

Identification of a *Mycobacterium leprae* Specific Protein Antigen(s) and its Possible Application for the Serodiagnosis of Leprosy¹

Harlan D. Caldwell, Waldemar F. Kirchheimer, and
Thomas M. Buchanan²

Knowledge of the antigenic character of *M. leprae* is limited due to inadequate quantities of bacilli available for antigenic analysis until recently. Previous investigations using lepromatous nodules as a limited source of bacilli or soluble antigen have suggested unique *M. leprae* protein antigens (^{1,2,11}) not shared by other mycobacteria. More recently, Abe,* utilizing indirect immunofluorescence with pooled lepromatous leprosy patients' sera adsorbed to remove cross reacting antibody to common mycobacterial antigens, has demonstrated further evidence for one or more antigens specific for *M. leprae*. Harboe, *et al.*, using a radioactive assay and adsorbed human sera, also obtained results suggesting *M. leprae* specific antigens (⁶).

The recent availability of large amounts of *M. leprae* recovered from systemically infected nine banded armadillos (⁸) provides an opportunity for the initiation of studies to isolate, purify and characterize specific *M. leprae* antigens. Unfortunately, most such studies using bacilli purified from armadillo tissue have not demonstrated *M. leprae* specific antigens. Harboe, *et al.* (⁵), using sonic extracts of purified organisms, were unsuccessful in identifying specific *M.*

leprae antigens using crossed immunoelectrophoresis for analysis. In addition, only three weakly staining protein bands were found when the material was analyzed by polyacrylamide gel electrophoresis. The source of purified *M. leprae* used for analysis by Harboe was obtained from the World Health Organization Immunology of Leprosy Program (IMMLEP). We have recently shown that surface protein antigens of *M. smegmatis* are released and at least partially destroyed when subjected to the protocol used by IMMLEP that employed proteases for the purification of *M. leprae* (³). This suggested that similar surface antigens of *M. leprae* may be destroyed or selectively removed from *M. leprae* organisms during purification from infected tissue using the IMMLEP procedure. In this study we report confirmation of a protein antigen(s) specific for *M. leprae* that was solubilized from organisms that were separated from armadillo liver tissue without employing any proteases. The antigen is strongly precipitated by treated lepromatous leprosy (LL) patients' sera and also recognized by serum from patients with tuberculoid leprosy. This indicates its possible importance for the serodiagnosis of leprosy.

MATERIALS AND METHODS

M. leprae organisms were separated from 200 gm of moderately infected armadillo liver (ca. 10⁹ organisms/gm tissue) by the method described by Prabhakaran, *et al.* (¹²). The liver was minced with scissors and 6 gm aliquots were homogenized in 20 ml of 0.2 M sucrose for 30 seconds at 4°C, using a press homogenizer (Braun, Model 853-203, B. Braun Instruments, San Francisco, California). The homogenate was centrifuged at 200 × g for 20 min. The supernatant was retained and the residue resuspended in 20

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² H. D. Caldwell, Ph.D., F. I. Proctor Foundation, Room 331, University of California, San Francisco, California 94113; W. F. Kirchheimer, M.D., Ph.D., Chief, Laboratory Research Branch, USPHS Hospital, Carville, Louisiana 70721; T. M. Buchanan, M.D., Head, Immunology Research Laboratory, USPHS Hospital, Seattle, Washington 98114; from the Immunology Research Laboratory, USPHS Hospital, Seattle, Washington and the Departments of Medicine and Pathobiology, University of Washington, Seattle, Washington.

