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Immunochemical Analysis of Soluble Antigens of *Mycobacterium lepraemurium* [1]

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Investigations concurrent with ours, by Navalkar *et al* (5), have revealed six antigens in the extracts obtained from disrupted *Mycobacterium lepraemurium*. These researchers also reported that one or two of these antigens were shared with a number of other mycobacteria, an observation made earlier by Kwapinski *et al* (4) and Stanford (7). In this paper, we report on our investigations on the immunochemical nature of soluble antigens of *M. lepraemurium* and their relationships to *Actinomycetales* and *Eubacteriales*.

MATERIALS AND METHODS

Source and method of purification of M. lepraemurium. The source of the soluble antigens was a concentrated suspension of partly purified *M. lepraemurium* grown in white rabbits, and kindly supplied by L. Kato, Institut d'Hygiene et Microbiologie, Université de Montréal. These microorganisms were further purified by centrifugation through 30% sucrose gradient as follows: 1 ml of concentrated, partly purified mycobacteria was added to 9 ml of 30% aqueous solution of sucrose and homogenized in the cold for two minutes in a Sorvall high-speed omni-mixer. The homogenate was centrifuged for 20 minutes at 12,500 × g in a Sorvall Superspeed centrifuge, to sediment possible fine cell debris. The supernatant was withdrawn, vacuum dialyzed to remove sucrose, concentrated in the cold to 1/5 original volume, and centrifuged at 12,000 × g for 15 minutes. The sediment thus obtained was examined microscopically at 1000X magnification upon staining by Ziehl-Neelsen's method (for the detection of mycobacteria), and with 0.1% malachite green (for the detection of any remaining cell debris). Additional amounts of *M. lepraemurium* were recovered from the first homogenate sediment, after the resuspension and homogenization in 30% to 35% sucrose, by the aid of a Sorvall omni-mixer. The latter homogenate was further processed as described above.

In order to ascertain that the final product contained no soluble constituents of rat tissues, the purified mycobacteria were suspended in a 0.06 M, pH 7.6, phosphate buffered saline (PBS) and placed in a 4 mm wide well cut in a layer of 1.1% agarose gel (pH 8.2), made in 0.01 M, barbiturate buffer containing 5% anti-rat antiserum and 1:25,000 merthiolate. The gel-covered slides were incubated at 37°C for 48 hours and at 4°C for a week, and inspected for the presence of precipitation bands around the wells. The wells on control slides received an extract of normal rat tissues made in PBS.

Antigen production. The purified mycobacteria, found free of contaminating rat-tissue components were suspended in a 0.02 M, pH 7.6 EDTA containing 1:25,000 merthiolate to form a dense suspension. They were homogenized for one minute in a Sorvall omni-mixer, placed on crushed ice, and subjected for ten minutes to ultrasonication, in a Biosonik III at 20 Hz. The breakage of the mycobacteria was ascertained in two ways: 1) by observation of Ziehl-Neelsen stained preparations at 1000X and finding that the broken bacteria lost acid-fastness, and 2) by finding empty or near-empty bacilli when observed by an electron microscope at 25,000X magnification. When it was found that between 90% to 95% mycobacteria were disrupted, the ultrasonicate was centrifuged for 15 minutes at 600 g in a Sorvall high-speed centrifuge, to sediment the bacterial debris.

The supernatant was carefully collected (later used for cell wall preparation), recentrifuged at 800 g for ten minutes, and examined microscopically at 1000X magnification. If no bacilli were observed, the liquid was diluted ten times in a 0.05 M, pH 7.4 PBS, passed through a 0.45 μ membrane and concentrated to 1/20 volume by vacuum di-

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ERRATUM: Vol. 44, #4, page 443, line 18 should read ". . . purified *M. lepraemurium* grown in white rats . . ." Authors' error not picked up by editor or authors' proofreading.

alysis. The resulting concentrate represented the cytoplasm of *M. lepraemurium.*

The cell wall preparations were made from the sedimented bacterial debris according to Kwapinski's method ([1]). In an identical manner, cytoplasms and cell walls were produced from *M. leprae,* which was recovered and purified as reported earlier ([4]).

Sources of control antigens. To examine possible antigenic relationships, soluble antigens were produced from the following microorganisms.

