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Comparison of 22 Species of Mycobacteria by Immunodiffusion Against an Absorbed Reference Leprosy Serum[1]

Thomas P. Gillis, Masahide Abe, Ward E. Bullock, Oscar Rojas-Espinosa, Ethel Garcia-Ortigoza, Philip Draper, Waldemar Kirchheimer, and Thomas M. Buchanan[2]

Little is known about the antigenic mosaic of *Mycobacterium leprae*. Even less is known about the antigens which may be important in the various immunopathological phases of Hansen's disease. To date the only antigen of *M. leprae* for which the chemical composition is known is the broadly cross-reactive arabinogalactan polymer described by Estrada-Parra (5). More information about the antigens of *M. leprae* will be required to characterize the immune response in a disease as clinically and immunobiologically diverse as leprosy.

Evidence is mounting for the existence of *M. leprae*-specific antigens (1, 2, 3, 7, 9). Primarily protein antigens have been implicated; however, a glycolipid antigen recently described by Brennan (3) shows promise for species specificity. Caldwell, *et al.* (4) reported the existence of a putative *M. leprae*-specific protein antigen detected by immunodiffusion precipitation with a pooled leprosy serum (ARLS) adsorbed to make it specific for *M. leprae* in an immunofluorescence test as described by Abe, *et al.* (2). Immunoreactivity with ARLS was not seen in identically prepared extracts of *M. smegmatis*, *M. vaccae*, *M. phlei*, *M. diernhoferi*, and *M. duvali*.

We were interested in comparing the immunodiffusion precipitation patterns of *M. leprae* antigens with identical antigen extracts of other mycobacteria using this adsorbed reference leprosy serum (ARLS). This study summarizes the immunoreactiv-

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[2] T. P. Gillis, Ph.D., Immunology Research Laboratory, USPHS Hospital, 1131 14th Avenue South, Seattle, Washington 98114, U.S.A.; M. Abe, National Institute for Leprosy Research, Tokyo, Japan; W. E. Bullock, M.D., Division of Infectious Diseases, Department of Medicine, University of Cincinnati Medical Center, Cincinnati, Ohio 45267, U.S.A.; O. Rojas-Espinosa, Ph.D.; E. Garcia-Ortigoza, Ph.D., Department of Immunology, National School of Biological Sciences, Mexico City, Mexico; P. Draper, Ph.D., National Institute for Medical Research, The Ridgeway, Mill Hill, London, England; W. F. Kirchheimer, M.D., Ph.D., Laboratory Research Branch, USPHS Hospital, Carville, Louisiana 70721, U.S.A.; T. M. Buchanan, M.D., Immunology Research Laboratory, USPHS Hospital, 1131 14th Avenue South, Seattle, Washington 98114, U.S.A.

ity of ARLS with extracts from 22 species of mycobacteria.

MATERIALS AND METHODS

Organisms. The Hawaiian strain of *Mycobacterium lepraemurium* (MLM) was serially propagated *in vivo* by intraperitoneal injection of 6–8 week old female C3H/Anf mice (Cumberline View Farms, Quinton, Tennessee) with 0.2 ml of an inoculum 1×10^8 organisms. After 3 months the mice were sacrificed; heavily infected pelvic fat pads were removed by sterile technique, pooled, and frozen at −70°C until used.

To obtain infected livers and spleens, 1.0 g of thawed fat pad was homogenized in 30 ml of phosphate buffered saline (PBS), pH 7.2; the inoculum was adjusted to 1×10^8 bacilli in 0.2 ml after quantitation of the organisms by the method of Shepard and McRae [12] and delivered intravenously into other mice via the tail vein. Groups of mice were sacrificed at either 19 or 20 weeks to recover heavily infected livers and spleens. The Douglas strain of *M. lepraemurium* was also used in some experiments. These organisms were propagated in outbred white mice (Parks strain) and were harvested as above from heavily infected spleens and livers. Some of these infected tissues were kindly provided by Dr. R. J. W. Rees, National Institute for Medical Research, Mill Hill, London, England.

