INFECTION AND IMMUNITY, Dec. 1977, p. 792-805

Vol. 18, No. 3
Printed in U.S.A.

Antibody Response in Rabbits to Immunization with *Mycobacterium leprae*

M. HARBOE,[1]* O. CLOSS,[1] B. BJORVATN,[2] G. KRONVALL,[2] AND N. H. AXELSEN[3]

University of Oslo, Institute for Experimental Medical Research, Ullevaal Hospital, Oslo 1, Norway[1]; The Armauer Hansen Research Institute, Addis Ababa, Ethiopia[2]; and The Protein Laboratory, University of Copenhagen, Copenhagen, Denmark[3]

Received for publication 11 March 1977

Mycobacterium leprae purified from liver tissue of an infected armadillo (the A/10 preparation) was tested for antigenic composition by immunization of rabbits and characterization of the antibody response by crossed immunoelectrophoresis. The rabbit antisera detected seven distinct components in the *M. leprae* preparation. This number is far lower than in similar experiments with other mycobacteria. The *M. leprae* sonic extract gave far fewer lines after polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue than sonic extracts prepared from BCG, *M. smegmatis*, and *M. phlei* adjusted to the same protein concentration based on the Folin assay. The seven components detected in *M. leprae* cross-reacted extensively with *M. avium*, BCG, *M. lepraemurium*, *M. smegmatis*, and *Nocardia asteroides*. The seven components are involved in immune reactions in leprosy; antibodies against all of them were demonstrated in sera from patients with lepromatous leprosy, but the specificity of the antibodies varied from patient to patient. The reason for the demonstration of so few antigenic components and some of the implications of these findings for the use of armadillo-grown *M. leprae* to develop specific skin test reagents and in other aspects of leprosy research are discussed.

Since *Mycobacterium leprae* cannot be grown in vitro, bacilli have been available in only limited amounts, and studies on the antigenic composition have previously been made on bacilli purified from nodules of patients with lepromatous leprosy in comparison with cultivable mycobacteria. Carbohydrate antigens cross-reacting with other mycobacteria have been identified, but there is only limited information on their molecular structure (1, 12, 13, 26, 34). By immunodiffusion tests on soluble components present in homogenates of lepromatous nodules, Abe has provided evidence of an *M. leprae*-specific protein antigen (1, 2).

Since the demonstration that *M. leprae* may grow and establish a severe systemic infection in the nine-banded armadillo (8, 18-20), larger amounts of *M. leprae* bacilli grown in vivo have become available, and this source of *M. leprae* is currently being intensively exploited to obtain more information on the basic properties of the leprosy bacillus. Increased information on its antigenic structure is greatly needed.

The immune response of the host is of decisive importance for the clinical course after infection with *M. leprae* (14, 25, 28, 29, 36, 37). If the response is effective after exposure to leprosy bacilli, the individual may develop a few skin lesions that heal spontaneously, or he may develop no symptoms at all. In other cases, the bacilli survive and multiply in host tissues, particularly in the skin and peripheral nerves. Immune reactions against bacillary components may then induce nerve damage and are thus responsible for the deformities often associated with leprosy. Lepromatous leprosy with apparent uninhibited growth of the bacilli is characterized by lack of cell-mediated immune reactions against *M. leprae* (25, 36), and suppression of cell-mediated immunity in *M. leprae*-infected mice leads to systemic disease very similar to lepromatous leprosy (27). Characterization of the immune response to the various antigens of *M. leprae* is of great interest, as it has been demonstrated that variation in specificity may be related to important clinical symptoms, particularly during reactions with induction of nerve damage (7, 9). Advances in this field require additional information concerning the antigenic composition of *M. leprae*.

Further development of reliable methods for diagnosis of subclinical infection, e.g., by skin testing, depends on the production of suitable antigenic preparations of *M. leprae* and better methods of characterizing their composition and specificity.

The taxonomic relationship of *M. leprae* to other mycobacteria (23, 31-33) has not been defined in detail, since most of the usual criteria for such analyses cannot be applied due to lack of growth in vitro. Additional information on this matter is of crucial importance if an attempt to develop a vaccine against leprosy is to be based on the use of a related mycobacterium. Studies on the antigenic structure of *M. leprae* are expected to be of particular importance in this respect.