Eubacteriales: *Alcaligenes faecalis* ATCC 8750; *Bacillus anthracis* No. 4, *B. cereus* B1, *B. subtilis* ATCC 6051; *Citrobacter freundii* ATCC 8090; *Clostridium perfringens* ATCC 19404; *Corynebacterium diphtheriae* PW8, *C. pseudodiphtheriticum* ATCC 10700; *Enterobacter aerogenes* E3; *Escherichia coli* ATCC 13224; *Klebsiella pneumoniae* ATCC 23883; *Leptospira pomona* ATCC 23478; *Neisseria catarrhalis* L576; *Proteus vulgaris* OX19 (U.M.), *Proteus vulgaris* ATCC 13315; *Psuedomonas aeruginosa* No. 1; *Salmonella gallinarum* ATCC 9184; *Spirillum serpens* No. 368; *Staphylococcus aureus* No. 231, *S. epidermidis* ATCC 153; *Streptococcus pneumoniae* No. 108, Serotype I, *Streptococcus mitis* No. 17, *S. pyogenes* ATCC 12202; *Treponema denticola* T-32A, and *T. scoliodontum.*

Actinomycetales: *Actinomadura* ATCC 13723 and 13724; *Am. pelletieri* 14816; *A. israelii* 561 and 656; *A. naeslundii* 45 and 447; *Arachnia propionica* 364; *Nocardia asteroides* 10056 and 8595; *N. brasiliensis* 11020; *N. caviae* 91; *N. corallina* 4273; *N. erythropolis* 17896; *N. turbata* 152; *N. polychromogenes* 3409; *N. rubra* 685 and 12006; *Mycobacterium aquae* 23422, *M. avium* 701 and 801; *M. borstelense* 23030; *M. bovis* 19210; *M. flavescens* 14474; *M. fortuitum* 23048; *M. friburgensis* 23245; *M. gallinarum* 19710; *M. intracellulare* 15985; *M. kansasii* 12478; *M. marinum* 927; *M. microti* 1001 and 11152; *M. rhodochrous* 13808; *M. scrofulaceum* 15080, 23419, and 23429; *M. salmoniphilum* 13936; *M. terrae* 15755; *M. tuberculosis* 202: *Mycococcus* 13556; *Oerskovia turbata* 891; and *Oerskovia xanthineolytica* Y13-3.

Fungi: *Aspergillus fumigatus* No. 56; *Candida albicans* No. 37; *Saccharomyces cerevisiae* No. 35; *Penicillium aculeatum* ATCC 10499 and *Trichophyton mentagrophytes* ATCC 598.[3]

All *Actinomycetales* strains, most of the *Eubacteriales* strains, and fungi were grown in KNM or KIM semisynthetic culture media ([1]) or in a thioglycollate broth. The cultures were incubated at 37°C until a full growth was obtained. After the purity and identity of the cultures were ascertained by the appropriate cultural and biochemical methods, the organisms were treated with 0.5% formalin, harvested, and washed with PBS. The soluble antigens were obtained from the cells in the manner identical to that used for *M. lepraemurium.*

Immunologic assays. The antigens were repeatedly examined by the following assays: immunodiffusion in 1.1% agarose, counterimmunoelectrophoresis ([6]), double-immunoelectrophoresis ([9]), and delayed-skin hypersensitivity assay. Antisera for the immunodiffusion tests were produced in adult albino rabbits by a series of injections ([1]).

The hypersensitivity assay was carried out in the following manner. Groups of albino guinea pigs weighing approximately 600 gm were first injected intracutaneously, on both sides of the neck, with one of the following materials: 0.2 ml of *M. lepraemurium* cytoplasm containing 20 μg solids and 20 μg tetracyclines; 0.2 ml of the cytoplasms obtained from *M. leprae* and five other mycobacteria, selected in accordance with the similarity of antigenic compositions revealed by previous studies ([2,4]); 0.2 ml of cell walls or whole cells of *M. lepraemurium* and *M. leprae* containing 40 μg solids; 0.2 ml lepromin containing 3×10^7 mycobacteria (Instituto de Leprologia, Rio de Janeiro); and 0.2 ml globulins recovered by 33% saturation with ammonium sulfate from two pools of sera obtained from lepromatous leprosy (received from J.O. Almeida, Ribeirao Preto).

Sixteen days after sensitization, the guinea pigs received injections of 0.1 ml of each of the above materials diluted 1:500 with

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[3]The strains marked as ATCC were obtained from American Type Culture Collection. The sources of other microorganisms were as follows: Department of Microbiology and Immunology, University of Western Ontario, London, Ontario; Faculdade de Medicina, Ribeirao Preto, Brazil; Department of Microbiology, University of Minnesota, Minneapolis; Department of Microbiology, Tulane Medical School, New Orleans, La.; and Department of Medical Microbiology, University of Manitoba, Winnipeg.

sterile distilled water in the closely shaved skin. As a control, 0.1 ml of a solution of tetracycline (200 μg/ml) was injected intracutaneously.