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ml of 0.2 M sucrose and rehomogenized and recentrifuged. The supernatant fractions were pooled and the residues discarded. Nine ml of the pooled supernatant fractions were carefully layered over 12 ml of 0.3 M sucrose and centrifuged at $6,000 \times g$ for 10 min. The supernatant was discarded and the residues resuspended in 0.2 M sucrose. Nine ml portions of the suspension were layered over 12 ml of 1.5 M KCl. The material was centrifuged at $6,000 \times g$ for 10 min. The supernatant was discarded, and the precipitate was again suspended in 0.2 M sucrose, layered over 1.5 M KCl, and centrifuged. The pooled bacilli pellets were washed twice with 0.05 M Na phosphate, 0.15 M NaCl pH 7.0 (buffered saline). Organisms were suspended in buffered saline and quantitated by the method of Hanks, *et al.* (⁴). The total number of bacilli recovered from 200 gm of tissue was 1.4×10^{11} (7×10^8 bacilli/gm of tissue). The suspension of bacilli was divided into four 10 ml aliquots and kept at -70°C until used for analysis. *M. leprae* prepared by this method contained a moderate amount of liver tissue debris. *M. smegmatis* ATCC 19420, *M. phlei* ATCC 11758, and *M. diernhoferi* ATCC 19340 were obtained from the American Type Culture Collection, Rockville, Maryland. *M. vaccae* and *M. duvali* were obtained from Dr. Theodosia M. Welch, United States Public Health Service (USPHS) Hospital, San Francisco, California. Organisms were grown on Middlebrook and Cohn 7H10 agar base supplemented with OADC enrichment (BBL Div., Becton, Dickinson & Co., Cockeysville, Maryland). Cultures were grown at 37°C with 5% CO_2 .

Sera from 10 treated lepromatous leprosy (LL) patients were obtained from Dr. Theodosia M. Welch, USPHS Hospital, San Francisco, California. Five sera were obtained from patients treated for lepromatous leprosy at the USPHS Hospital, Seattle, Washington. A pool of sera from LL patients adsorbed to make it specific for *M. leprae* by indirect immunofluorescence was a gift kindly supplied by Dr. Masahide Abe, National Institute for Leprosy Research, Tokyo, Japan. The indirect fluorescent antibody titer to *M. leprae* of this serum pool was 1:2,560. Cross reacting antibody to other mycobacterial species was not de-

tectable. The sera was made *M. leprae* specific by Dr. Abe by adsorption with sonicated suspensions of *M. bovis* (BCG), *M. vaccae*, cardiolipin and lecithin. Control sera were obtained from 13 patients with chlamydial urethritis, from two patients with brucellosis and from 16 healthy persons after immunization with BCG (Baccillus Calmette Guérin). Eleven of the 16 persons converted to tuberculin (PPD) skin test positivity after BCG immunization. Tube agglutination antibody titers to the brucella antigen for the brucellosis sera were 1:2,500 and 1:20,000.

Armadillo sera. Sera from four armadillos systemically infected with *M. leprae* and two uninfected armadillo controls were provided by one of the authors (WFK) from the armadillo colony at USPHS Hospital, Carville, Louisiana. Infected armadillos were bled immediately before they were sacrificed for collection of infected tissue.

Antigen preparation. *M. leprae* (3.0×10^{10}) in buffered saline were mixed with 10 volumes of -20°C acetone. The suspension was kept at 4°C for 16 hours and then centrifuged at $10,000 \times g$ for 10 min. Acetone treated organisms were washed $\times 2$ in buffered saline and then pelleted bacilli were resuspended in 25 ml of 0.2 M lithium acetate containing 20 mM EDTA, pH 8.8. The solution was transferred to a 100 ml glass screw cap bottle. Thirty gm of glass beads 5 mm in diameter were added, and the mixture was agitated in a shaker water bath at 45°C for 2 hours. The liquid suspension was then centrifuged at $30,000 \times g$ for 20 min. at 4°C . The supernatant was removed and dialyzed at 4°C for 24 hr against 200 volumes of distilled water, followed by dialysis against phosphate buffered saline containing 0.02% sodium azide. The dialyzed supernatant was concentrated by vacuum dialysis to a final volume of one ml. Antigen extracts prepared from 600 mg wet weight of *M. smegmatis*, *M. phlei*, *M. diernhoferi*, *M. vaccae* and *M. duvali* were prepared identically to the procedure used for extraction of *M. leprae* antigen. Twenty gm of normal armadillo liver was homogenized in 60 ml of buffered saline containing 1% Triton X-100 and 20 mM EDTA, using an Ultra-Turrax homogenizer (Tekmar Co., Cincinnati, Ohio). The homogenate was centrifuged at $10,000 \times g$ for 20 min., and

the supernatant was retained as a control antigen. Protein concentration of antigen extracts was determined by the method of Lowry, et al. (¹⁰).