The purpose of this investigation was to study the antigenic composition of *M. leprae* by characterization of the specificity of the antibody response in rabbits during immunization with purified *M. leprae* obtained from infected armadillos. To obtain as much information as possible, the specificity of the antibodies was carefully followed during the early immunization. Cross-reactions between the demonstrated antigenic components of *M. leprae* and other mycobacteria were studied. Finally, evidence was sought on whether the demonstrated antigenic components are involved in antibody production against the leprosy bacillus during clinical disease in man.

MATERIALS AND METHODS

Mycobacteria and related bacilli. *M. leprae* was provided from the World Health Organization's Immunology of Leprosy (IMMLEP) program by R. J. W. Rees and P. Draper as freeze-dried bacilli (the A/10 preparation). The bacilli were purified by the Draper method (11) from liver tissue of armadillo no. 29 received by IMMLEP from W. F. Kirchheimer in February 1975. In the liver, 18% of the acid-fast bacteria were solid staining. Armadillo no. 29 was inoculated with bacilli prepared from a skin biopsy from a leprosy patient from the Phillipines. The animal developed a severe systemic infection with acid-fast bacilli having features characteristic of the species *M. leprae*. They were noncultivable on regular media, and two separate inoculations into mouse footpads gave histologically typical lesions with growth of acid-fast bacilli within nerves. Among other armadillos inoculated with the same material, three have developed severe systemic infection with noncultivable mycobacteria showing characteristic features of *M. leprae* upon mouse foot-pad inoculation and in the pyridine extraction test (W. F. Kirchheimer, personal communication).

M. bovis (BCG) was obtained as 14-day cultures on Sauton medium from K. Bunch-Christensen, Statens Seruminstitut, Copenhagen, and was identical to the vaccine strain distributed by this laboratory.

M. avium (Maren Cecilie), *M. smegmatis* (NCTC 8159), and *Nocardia asteroides* (no. 9974, Emmons, U.S.A.) were obtained from Mogens Magnusson, Statens Seruminstitut, Copenhagen.

M. duvalii (NCTC 358) was obtained from Bjørn Myrvang, Armauer Hansen Research Institute, Addis Ababa, Ethiopia. The bacilli were cultured on Sauton medium, harvested as soon as sufficient growth had been obtained, and stored at −20°C until they were used for antigen preparation.

***M. leprae* antigen preparations for immunization and testing.** Sixty milligrams of freeze-dried *M. leprae* was added to 10 ml of 0.9% NaCl and sonified for 15 min under cooling on ice, using the Branson B12 Sonifier (Branson Sonic Power Co., Banbury, Conn.) with 80 W of effect. The sonic extract was centrifuged at 20,000 × *g* for 15 min at 4°C. Three milliliters of the supernatant fluid were pipetted off to be used as antigen in crossed immunoelectrophoresis (CIE). It contained 1 mg of protein per ml, as determined by a modified Folin technique (24) with human serum as standard, and was stored at 4°C with 15 mM NaN_3. The pellet was suspended in the remaining supernatant, briefly sonified to ensure complete mixing, and then used for immunization.

The *M. leprae* antigen was mixed with Freund incomplete adjuvant (1/1, vol/vol) and injected intradermally in five rabbits (3200, 3204, 3205, 3207, and 3217) in doses of 100 μl delivered at multiple sites in the neck region and above the scapulae at days 2, 16, 30, 44, 72, and 100 and every 4th week from then on. About 50 ml of blood was taken from the ear vein at day 0 (preimmune sample), 14 (tap 1), 28 (tap 2), 42 (tap 3), 56 (tap 4), 84 (tap 5), and 10 days after each of the succeeding immunizations. The sera were stored at 4°C with 15 mM NaN_3.

Immunization with antigen preparations from other mycobacteria and *N. asteroides*. Antigen was prepared from *M. avium, M. smegmatis, M. duvalii, M. bovis* (BCG), and *N. asteroides* as follows: 600 mg (wet weight) of bacilli was suspended in 10 ml of 0.9% NaCl and sonified in the same way as *M. leprae*. After centrifugation, 3-ml portions of the supernatants were taken off to be used as antigens in CIE. They contained 1.0 to 1.4 mg of soluble proteins per ml, as determined by the Folin technique, and were stored at 4°C with 15 mM NaN_3. The remaining materials were mixed and briefly sonified. They were then mixed with Freund incomplete adjuvant (1/1, vol/vol) and injected intradermally in rabbits in doses of 100 μl, delivered at multiple sites on days 0, 14, 28, 42, and 56 and thereafter once a month. The rabbits were bled 8 days after injection 4 and each of the subsequent injections. For each mycobacterial species, four or five rabbits were immunized.