MLM were purified from mouse livers and spleens by gentle disruption of the infected tissue in a Potter-Elvenjheim homogenizer in PBS. The homogenate from 5 g of tissue was centrifuged at $500 \times g \times 5$ min and the supernatant fluid was removed and saved. The pellet was resuspended in 20 ml of PBS and the homogenization and centrifugation procedure was repeated. After three cycles of homogenization and centrifugation the three supernates were pooled and centrifuged at $10{,}000 \times g$ for 10 min at 4°C. The pelleted MLM were washed two times by repeat centrifugation with PBS and once with deionized water. Purified organisms were stained [11] and those preparations demonstrating minimal contamination by host tissues were frozen at −70°C until the antigen extracts were prepared. *M. leprae* were purified from experimentally infected armadillo livers by the method of Prabhakaran, *et al.* [10], and some additional *M. leprae* were purified by Dr. Philip Draper.

Strains of *Mycobacterium nonchromogenicum* (ATCC 19530), *Mycobacterium flavescens* (ATCC 14474), *Mycobacterium gastri* (ATCC 15754), *Mycobacterium tuberculosis* $H_{37}Ra$ (ATCC 25177), *Mycobacterium phlei* (ATCC 11758), *Mycobacterium diernhoferi* (ATCC 19340), *Mycobacterium smegmatis* (ATCC 19420), *Mycobacterium marinum* (ATCC 927), *Mycobacterium triviale* (ATCC 23290), *Mycobacterium thamnopheos* (ATCC 4445), *Mycobacterium peregrinum* (ATCC 14467), *Mycobacterium bovis* BCG (ATCC 19015), *Mycobacterium terrae* (ATCC 15755), *Mycobacterium gordonae* (ATCC 14470), and strains of *Mycobacterium vaccae* and *Mycobacterium duvali* obtained from Dr. Theodosia M. Welch, U.S. Public Health Service Hospital, San Francisco, California, were grown under appropriate environmental conditions in modified Sauton's media [13] with pyruvate added to a final concentration of 0.2%. The organisms were collected by centrifugation at $10{,}000 \times g \times 20$ min and washed twice with PBS and once with deionized water before storage at −70°C as a pellet. Maintenance of acid-fast staining, microscopic morphology and colonial morphology on solid media, as well as biochemical tests, were used as criteria for purity.

M. tuberculosis H37Rv (TMC 102), *M. intracellulare* (TMC 1403, "mais complex 16"), *M. microti* (TMC 1601), *M. scrofulaceum* (TMC 1321), and *M. kansasii* (TMC 1203) were obtained from the Trudeau Mycobacteria Collection (Trudeau Institute, Inc., Saranac Lake, New York) and were grown on Proskauer and Baker media (Difco) under appropriate environmental conditions. Cells were collected and washed as above and lyophilized before extraction.

Antigen Preparation. All organisms were extracted with lithium acetate as previously described [4] with the following modifications. The final $30{,}000 \times g \times 20$ min supernatant fluid was dialyzed against deionized water, lyophilized, and resuspended in a small volume of PBS. This preparation was designated 30 KS. When organisms had been lyophilized prior to extraction,