Enzyme treatment of *M. leprae* antigen was done using trypsin-TPCK, alpha-chymotrypsin (Worthington Biochemical Corporation, Freehold, New Jersey) and a pronase equivalent (protease from *Bacillus amyloliquefaciens*, Sigma Chemical Company, St. Louis, Missouri). Concentrated enzyme solutions were added to 45 μ l *M. leprae* antigen (5.6 mg/ml) to give a final concentration of 100 μ g/ml. Enzyme-antigen mixtures were incubated at 37°C for 24 hours. The effect of treatment on antigenicity was determined by immunodiffusion analysis performed at 4°C. The effect of weak alkaline hydrolysis on antigenicity was determined by adding 5 μ l of a 1 N NaOH to 45 μ l *M. leprae* antigen. The solution was kept at 20°C for 2 hours and was neutralized by the addition of 5 μ l of 1 N HCl.

Ouchterlony immunodiffusion analysis. The micro-technique of Sharpless and Le Grippio was used (¹³). Agarose (0.5%) in 10 mM N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES), containing 0.5% NaCl pH 7.3, was used as the support matrix. The surface of glass microslides (25 \times 75 mm) was evenly covered with 1.4 ml of the agarose-matrix. After the agarose had solidified, plastic templates were placed on the agarose surface, and 20 μ l of antigen or antiserum were added to the wells. The slides were incubated in a humidified chamber at 4 or 22°C for 48 hours. Slides were washed for 12 hours in 10 mM TES saline, overnight in distilled water, and then photographed.

RESULTS

Figure 1 illustrates that a single immunoprecipitate was formed when *M. leprae* antigen was reacted against sera from pooled patients made specific for *M. leprae* by adsorption. This same antigen was also precipitated by serum from an infected armadillo and a treated LL patient since a single line of identity was observed between these sera when tested against the *M. leprae* antigen extract (Fig. 1). Antibody specific for this antigen, as determined by a reaction of identity with the ad-

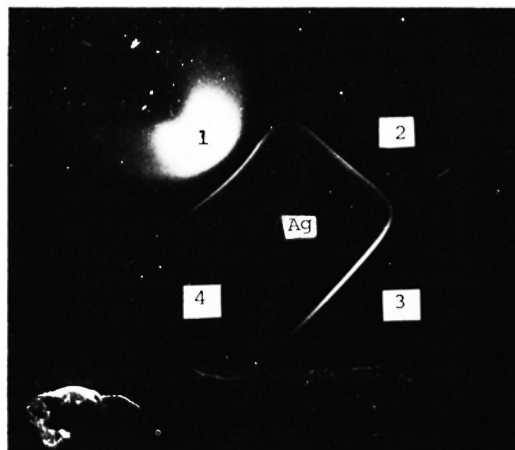


FIG. 1. Ouchterlony Immunodiffusion Precipitation. Ag = antigen of *M. leprae* extracted with LiAc-EDTA and concentrated. 1 = serum from *M. leprae* infected armadillo; 2 = serum from patient with treated lepromatous leprosy (LL); 3 = serum from LL patients adsorbed by Abe to make it monospecific for *M. leprae*; 4 = serum from uninfected control subject.