Other antibodies. Four monospecific antisera were made against selected bacterial antigens by immunization of rabbits with immune precipitates cut out of gels after CIE. Such antisera were made against two antigens of BCG (15, 16), two antigens of *N. asteroides*, and antigen 13 of *M. lepraemurium* (MLM) (10).

Lepromatous sera. The sera were obtained at the Armauer Hansen Research Institute from patients attending the Addis Ababa Leprosy Hospital. Age, sex, and severity of disease were not taken into account during selection of patients. Individual sera were obtained from 21 previously untreated patients whose disease had been clinically and histopathologically classified as lepromatous leprosy (LL, LI, and BL) (21, 29, 30). The lepromatous pool was made from 43 individual sera.

CIE. CIE with intermediate gel was carried out and the plates were washed, pressed, and stained with Coomassie brilliant blue R-250 as described in detail elsewhere (6). Briefly, CIE was performed on glass plates (5 by 7 cm) in 1% agarose, Litex type HSA with moderate electro-endosmotic flow, batch no. 0264 (Litex, Glostrup, Denmark). The top gel contained 200 μl of antiserum, and the intermediate gel contained 100 μl of buffer, preimmune serum, concentrated antimycobacterial immunoglobulin, or antiserum, as specified in the text. The circular antigen well contained 10 μl of antigen solution prepared as described above.

Polyacrylamide gradient gel electrophoresis. This was kindly performed by B. G. Johansson at the Clinical Chemistry Laboratory, University Hospital, Lund, Sweden, using Pharmacia electrophoresis apparatus GE-4 and prefabricated Pharmacia gel PAA 4/30. After electrophoresis, the gels were fixed and stained with Coomassie brilliant blue.

Nomenclature. Various principles may be used for designation of components in CIE when several complex and partly cross-reacting systems are established and compared with each other. We have decided to number each system logically and independent of the others and to specify the components that correspond to each other as this is established. It is probable that *M. leprae* contains more antigenic components than those presently detected, but, to use the system, each component must be given its own designation even though it is provisional. Numbers were therefore assigned to the different components, starting with 1 for the most anodic component, and independent of previous designations of antigenic components of *M. leprae*. Table 1 indicates how these numbers correspond to defined components of other mycobacteria.

RESULTS

Antibody response in rabbits. Preimmune serum and sera from the subsequent bleedings of each of the five rabbits immunized with *M. leprae* were incorporated in the upper gel of CIE plates to characterize the antibody response. When preimmune serum was used, a faint fuzzy and skew precipitin line extended from the antigen well in some instances. In CIE of sonic extracts of *M. bovis* (BCG) with normal rabbit serum in the upper gel, a similar fuzzy precipitate often appears, probably due to interaction between BCG antigen 60 and normally occurring antibodies in rabbit serum (16). A cross-reacting antigen occurs in *M. leprae* sonic extracts and is probably responsible for the fuzzy precipitate seen in Fig. 1A, which is clearly different from all precipitates that appeared later during immunization.

TABLE 1. *Designation of M. leprae antigens*

Component	Other designations and known cross-reactions with defined components of other mycobacteria
1	
2	
3	
4	Called *M. leprae* antigen 21 by Kronvall et al. (21). Cross-reacts with *M. smegmatis* antigen 21 (21, 22) and *MLM* antigen 13 (10) as defined by incorporation of monospecific anti-MLM 13 in intermediate gels of CIE.
5	Called *M. leprae* antigen 1 by Kronvall et al. (21). Cross-reacts with *M. smegmatis* antigen 1 and *MLM* antigen 14.
6	Probably a carbohydrate antigen.
7	Cross-reacts with BCG antigen 60 (16) and *MLM* antigen 38 (10) as defined by incorporation of monospecific anti-BCG 60 (16) in intermediate gels of CIE.