Observations and recordings of local signs in the skin, and measurements of the areas of induration, in two perpendicular directions were made at six hour intervals for 72 hours. The animals were further observed for two weeks.

Physiochemical analysis of antigens. The various types of polymeric components present in the cytoplasm of *M. lepraemurium* and *M. leprae* were resolved by the electric current using a polyacrylamide electrophoresis technic ([8]). A total of 60 samples of the soluble antigen preparations were resolved, and four gel slabs were used for each staining procedure ([1]), in order to reveal the presence of proteins, carbohydrates, nucleic acids, and lipids.

RESULTS

The soluble antigens of *M. lepraemurium* were resolved on the polyacrylamide gel electrophoresis into six polymeric components, four proteins and two polysaccharide-polypeptides, their respective molecular weights being approximately: 1×10^4, 1×10^5, 4×10^6 for proteins, and 20,000 and 35,000 for polysaccharide-polypeptides.

Studied by various immunodiffusion procedures, with and without electric current, *M. lepraemurium* proved to contain at least six different antigens. Except one, all the antigens were resistant to heating at 100° C for 20 minutes. Two or possibly three of these antigens were found to cross-react with anti-*M. leprae* antiserum, and one antigen proved to be immunologically related with soluble antigens of *M. tuberculosis* 202, *M. simiae* 3056 and 3015, *M. balnei* 11565, *M. scrofulaceum* 15080, 23429, and 23419, *M. aquae* 23422, *M. microti* 1601 and 11152, *M. intracellulare* 15985, *M. marinum* 927, *M. avium* 701 and 801 (2 lines), *M. gallinarum* 19710, and *M. kansasii* 12478, as well as with the soluble antigens of *Actinomyces israelii* 561. No cross-reactions were found between *M. lepraemurium* and other *Actinomycetales, Eubacteriales* and fungi.

The soluble antigens of *M. lepraemurium* (as well as those of *M. leprae)* were found to sensitize guinea pigs which responded vigorously to the intradermal challenges with minute amounts of these antigens (Table 1). Guinea pigs sensitized with the soluble antigens of *M. lepraemurium* showed strong delayed hypersensitivity only to the soluble antigens of *M. lepraemurium* and *M. leprae,* and to a lesser extent to the antigens of *M. scrofulaceum* 23419. The animals sensitized with cell walls of *M. lepraemurium* showed strong delayed hypersensitivity to both *M. lepraemurium* and *M. leprae* and a less intense reaction to the cytoplasmic antigens of *M. scrofulaceum* 23419 and *M. aquae* 23422. Surprisingly, no reaction was observed on the challenge with lepromin and with the globulins obtained from pooled leprous human sera. The guinea pigs sensitized with the soluble antigens of *M. salmoniphilum* 13936 and *M. aquae* 23422 reacted rather strongly to the soluble antigens of *M. lepraemurium* and *M. leprae* but not to lepromin.

DISCUSSION

The presence of six different soluble antigens in *M. lepraemurium* revealed in these studies coincides with the data published earlier by Navalkar *et al* ([5]), Kwapinski *et al* ([4]) and Stanford ([7]), but these antigens have now been physicochemically identified. There is also a good agreement between our data and those published by Navalkar *et al* ([5]) relevant to the antigens shared between *M. lepraemurium* and some other mycobacteria. In addition, we have found that an antigenic relationship exists between *M. lepraemurium* and *A. israelii,* which is in line with the observations made earlier ([4]) in reference to *M. leprae* and *A. israelii.* Since the antigens of *M. lepraemurium* have been found to be closely related to those of mycobacteria, but not to any antigens of *Eubacteriales* and fungi, the taxonomic position of this bacterium within the order *Actinomycetales* has thus been reinforced by immunologic studies.