sorbed LL sera specific for *M. leprae*, was detected in 15 separate sera from patients with treated LL (Fig. 2). Additional antigens were also precipitated by some LL patients' sera. However, these antigens did not react with the LL sera made specific for *M. leprae* by adsorption, suggesting that they are not antigens unique for *M. leprae* organisms. Sera from 3 of 4 *M. leprae* infected armadillos precipitated the antigen whereas two sera from uninfected armadillos were negative. No precipitin reaction was observed from 15 human control sera from patients with either brucellosis or chlamydial urethritis. Serum from one of 16 persons immunized with BCG showed a weak positive reaction with the precipitin line near the serum well. Since the bacilli used for antigen preparation contained armadillo liver tissue, extracts from normal armadillo livers were also tested against the LL sera specific for *M. leprae*. Normal liver extracts were tested at protein concentrations equal to the *M. leprae* antigen extract (5 mg/ml), and at 1 and 10 mg/ml. No reactions were observed with any of the concentrations tested, indicating that the antigen was not of host origin.

Further evidence of an antigen(s) specific for *M. leprae* is shown in Fig. 3. Identically prepared lithium-acetate-EDTA extracts of

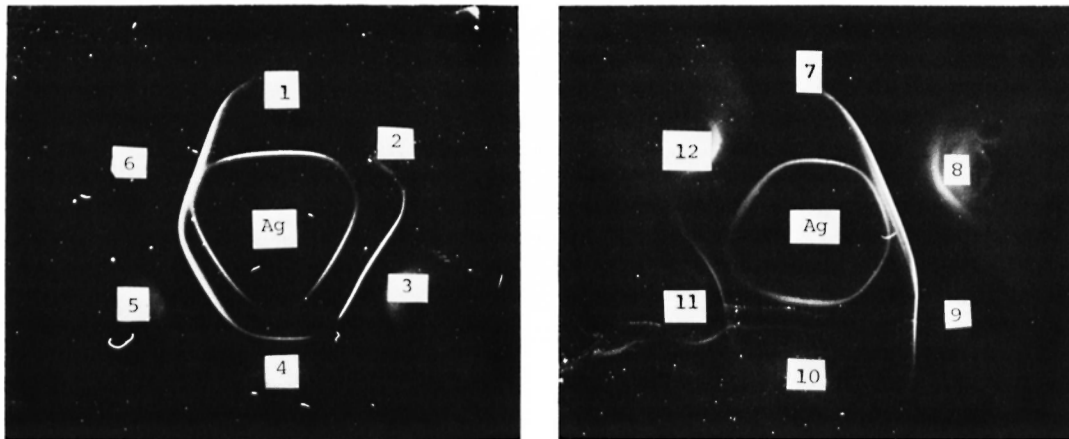


FIG. 2. Ouchterlony Immunodiffusion Precipitation. Ag = antigen of *M. leprae* extracted with lithium acetate-EDTA and concentrated (5.6 mg/ml protein); 1 and 7 = adsorbed pooled patients' serum, specific for *M. leprae* by immunofluorescence; 2-6 and 8-12 = sera from 10 patients with treated LL.

M. smegmatis, *M. phlei*, *M. diernhoferi*, *M. vaccae* and *M. duvali* did not react with the adsorbed LL serum for *M. leprae*. Extracts from each species were tested at protein concentrations at two-fold dilutions varying from 8 to 0.5 mg/ml, and this did not change the results.

Figure 4 illustrates that treatment of the *M. leprae* antigen with pronase, 0.1 N NaOH, or heating at 80°C for one hour de-

stroyed antigenicity. This suggests a protein nature for the *M. leprae* specific antigen. Irradiation (2×10^6 rads), trypsin, or alpha-chymotrypsin did not affect antigenicity.