Serum from tap 1 of four rabbits gave two to three distinct but rather faint precipitin lines. From tap 2, strong precipitin lines appeared. The overall picture was that the precipitin lines became rapidly stronger and increased in number to five to seven distinct lines, and this number did not increase further during 3 to 4 months of immunization. There was a striking similarity in the response of the various rabbits. Precipitin lines 2, 5, and 7 were very prominent, appeared early in the response, and were developed with serum from all of the five rabbits immunized with *M. leprae*. Two additional lines were seen with some of the antisera, but they were too weak to permit further analysis.

Figure 1 illustrates the typical development of antibody activity in rabbit serum during immunization with the *M. leprae* antigen. This immune response was distinctly different from the response during similar immunization of rabbits with a series of cultivable mycobacteria. In the latter case, antisera obtained 50 days after beginning of immunization usually gave more than 20 precipitin lines, and, after prolonged immunization, usually precipitated more than 40 antigenic components in the mycobacterial extracts. The difference in response to immunization with *M. leprae* and *M. smegmatis* antigen is illustrated in Fig. 2. Concentrated immunoglobulin from a pool of taps 3 and 4 of our anti-*M. leprae* antisera reacted with the same but not with more antigenic components than in the A/10 preparation in sonic extracts prepared from five different batches of armadillo-grown *M. leprae*. The concentration of the various antigens varied somewhat in the different sonic extracts. The line of component 3 is quite weak, and this antigen was not demonstrated in all sonic extracts.

We chose to characterize the specificity of the response to *M. leprae* in the five rabbits by comparison with the pattern given by serum from tap 5 of rabbit 3204. This serum gave the maximal number of precipitin lines, and all of