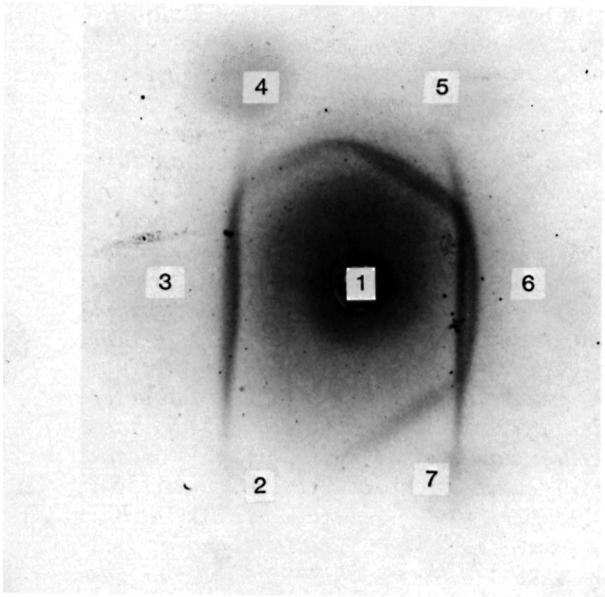

FIG. 1. Immunodiffusion precipitation between ARLS and pellet (P) or supernatant (S) fractions after centrifugation at 100,000 × *g* × 90 min of lithium acetate extracts of *M. leprae*, *M. bovis* BCG and *M. lepraemurium* (MLM). Wells contained 1—ARLS, 2—BCG(S) (100 μg/ml), 3—*M. leprae*(S) (266 μg/ml), 4—MLM(S) (100 μg/ml), 5—MLM (P) (75 μg/ml), 6—*M. leprae* (P) (75 μg/ml), and 7—BCG (P) (75 μg/ml).

TABLE 1. *Immunoreactivity of mycobacterial antigen extracts with ARLS*[a]

Organism	Immunoreactivity
M. lepraemurium	+[b]
M. bovis BCG (ATCC 19015)	+
M. nonchromogenicum (ATCC 19530)	I[c]
M. flavescens (ATCC 14474)	I
M. gastri (ATCC 15754)	I
M. gordonae (ATCC 14470)	I
M. tuberculosis H37Ra (ATCC 25177)	–[d]
M. tuberculosis H37Rv (TMC 102)	–
M. phlei (ATCC 11758)	–
M. vaccae	–
M. diernhoferi (ATCC 19340)	–
M. smegmatis (ATCC 19420)	–
M. duvali	–
M. intracellulare (TMC 1403)	–
M. microti (TMC 1601)	–
M. scrofulaceum (TMC 1321)	–
M. kansasii (TMC 1203)	–
M. marinum (ATCC 927)	–
M. triviale (ATCC 23290)	–
M. thamnopheos (ATCC 4445)	–
M. peregrinum (ATCC 14467)	–
M. terrae (ATCC 15755)	–

[a] ARLS refers to adsorbed reference leprosy serum pool, see Materials and Methods.

[b] + refers to one line of identity with *M. leprae* extracts.

[c] I refers to immunoreactivity, no clear line of identity.

[d] – refers to no immunoreactivity.

the dried material (200 mg) was suspended in 100 ml of acetone (−20°C) and held at 4°C with constant stirring for 18 hr. Cells were collected by centrifugation, washed in PBS, and extracted as described above. Protein concentrations of antigen extracts were determined by the method of Lowry, *et al.* ([8]). In some experiments, extracts of *M. leprae*, MLM, and *M. bovis* were further purified by centrifugation at 100,000 × *g* × 90 min at 4°C. The pellet (P) and supernatant (S) fractions were then analyzed by immunodiffusion precipitation against ARLS (see Fig. 1).

Sera. The reference serum (ARLS) used in this study was prepared by M. Abe as described previously ([4]). Briefly, a pool of sera from lepromatous leprosy patients was adsorbed with sonicates prepared from *M. vaccae*, *M. bovis* BCG, and an aqueous solution of cardiolipin/lecithin ([2]). The final adsorbed sera (diluted 1:10) was lyophilized and concentrated 10 fold by resuspension in deionized water to the original serum volume. Insoluble material was removed by centrifugation at 10,000 × *g* × 10 min. Immunodiffusion was performed as previously described ([4]). In addition, sera from 14 mice infected for 19 and 20 weeks with MLM were tested by immunodiffusion precipitation against the antigen extract of *M. leprae*.

