these investigators were able to obtain some individual antigens, only two tested the biological activity of these (Baer and Chaparas, 1966; Abe *et al.*, 1972). Only Abe and his co-workers (1972) studied antigens separated from *M. leprae*; they reported that their fractions elicited cross-reactions in BCG-sensitised animals that were about equal in intensity to the reactions elicited by a lepromin preparation prepared in their laboratory, and of about the same intensity as those elicited in *M. leprae*-sensitised animals. Our studies are in agreement with those of Abe *et al.* (1972), although our antigens did not produce reactions in BCG-sensitised animals as large as those observed by them. Failure of our Dharmendra antigen to elicit good reactions may be explained by the fact that our antigen had been in storage for several years before it was used and hence its potency may have diminished considerably. Abe *et al.* (1970) state that the lepromin preparation made in their laboratory and maintained in the cold for 5 years had a diminished potency—as judged by the ability to detect the late reaction in patients in comparison with a portion of the same preparation lyophilised and kept for the same period. It is very likely that this may also be true of Dharmendra lepromin.

Of the three antigen-positive fractions tested, one, fraction C, appeared to be specific, whereas the others appeared to contain cross-reacting antigens. Only one antigen was demonstrated by comparative immunodiffusion analysis of fraction C, whereas the other two fractions contained more than one antigen. Protein and carbohydrate assays made on our fractions have indicated that fraction C is a protein and the other two contain both protein and polysaccharide components. Control studies of an extract from normal human tissue as a skin-test antigen gave negative results in all animals tested, indicating that the antigen-positive fractions either did not contain any tissue-derived antigens, or that if they did, the concentration of these antigens may be of a very low order. Our earlier studies (Navalkar, 1971) had demonstrated the presence in *M. leprae* of antigens common to other mycobacteria; the present study confirms this.

Because the PCA test is much more sensitive than the standard precipitin tests in its ability to detect minute quantities of antibodies, it was used to confirm the specificity of fraction C. The studies on the reactivity of PCA yielded results similar to those of skin-test reactivity. When animals were challenged by the intracardiac injection of the antigen-positive fractions, only fraction C produced a positive reaction, and only at the site at which serum from *M. leprae*-sensitised animals had been placed, whereas the other fractions elicited positive PCA reactions at sites at which sera from animals sensitised with other mycobacteria had been injected. This is further evidence of specificity. No positive PCA reactions were elicited when animals were challenged with the two lepromins and PPD at the site at which the serum from *M. leprae*-sensitised

animals had been injected, although positive reactions were elicited at other sites.

The reactivity of fraction C in the PCA studies is of significance. In general, cell-mediated immunity cannot be transferred passively through serum by a humoral factor; hence the positive PCA reaction exhibited by fraction C, which apparently contained only a single antigen, was somewhat unexpected. A possible explanation of this may be that fraction C possesses two antigenic determinants. Alternatively, it may be composed of two distinct antigens that were not easily distinguishable by immunodiffusion methods.

SUMMARY

Chromatographically separated antigens of *Mycobacterium leprae* were tested for their ability to elicit skin reactions in guinea-pigs sensitised with homologous and heterologous mycobacteria. Of the three antigen-positive fractions obtained, one showed specific activity and the other two cross-reactivity, as indicated by studies of hypersensitivity and passive cutaneous anaphylaxis.

The fraction exhibiting specificity contained only one antigen, which was protein in nature, whereas the other two fractions contained more than one antigen and possessed both protein and polysaccharide constituents. Because the single-antigen-containing fraction showed both positive skin and PCA reactivity, the suggestion is made that this fraction may contain either an antigen with two determinants or may contain two antigens that are not easily distinguishable by immunodiffusion methods.

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