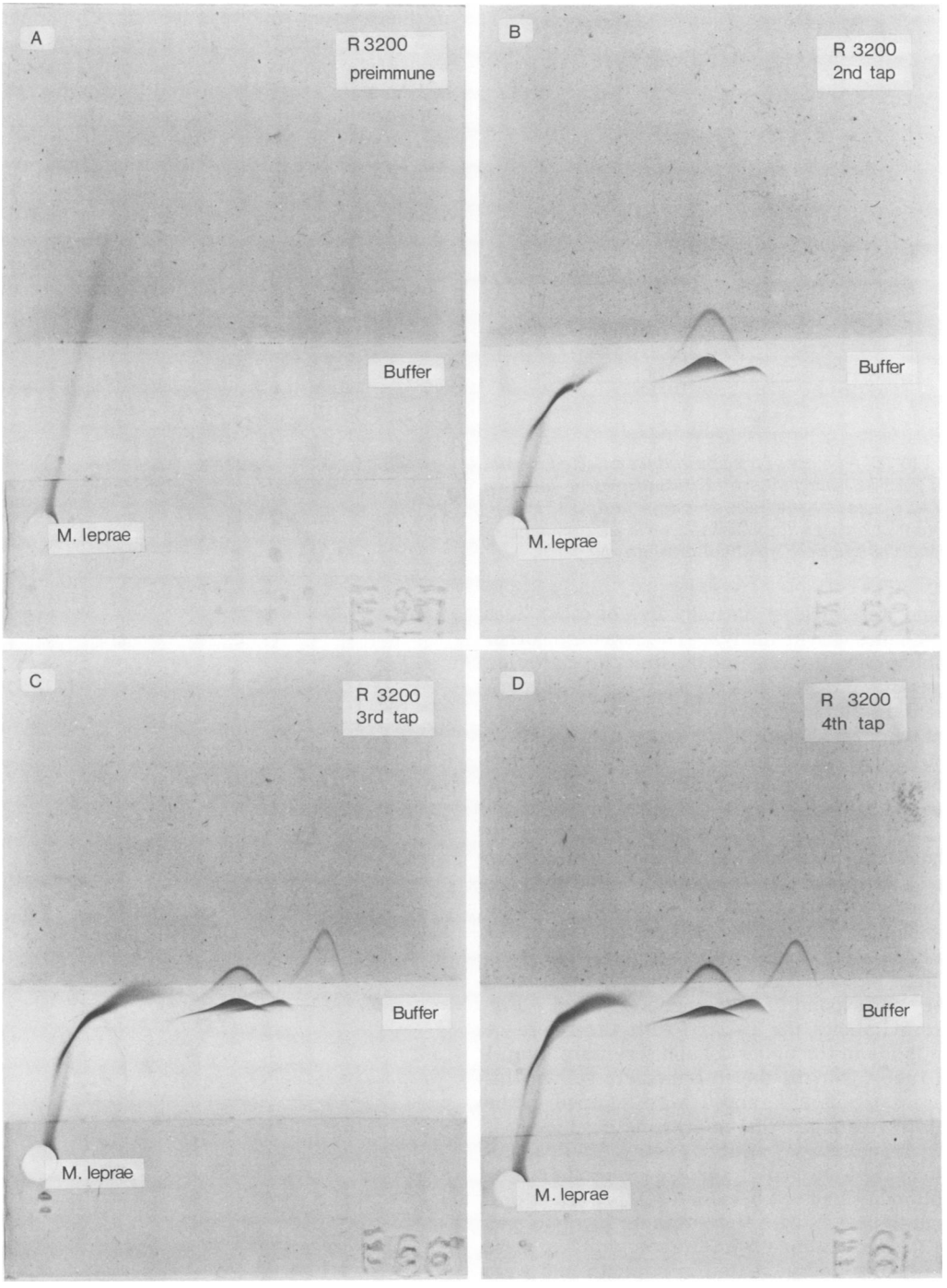

FIG. 1. *Development of antibody activity in rabbit 3200 during immunization with M. leprae. The sera were tested by direct precipitation in CIE against M. leprae sonic extract. The anode is to the right in the first-dimension electrophoresis and at the top in the second-dimension electrophoresis in all CIE plates shown.*

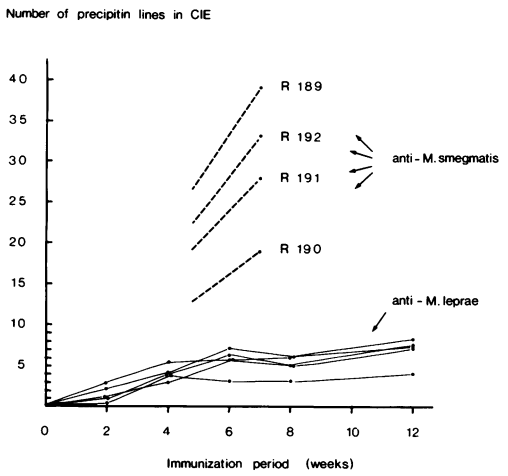

FIG. 2. *Number of precipitin lines detected by rabbit anti-M. smegmatis and anti-M. leprae antisera in CIE against the corresponding antigens. Preparation of antigen from mycobacteria and immunization schedule were identical until day 50.*

them were at least partially free of other lines and hence easy to analyze. The sera of the other rabbits were investigated by direct precipitation tests and intermediate gel analysis. The experimental setup is illustrated in Fig. 3.

Figure 3A shows the reference pattern where the upper gel contained the selected reference antibody, the intermediate gel contained the normal rabbit serum, and the antigen well contained the *M. leprae* sonic extract. Figure 3B shows the pattern obtained when serum from tap 1 of rabbit 3207 was incorporated into the upper gel, with normal rabbit serum in the intermediate gel and the A/10 preparation in the antigen well. Three precipitin lines are seen. Their position and appearance indicate that they correspond to components 2, 5, and 7. Figure 3C shows the pattern with the reference antibody in the upper gel and serum from tap 1 of rabbit 3207 in the intermediate gel. In this case antigens 2, 5, and 7 were retained in the intermediate gel. The intermediate gel analysis also revealed weak antibody activity in this sample against antigens 1 and 4, even though these antibodies had not been detected by direct precipitation tests with the serum in the upper gel. In the next sample from the same rabbit, these antibodies had increased in strength and were now detectable by direct precipitation.

The antibodies of the other rabbits reacted with the same components of the A/10 *M. leprae* antigen as the precipitating antibodies in tap 5 of rabbit 3204. Table 2 summarizes the findings concerning the specificity of the antibodies. The similarity in the antibody response in the different rabbits is striking. Components 2, 5, and 7 induced strong and early responses in all of the rabbits. Antibodies against the other components usually appeared later; when they appeared in an individual rabbit, they usually increased in strength in the next bleeding and were detectable in later bleedings, but exceptions to this rule were noted. The sera obtained from rabbit 3204 gave a double precipitin line with component 4, whereas all of the other antisera gave a sharp line with this component. Control experiments were made to ascertain that