DISCUSSION

The antigen describes at least one antigen that appears relatively specific for *M. leprae*. This study was not found in extracts of other mycobacteria and was precipitated by a

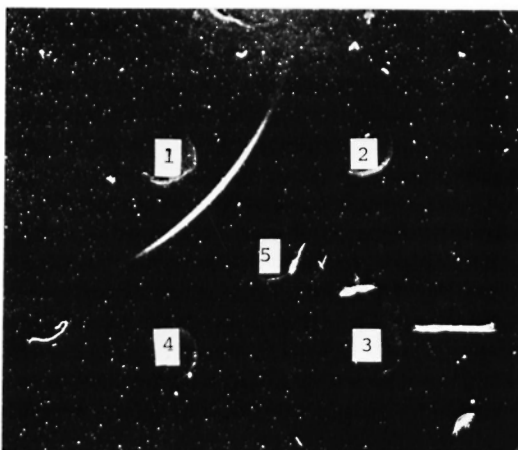


FIG. 3. Ouchterlony Immunodiffusion Precipitation. 1 = *M. leprae* antigen (5.6 mg/ml); 2 = *M. smegmatis* antigen (5.8 mg/ml); 3 = *M. phlei* antigen (5.4 mg/ml); 4 = *M. diernhoferi* antigen (5.0 mg/ml). Antigens were lithium acetate-EDTA, pH 8.8 concentrated extracts; 5 = pooled adsorbed serum from LL patients that is specific for *M. leprae* by immunofluorescence.

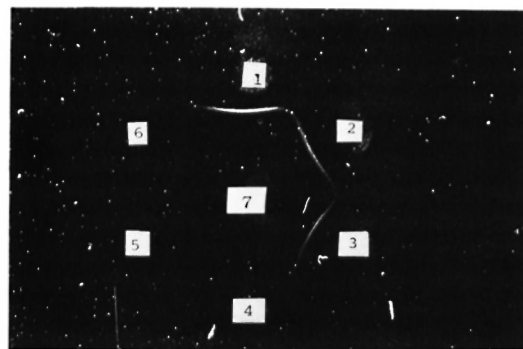


FIG. 4. Ouchterlony Immunodiffusion Precipitation. 1 = *M. leprae* antigen 2×10^6 rads irradiation; 2 = *M. leprae* antigen treated with trypsin 100 μ g/ml 37°C for 24 hr; 3 = *M. leprae* antigen treated with alpha-chymotrypsin 100 μ g/ml 37°C for 24 hr; 4 = *M. leprae* antigen treated with pronase 100 μ g/ml 37°C for 24 hr; 5 = *M. leprae* antigen treated with 0.1 N NaOH 20°C for 2 hr; 6 = *M. leprae* heated at 80°C for 1 hr; 7 = pooled adsorbed serum from LL patients that is specific for *M. leprae* by immunofluorescence. Immunodiffusion done at 4°C for 48 hours.

pool of sera from LL patients adsorbed to make it highly specific for *M. leprae*. Sera from 31 control subjects were non-reactive with the antigen except for one BCG immunized person, and further studies will determine whether the *M. leprae* protein antigen(s) shares any determinants with antigens of BCG. The antigen is a protein and is likely a surface component of *M. leprae* organisms. Evidence in support of the antigen's surface location are: a) the antigen was extracted from organisms using a hypertonic lithium-acetate-EDTA solution, a procedure used to selectively release outer-membranes from gonococci (⁷), and b) *M. leprae* exhibits a distinct "ring-like" fluorescence (personal communication from Dr. Abe) on the surface of the bacilli in addition to slight cytoplasmic fluorescence when stained with adsorbed antiserum specific for *M. leprae*. This indicates that at least some of the *M. leprae* specific protein antigen(s) is expressed on the organism's surface.

The relationship of the antigen in this study to the protein antigens described by Abe, et al., (²) and Navalkar (¹¹) obtained from homogenates of lepromatous nodules is not known. It is possible that the antigens are the same; however neither Abe nor Navalkar was successful in consistently demonstrating antibody in 15 of 15 patients which was specific for the antigen. It is therefore possible that the antigen(s) we have identified is different from those described previously. Kronvall, et al. (⁹) detected antigenic determinants shared with *M. avium-intracellulare* and *M. smegmatis*. The *M. leprae* specific antigen described here was not found in extracts of *M. smegmatis*, suggesting that the antigen(s) described by Kronvall, et al. is different. The recent publication of Harboe, et al. is consistent with our results and suggests that a protein antigen exists that is specific for *M. leprae* and that antibody to this antigen can be detected with a high frequency in patients with leprosy using a radioactive antigen binding assay (⁶).