RESULTS

Table 1 summarizes the immunoreactivity of lithium acetate antigen extracts from the 21 species of mycobacteria with ARLS. Only extracts of *M. lepraemurium* and *M. bovis* BCG showed an immunoprecipitation line of identity with the extracts from *M. leprae* when reacted with ARLS (Fig. 1). A second immunoprecipitation line formed between ARLS and the *M. leprae* antigen extract was not present with the extracts from *M. bovis* and MLM. Other antigen recognition, not clearly demonstrating lines

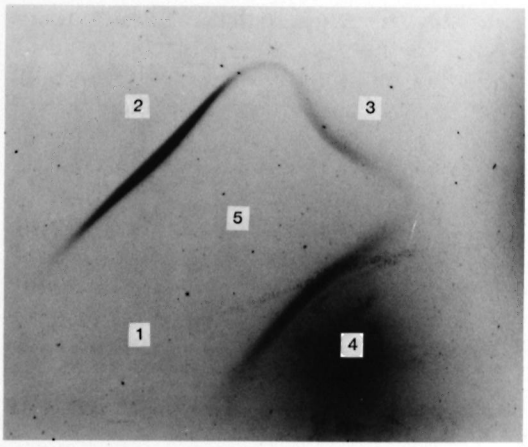

FIG. 2. Immunodiffusion precipitation reaction of lithium acetate extract (30 KS) of *M. leprae* (500 μg/ml, well 5) with ARLS (well 3), or with mouse sera (serum 8—well 1, serum 2—well 2, and serum 3—well 4).

of identity, occurred between ARLS and extracts of *M. nonchromogenicum, M. flavescens, M. gordonae,* and *M. gastri.* No immunoreactivity with ARLS was observed with any of the other 15 species of mycobacteria, including significant human pathogens such as *M. tuberculosis, M. intracellulare, M. scrofulaceum, M. kansasii,* and *M. marinum.*

Table 2 summarizes the immunoreactivity of the *M. leprae* antigen extract (30 KS) with sera from MLM infected mice. Four of the 14 sera produced an immunoprecipitation band that fused to form a line of identity with the precipitation band formed between ARLS and the same *M. leprae* antigen extract (Fig. 2). Three other mouse sera recognized different antigens in the *M. leprae* extract than in the ARLS serum, and seven other mouse sera were nonreactive in the test system (Table 2). Recognition of the ARLS identified antigens of *M. leprae* was more pronounced from sera of mice infected with MLM for 20 weeks (three of six sera positive) than after 19 weeks of infection (one of eight sera positive, Table 2).

TABLE 2. *Immunoreactivity of serum from MLM infected mice tested against* M. leprae *extract.*

Mouse sera	Length of infection (weeks)	Immunoreactivity		
		M. leprae identity[a]	Other antigens[b]	None
1	20	+		
2	20	+		
3	20	+		
4	19	+		
5	20		+	
6	20		+	
7	19		+	
8	20			+
9	19			+
10	19			+
11	19			+
12	19			+
13	19			+
14	19			+

[a] Serum recognized one or more of the same antigens identified by ARLS.

[b] Reaction with antigens other than *M. leprae* antigen(s) defined by ARLS.

DISCUSSION

The choice of the adsorbed reference leprosy serum (ARLS) used in this study was based upon the pioneering research of Abe, *et al.* ([1,2]). They found that most of the antibodies that recognize antigens shared between *M. leprae* and other mycobacteria could be removed from the serum of patients with lepromatous leprosy by adsorption with *M. bovis, M. vaccae,* and a mixture of cardiolipin and lecithin. The remaining antibodies were specific for *M. leprae,* and field test results from the Miyako Islands of Japan suggest that these antibodies, as detected in an indirect immunofluorescence assay (IFA), may indicate early infection with *M. leprae* ([2]). Since we were interested in defining *M. leprae* specific antigens, the ARLS reference serum was a logical starting point, though it may be less specific in immunodiffusion precipitation reactions after 10 fold concentration than it is in the IFA test.

Previously, using ARLS in an immunodiffusion precipitation test, we found reactivity with antigen(s) within a lithium acetate extract of *M. leprae* that was not present in four other species of mycobacteria ([4]). This antigen(s) was destroyed by heat, proteases, or NaOH, suggesting that it was protein in nature. Subsequent studies indicated that MLM shared some antigenic determinants with *M. leprae* as identified by ARLS ([6]). The present study increased