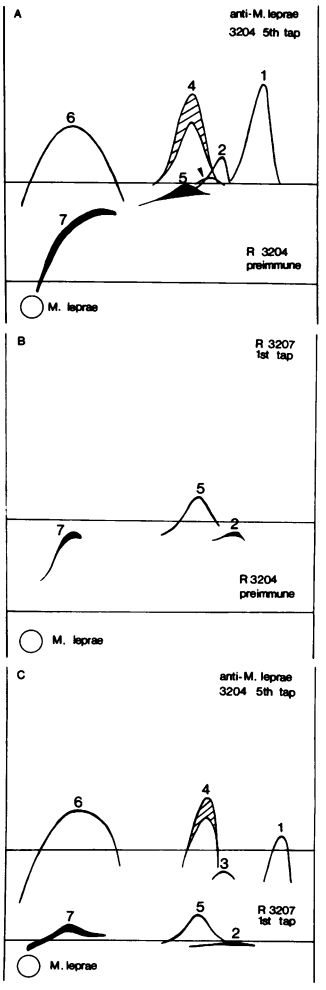

FIG. 3. *Demonstration of antibody activity in serum 3207 by CIE. 3A shows the reference pattern. The different components have been numbered; for the sake of clarity, component 3 is indicated with a small pointer. 3B shows that three precipitin lines were developed with tap 1 of serum 3207 in the top gel. 3C shows that additional weak antibodies against M. leprae antigens 1, 3, and 4 were detectable by incorporation of the same serum in the intermediate gel.*

TABLE 2. *Development of anti-M. leprae antibodies as shown by direct precipitation in CIE with serum in the top gel and M. leprae sonic extract (A/10) in the antigen well*

Component	Rabbit no.																									Reaction
	3200					3204					3205					3207					3217					
	1[a]	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	
1	−	−	+	+	+	−	−	−	−	+	−	−	−	−	+	−	+	+	+	?	−	−	−	−	−	
2	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Very strong line
3	−	−	+	?	?	−	−	+	+	+	−	−	+	+	+	−	−	−	−	?	−	−	−	−	+	
4[b]	−	+	+	+	+	−	−	+	+	+	−	+	+	+	+	−	+	+	+	+	−	+	−	−	−	Strong line in some
5	(+)	+	+	+	+	−	+	+	+	+	−	+	+	+	+	+	+	+	+	+	−	−	+	+	+	Very strong line
6	−	−	+	−	−	−	−	−	+	+	−	−	+	−	−	−	−	−	−	−	−	+	−	−	−	Diffuse
7	−	+	+	+	+	−	(+)	+	+	+	−	+	+	+	+	+	+	+	+	+	−	+	+	+	+	Very strong line

[a] Tap number.

[b] Serum 3204 gave a double precipitate with this component.

none of these seven components were contaminating antigens of armadillo origin. Incorporation of potent antibodies against armadillo liver or armadillo liver homogenates in the intermediate gel of CIE did not affect any of these precipitin lines.

Polyacrylamide gel electrophoresis of sonic extracts of *M. leprae* and other mycobacteria. Since the rabbit antisera detected far fewer components in the ultrasonic extract made from the A/10 preparation of *M. leprae* than in sonic extracts prepared from other mycobacteria, the *M. leprae* sonic extract was compared with other sonic extracts by a completely independent technique. Sonic extracts were made in the same way from BCG, *M. smegmatis*, *M. phlei*, and *M. leprae* suspended in saline. The protein concentration determined by the Folin technique varied from 1.0 to 1.4 mg/ml; it decreased about 20% upon dialysis against phosphate-buffered saline probably due to removal of peptides of low molecular weight. The concentration was adjusted to 1 mg/ml before discontinuous polyacrylamide gel electrophoresis. Figure 4 shows the pattern obtained after staining with Coomassie brilliant blue. BCG, *M. smegmatis*, and *M. phlei* sonic extracts gave more than 30 distinct bands. The *M. leprae* preparation showed only three bands (indicated by the arrows); one line was very weak and did not reproduce on the photograph. By similar electrophoresis of *M. leprae*, with the sonic extract concentrated ×5, the same pattern with three lines was obtained. The experiment thus confirmed that the *M. leprae* preparation contained far fewer components than the other sonic extracts.