The methods used to purify *M. leprae* from infected armadillo tissue have often utilized pronase,** which destroys this an-

tigen. Recently, pronase treatment has been eliminated in the purification of *M. leprae*. However, sodium hydroxide, trypsin, and chymotrypsin treatment have been recently employed.*** We found that sodium hydroxide treatment also destroys the antigen. Therefore, much of the immunologic research conducted using *M. leprae* purified by methods employing NaOH or pronase almost certainly did not evaluate the role of this protein antigen. We found no loss of antigenicity after trypsin or chymotrypsin treatment under the conditions utilized. Since the amino acid cleavage sites for trypsin and chymotrypsin are very specific, the cleavage products produced may be large polypeptides that retain their antigenicity as measured by Ouchterlony immunodiffusion. Trypsin or chymotrypsin may, however, cleave exposed surface proteins from *M. leprae* organisms during purification from infected tissue and thus might be harmful if used during *M. leprae* purification. There is therefore a need to develop improved methods for the purification of *M. leprae* that do not employ conditions that are likely to remove or destroy this antigen or other antigens that may be important in the understanding of the immunological response to *M. leprae*.

Since antibody to this antigen is detectable in the sera of treated LL patients, tuberculoid patients, and in sera from *M. leprae* infected armadillos, this protein may be important for an understanding of the immunologic response to *M. leprae* infections. If this protein antigen is indeed specific for *M. leprae*, use of this protein in a sensitive and quantitative immunoassay may allow detection of *M. leprae* infection prior to development of clinical leprosy. Such an assay would be a definite aid to strategies for the eradication of leprosy. Treatment early after infection might prevent development of lepromatous leprosy, remove the patient as a source of infection to others, and decrease the likelihood of the development of dapsone or other drug resistance. A quantitative assay for this *M. leprae* specific protein antigen(s) may clarify its role in the development of reversal or erythema nodosum leprosum reactions. Im-

** World Health Organization, Report of the Third IMMLEP Scientific Working Group Meeting, 21-25 February 1977, Geneva, Switzerland.

*** World Health Organization, Report of the Second IMMLEP Meeting, December 1977, Geneva, Switzerland.

munochemical characterization of this antigen(s) and an understanding of its optimum purification may allow sufficient quantities of it to be prepared for studies of cell-mediated immunity, such as the suitability of this antigen for skin testing, or as a vaccine. Finally, an assay for an *M. leprae* specific antigen and its antibody may help to characterize whether armadillos captured in the wild and found to have mycobacterial infections have, in some cases, infection with *M. leprae*.

SUMMARY

Acetone-killed *Mycobacterium leprae* separated from infected armadillo liver tissue without the use of proteases were treated with 0.2 M lithium acetate, 20 mM EDTA, pH 8.8 solution, and the concentrated antigen extract was analyzed by Ouchterlony immunodiffusion. The antigen extract gave a single immunoprecipitate when reacted with pooled lepromatous leprosy (LL) patients' sera made highly specific for *M. leprae* by adsorption. Apparently identical precipitates were produced by reacting the antigen extract with sera of each of 15 treated LL patients, 5 of 7 patients with tuberculoid leprosy, and 3 of 4 *M. leprae* infected armadillos. Serum from 1 of 16 persons immunized with BCG and from none of 15 patients with chlamydial urethritis or brucellosis reacted with the antigen. Identically prepared extracts of *M. smegmatis*, *M. phlei*, *M. vaccae*, *M. duvali* and *M. diernhoferi* gave no immunoprecipitates with sera from LL patients or infected armadillos. Preliminary characterization indicates the antigen is protein since antigenicity was destroyed by pronase and/or heat treatment. The relative specificity of the protein antigen for *M. leprae* and the presence of antibody to this antigen in patients with leprosy suggest a possible role for this antigen in the serodiagnosis of leprosy.