to 22 the number of species of mycobacteria that have been examined for antigens that react with ARLS in an immunodiffusion precipitation test. Two species, *M. bovis* (BCG) and *M. lepraemurium*, appeared to share antigens with *M. leprae*, as detected by ARLS in immunodiffusion precipitation. One of these, *M. bovis* (BCG), was used to produce the adsorbed ARLS. This adsorption decreased antibody levels sufficiently to remove false positive reactions in the IFA test among BCG vaccine recipients ([2]), providing the IFA test was performed with a fluoresceinated rabbit antihuman imunoglobulin that had been mixed 1:1 with a 5% (w/v) suspension of *M. bovis* BCG. In contrast, using immunodiffusion precipitation analysis under the conditions described, it is evident that some antibodies are able to precipitate BCG antigen(s) in agarose at antibody concentrations much higher than would be used for analysis in indirect immunofluorescence (IFA). In addition, it is conceivable that antibodies remaining to BCG in patients' sera post-adsorption may be competed for by BCG antigens in the second antibody reagent (fluoresceinated antihuman Igs) used in IFA.

Alternative explanations for BCG reactive antibodies in ARLS that are detected by immunodiffusion precipitation, but not by IFA, are: a) the antigen recognized by immunodiffusion precipitation is not externally located on *M. leprae* and therefore not recognizable by immunofluorescence; or b) the immune complexes present in ARLS contribute to the precipitin line formed with the BCG extract by immunodiffusion precipitation but are washed away prior to adding the fluorescent reagent in the IFA. The immunodiffusion precipitation pattern defined by ARLS with antigens in the lithium acetate extracts of *M. gastri, M. flavescens, M. gordonae*, and *M. nonchromogenicum* was one of non-identity, as compared to the precipitin line formed between ARLS and the *M. leprae* extract. Characterization of the precise nature of these antigens, and whether they are also found in *M. leprae*, will await further immunochemical identification of the antigens from each species.

These immunodiffusion precipitation results strongly suggest that ARLS contains antibodies that recognize antigens found on *M. leprae* and on a limited number of nonpathogenic mycobacteria. Specifically, 6 of 21 (29%) mycobacterial species other than *M. leprae* were recognized by ARLS (Table 1). The results further suggest that the antigenic determinants recognized by ARLS in lithium acetate extracts of MLM and *M. bovis* BCG are more closely related to determinants in identical extracts of *M. leprae* than to determinants recognized by ARLS in extracts of *M. flavescens, M. gastri, M. gordonae*, and *M. nonchromogenicum*. In addition, the reaction of partial identity indicated by the "spur" reaction between *M. leprae* and MLM suggests the existence of other *M. leprae*-specific antigenic determinants. However, definitive proof of whether *M. leprae* contains additional unique antigenic determinants not shared with any of the 6 cross-reactive or 15 non-cross-reactive species of mycobacteria is unlikely to be provided by immunodiffusion precipitation. Highly purified molecules of *M. leprae* and other mycobacteria, or monoclonal antibodies to *M. leprae*, will likely be required to indicate the existence of antigenic determinants specific to *M. leprae*.

A quantitative immunoassay based upon the protein antigen(s) of *M. leprae* or MLM that is recognized by ARLS might prove useful for the serodiagnosis of subclinical infection with *M. leprae*. If the mycobacteria that contain cross-reactive antigens with *M. leprae* do not cause sufficient human infection to stimulate an antibody response, the presence of antibody would suggest infection with *M. leprae*. The cross-reactive mycobacteria in this study, MLM, *M. bovis* BCG, *M. flavescens, M. gastri, M. gordonae*, and *M. nonchromogenicum*, are not common human pathogens and thus might infrequently cause cross-reacting antibodies to *M. leprae*. In contrast, significant human pathogens, including *M. tuberculosis, M. intracellulare, M. scrofulaceum, M. kansasii*, and *M. marinum*, showed no cross-reactive antigens recognizable by ARLS. Many further tests of sera from leprosy patients and their contacts, as well as control sera from patients with other mycobacterial infections

and persons immunized with BCG, will be required to accurately define the serodiagnostic usefulness of an immunoassay based upon the protein antigen(s) of MLM or *M. leprae*.