Cross-reactions with other mycobacteria and *N. asteroides*. Each of the antigenic components detected in *M. leprae* was analyzed for cross-reactions with other mycobacteria. As shown previously (10), cross-reactions may be detected by incorporating antibodies against other mycobacteria in the intermediate gel. If an antigenic component cross-reacts with another mycobacterial species, and if it has induced an antibody response during immunization of rabbits with the other mycobacterium, an antiserum against the second species will retain the relevant antigen when it is incorporated into an intermediate gel in CIE. The results of such experiments are summarized in Table 3. Table 3 shows how various antibodies reacted with the individual antigenic components of *M. leprae*.

Anti-BCG 60 antiserum (16) had a profound effect on *M. leprae* antigen 7. Antigen 6 was also affected. A similar pattern is seen in several mycobacterial species. Our current evidence indicates that line 7 is due to reaction with a glycoprotein that shows extensive cross-reactions between various mycobacterial species, whereas antigen 6 is of carbohydrate nature and contains only some of the antigenic determinants that are present on antigen 7. Serum 738 reacts with an antigen of BCG (15) that cross-reacts with *M. tuberculosis* but few other mycobacteria. Serum 662 made against a precipitate with an antigen from *N. asteroides* reacts with a single antigen present in almost all mycobacteria tested (unpublished observations). These two antisera did not affect any precipitin lines in the *M. leprae* pattern. Monospecific antiserum against *N. asteroides* antigen 10 reacted strongly with *M. leprae* antigen 4, as did monospecific antiserum against MLM antigen 13 (10). Extensive separate experiments have indicated that *N. asteroides* antigen 10 corresponds to MLM antigen 13.

Table 3 also shows the reactions obtained when antisera against five other mycobacterial species and *N. asteroides* were incorporated into the intermediate gel. These findings indicate that the components detected in *M. leprae* show extensive cross-reactions with the other bacteria. Antigens 3, 5, 6, and 7 were in this way shown to cross-react with all of the six other

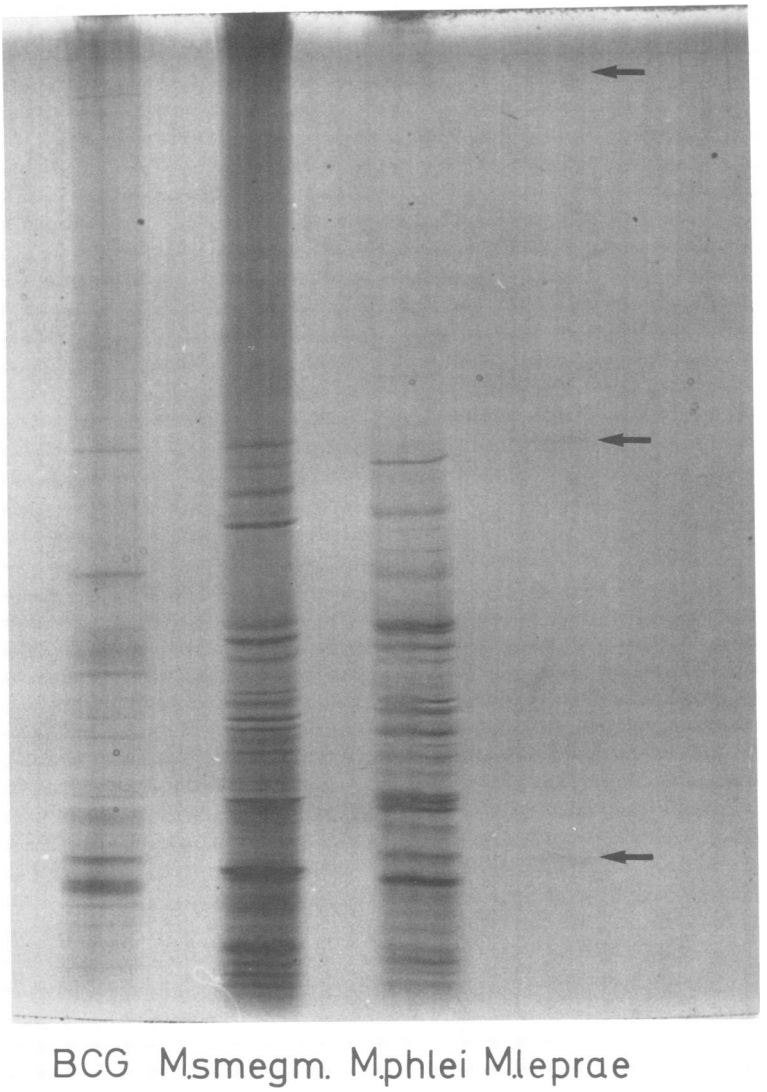

FIG. 4. *Polyacrylamide gel electrophoresis of sonic extracts from BCG, M. smegmatis, M. phlei, and M. leprae adjusted to the same protein concentration based on the Folin test. After staining with Coomassie brilliant blue, far fewer lines are seen in the M. leprae sonic extract than in the others.*

species tested. Additional experiments showed that these components were also detectable as antigens by direct precipitation tests against the bacterial species concerned.