RESUMEN

Se separó el *Mycobacterium leprae* a partir de tejido hepático de armadillo, evitando el uso de proteasas. Los bacilos, matados con acetona, se trataron con una solución de pH 8.8, conteniendo acetato de litio 0.2 M y EDTA 20 mM. El extracto antigénico concentrado se analizó por inmunodifusión de Ouchterlony. El ex-

tracto dió una sola banda de precipitación cuando se hizo reaccionar con una mezcla de sueros hecha, por adsorción, altamente específica para el *M. leprae*. También se obtuvieron precipitados, aparentemente idénticos, cuando el extracto antigénico se hizo reaccionar con cada uno de los sueros de 15 pacientes con LL tratada, con 5 de 7 sueros de pacientes con lepra tuberculoide y con 3 de 4 sueros de armadillos infectados con *M. leprae*. Uno de 16 sueros de personas inmunizadas con BCG y ninguno de 15 pacientes con uretritis por clamidias o con brucelosis, reaccionaron con el antígeno. Los extractos de *M. smegmatis*, *M. phlei*, *M. vaccae*, *M. duvali* y *M. diernhoferi*, preparados de manera idéntica, no dieron inmunoprecipitados con los sueros de pacientes con LL o de armadillos infectados. La caracterización preliminar indica que el antígeno es protéico puesto que su antigenicidad se destruye con pronasa y/o por calentamiento. La asociación, relativamente específica, de este antígeno con el *M. leprae* y la presencia de anticuerpo contra este antígeno protéico en pacientes con lepra, sugiere un posible papel para este antígeno en el serodiagnóstico de la lepra.

RÉSUMÉ

On a traité par de l'acetate de lithium à 0,2 M, et 20 mM EDTA, en solution au pH 8,8, des bacilles *M. leprae* tués par l'acétone, et recueillis à partir du tissu hépatique de tatous, sans qu'il n'ait été fait usage de protéases. L'extrait antigénique concentré a été analysé par la méthode d'immuno-diffusion d'Ouchterlony. L'extrait antigénique a fourni un immuno-précipité unique, lorsqu'on a fait réagir l'extrait avec un mélange de sérum obtenu à partir de malades souffrant de lèpre lépromateuse, et rendu hautement spécifique pour *M. leprae* par adsorption. Des précipités apparemment identiques ont été produits à la suite de la réaction de l'extrait antigénique avec le sérum testé isolément, recueilli chez 15 malades traités souffrant de lèpre LL, chez 5 parmi 7 malades présentant une lèpre tuberculoide, et chez 3 de 4 tatous infectés par *M. leprae*. Par contre, un seul sérum sur 16, chez des malades immunisés par du BCG, et aucun sérum recueilli chez 15 malades souffrant d'urétrite chlamydiale ou de brucellose, n'a réagi avec l'antigène. Des extraits préparés de façon identique à partir de *M. smegmatis*, de *M. phlei*, de *M. vaccae*, de *M. duvali* et *M. diernhoferi*, n'ont fourni aucun immuno-précipité, lorsqu'on les a mis en contact avec le sérum de malades atteints de lèpre LL, ou le sérum de tatous infectés. Le relevé préliminaire des caractéristiques de cet antigène a indiqué qu'il s'agissait d'une protéine, car l'antigénicité est détruite par la pronase, ainsi que par le traitement par la chaleur. La spécificité relative de l'antigène protéinique pour *M. leprae*, de même que la présence d'anticorps à cet antigène chez des malades souffrant de lèpre, suggèrent que cet antigène pourrait jouer un rôle dans le sérodiagnostic de la lepre.

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