SUMMARY

Lithium acetate antigenic extracts of 22 species of acetone-treated mycobacteria were tested by immunodiffusion precipitation for reactivity with a pool of sera from treated lepromatous leprosy patients (ARLS). This ARLS had been adsorbed with *M. bovis* (BCG), *M. vaccae*, cardiolipin, and lecithin to make it specific for *M. leprae* when used in an indirect immunofluorescence test. The ARLS produced two precipitin lines with *M. leprae* extract, one of which formed a line of identity with extracts of *M. lepraemurium* and *M. bovis* (BCG). Also, recognition without reactions of identity was produced between ARLS and *M. flavescens*, *M. gastri*, *M. gordonae*, and *M. nonchromogenicum*. The ARLS did not recognize the 15 other species including the human pathogens, *M. tuberculosis*, *M. intracellulare*, *M. kansasii*, *M. scrofulaceum*, and *M. marinum*. These data suggest that serologic tests for *M. leprae* infection might be affected by antibodies to antigens shared by *M. leprae* and other mycobacteria. The significance of these shared antigens will depend upon the prevalence of human immune responses to mycobacteria containing the shared antigens in any given community.

RESUMEN

Los antígenos extraídos con acetato de litio a partir de 22 especies de microbacterias tratadas con acetona, se hicieron reaccionar, por inmunodifusión en gel, contra una mezcla de sueros de pacientes con lepra lepromatosa tratada. Este antisuero (ARLS) se hizo especifico para *M. leprae* por absorción con *M. bovis* (BCG), *M. vaccae*, cardiolipina y lecitina, según una prueba de inmunofluorescencia indirecta. El ARLS produjo dos lineas de precipitación con el extracto de *M. leprae*, una de ellas fue de identidad con los extractos de *M. lepraemurium* y *M. bovis* (BCG). También hubo reconocimiento sin reacciones de identidad con *M. flavescens*, *M. gastri*, *M. gordonae* y *M. nonchromogenicum*. El ARLS no reconoció a las otras 15 especies incluyendo a los patógenos del hombre *M. tuberculosis*, *M. intracellulare*, *M. kansasii*, *M. scrofulaceum*, y *M. marinum*. Estos datos sugieren que las pruebas serológicas diseñadas para detectar la infección con *M. leprae* pueden ser afectadas por la presencia de anticuerpos contra antígenos compartidos por *M. leprae* y otras micobacterias. La relevancia de estos antígenos compartidos dependerá de la reactividad inmune de los humanos hacia las micobacterias que contienen los antígenos compartidos, en una comunidad dada.

RÉSUMÉ

Au moyen d'une méthode de précipitation par immunodiffusion, on a étudié des extraits antigéniques traités par l'acétate de lithium, et provenant de 22 espèces de mycobactéries traitées par l'acétone, quant à leur capacité de réaction à l'égard d'un pool d'échantillons de sérums obtenus chez des malades lépromateux traités (ARLS). Cette ARLS a été absorbée par *M. bovis* (BCG), *M. vaccae*, la cardiolipine, ainsi que la lécithine, afin de la rendre spécifique pour *M. leprae* lors de l'utilisation d'une épreuve d'immunofluorescence indirecte. Cette ARLS a produit deux lignes de précipitation avec l'extrait de *M. leprae*, dont l'une est identique à la bande formée par des extraits de *M. lepraemurium* et de *M. bovis* (BCG). On a également observé une reconnaissance, mais sans réactions identiques, entre ARLS et *M. flavescens*, *M. gastris*, *M. gordonae*, et *M. nonchromogenicum*. Cette ARLS ne permet pas de reconnaître 15 autres espèces, y compris les pathogènes humains, *M. tuberculosis*, *M. intracellulare*, *M. kansasii*, *M. scrofulaceum*, et *M. marinum*. Ces données suggèrent que les épreuves sérologiques utilisées pour reconnaître l'infection par *M. leprae*, peuvent être modifiées par des antigènes dirigés par des anticorps partagés par *M. leprae* et par d'autres mycobactéries. La signification de ces antigènes communs dépendra de la prévalence des réponses immunitaires humaines aux mycobactéries contenant les antigènes communs, dans une communauté donnée.

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