The soluble antigens of *M. lepraemurium* appear as potent elicitors of a highly specific cell-mediated immunity. Conspicuously, this specific immunity seems to be remote from the antigens occurring in lepromin and in leprous sera. It may perhaps be hypothesized that the antigens of *M. lepraemurium* and lepromin are incompatible within a nost, an effect postulated on the basis of the previous studies ([3]) where the incidence of lepromin reactivity was found to be significantly decreased in children preinjected with the soluble antigens of *M. leprae.*

TABLE 1. *Mean diameters (mm) of skin induration at the site of challenge injections after 48–72 hours, in guinea pig.*

	Challenged With												
ed With	*Mlm* cytopl.	*Mlm* walls	*Ml* cytopl.	*Ml* walls	Lepro-min	Leprous sera	Normal sera	13936	23422	23419	202	3056	11
aemurium cytoplasm	23	4	23	0	0	0	0	7	12	20	11	0	
aemurium walls	15	23N	17	13	0	0	0	5	12	18	0	0	
ae cytoplasm	10	0	12	0	0	18	0	14	18	22	12	22	
ae walls	17	0	17	0	0	12	0	0	23	0	0	0	
in	0	0	0	0	14	0	0	0	0	0	0	0	
s sera	0	0	0	0	U	12	19	0	0	0	0	0	
serum	0	0	0	0	0	21	20	0	0	0	0	0	
noniphilum 13936	14N	0	19	0	0	16	0	15	0	10	0	0	
ae 23422	24N	0	12	0	0	0	0	0	24	16	0	22	
fulaceum 23419	18	6	12	12	0	0	0	0	18	21	0	18	
rculosis 202	20	0	18	0	0	16	0	0	0	0	20	0	
ae 3056	0	0	22	0	0	20	0	16	15	0	14	20	1
ei 11565	0	0	0	0	0	0	0	0	0	0	0	14	1

crosis developed in 60-80 hours.
cer found in one to three weeks.
= *Mycobacterium lepraemurium.*
Mycobacterium leprae.

SUMMARY

Soluble antigens obtained from purified *M. lepraemurium* were identified by SDS polyacrylamide gel electrophoresis as three different proteins, $M_{\overline{w}}$ 1×10^4, 1×10^5, and 4×10^6, and two polysaccharide-polypeptides. Three antigens were found to react with anti-*M. leprae* antiserum; a single antigen proved to be immunologically related to *M. tuberculosis, M. simiae, M. balnei* and a few other mycobacteria, but not to *Eubacteriales* and fungi, as examined by immunoelectrophoresis.

In the dermal hypersensitivity assay, the guinea pigs sensitized with soluble antigens of *M. lepraemurium* only responded to the antigens of *M. lepraemurium, M. leprae* and partly to *M. scrofulaceum,* but not to other antigens, including lepromin and leprous serum globulins.

RESUMEN

Los antígenos solubles conseguidos de *M. lepraemurium* purificado, fueron identificados por método de electroforésis con gel SDS de polyacrylamide como tres proteinas diferentes, $M_{\overline{w}}$ 1×10^4, 1×10^5 y 4×10^6, y dos polypeptidos-polisacáridos. Tres antígenos fueron descubridos a reaccionar con antisuero de anti-*M. leprae;* un antígeno singular demostro a ser inmunologicamente relacionado a *M. tuberculosis, M. simiae, M. balnei* y unos cuantos otros micobacteria, pero no a *Eubacteriales* y hongos, cuando examindos por inmunoelectroforésis.

En la prueba de dermico hypersensitivo, los conejillos de Indias sensibilizados con antígenos solubles de *M. lepraemurium* solamente respondieron a los antígenos de *M. lepraemurium, M. leprae* y en parte a *M. scrofulaceum,* pero no a los otros antígenos, incluyente lepromino y suero de globulina leprosa.

RÉSUMÉ

Des antigènes solubles obtenus à partir de fractions purifiées de *M. lepraemurium* ont été identifiés par des méthodes d'électrophorèse sur gel SDS de polyacrylamide, comme appartenant à trois protéines différentes, $M_{\overline{w}}$ 1×10^4, 1×10^5, et 4×10^6, et comme deux polypeptides polysaccharidiques. On a observé que trois antigènes réagissaient avec un antiserum anti-*M. leprae.* Un seul antigène s'est révélé immunologiquement parent de *M. tuberculosis,* de *M. simiae,* de *M. balnei* et de quelques autres mycobactéries; par contre, il n'était pas lié aux *Eubactériales* et aux fungi, ainsi qu'il ressort des examens d'immunoelectrophorèse.

Dans les épreuves d'hypersensibilité cutanée, les cobayes sensibilisés par des antigènes solubles de *M. lepraemurium* n'ont répondu qu'aux antigènes de *M. lepraemurium,* de *M. leprae,* et partiellement à ceux de *M. scrofulaceum.* Par contre, ils ne répondaient pas aux autres antigènes parmi lesquels la lépromine, et les globulines de sérum de malades atteints de lèpre.

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