Retention of a particular antigen in the intermediate gel is a very strong indicator of cross-reactivity. Lack of effect does not, however, exclude cross-reactivity, e.g., because the corresponding antigenic component has not induced formation of precipitating antibodies upon immunization with the cross-reacting bacterium. Further experiments were therefore set up to evaluate whether cross-reactions might occur in the situations where it had not been demonstrated during the experiments summarized in Table 3.

Figure 5 shows an example of experiments made to evaluate antigen 2 with regard to BCG and antigen 4 with regard to cross-reaction with *M. duvalii*.

Figure 3A shows the reference pattern with anti-*M. leprae* in the upper gel, Fig. 5A shows the pattern with anti-BCG in the upper gel, and Fig. 5B shows the pattern with anti-*M. leprae* in the upper gel and anti-BCG in the intermediate gel. By direct precipitation with anti-BCG,

precipitin lines were developed whose position indicated that they corresponded to antigens 1, 4, 5, 6, and 7, whereas there was no line corresponding to antigen 2. Figure 5B shows that anti-BCG in the intermediate gel affected all precipitin lines in the reference pattern except line 2. In some cases, the antigens were completely retained in the intermediate gel, whereas in others, the effect was definite but less marked. Figures 5C and D illustrate similar experiments with anti-*M. duvalii* in the upper and the intermediate gel, respectively. In this case, we see components corresponding to antigens 1, 5, 6, and 7 in the direct precipitation test. By incorporation of anti-*M. duvalii* in the intermediate gel, all of the precipitin lines of the reference pattern were affected except line 4. It was concluded that antigen 2 of *M. leprae* could not be shown to cross-react with BCG and that antigen 4 could not be shown to cross-react with *M. duvalii*, whereas these two components cross-reacted with the other mycobacteria tested and with *N. asteroides*.

As shown in Table 3, cross-reactions were not demonstrated between antigen 1 of *M. leprae* and *M. avium*, *M. smegmatis*, or *N. asteroides* in tests where the corresponding antibodies were incorporated in the intermediate gel. Attempts were then made to demonstrate the corresponding antigen directly in the species concerned, as illustrated in Fig. 6. Fig. 6A shows CIE with anti-*M. leprae* antibody in the upper gel and 5 μl of the A/10 *M. leprae* antigen preparation plus 5 μl of buffer in the antigen well to produce a reference pattern. In this case, the anti-*M. leprae* antibody was a pool of antisera obtained from four of the five immunized rabbits. In the plate shown in Fig. 6B, the antigen well contained a mixture of 5 μl of the *M. leprae* antigen plus 5 μl of an ultrasonic extract of *M. smegmatis*. In the upper gel, the area below several precipitin lines is increased, indicating increased antigen concentration. This is clearly the case for lines 2, 4, 5, and 7. Line 1 shows another change, namely, an additional component with slower electrophoretic mobility that fuses with antigen 1 of the reference pattern. This shows that a cross-reacting antigenic component with different electrophoretic mobility is present in *M. smegmatis*. Figure 6C is a control plate where *M. leprae* antigen was mixed with diluted *M. smegmatis* sonic extract. The same effect is seen, but the line caused by reaction with *M. smegmatis* antigen is lower due to decreased concentration. A similar effect on line 2 is also shown with an arrow. Cross-reaction with regard to component 2 was demonstrated by the antibody in the intermediate gel technique (Table 3), but the experiment of Fig. 6 shows that the cross-reacting component in *M. smegmatis* has a faster electrophoretic mobility than component 2 of *M. leprae*.

By similar experiments, cross-reacting antigen corresponding to antigen 1 of *M. leprae* was also found in *M. avium* and *N. asteroides*.

It was concluded that the antigenic components detected in *M. leprae* by the present rabbit antisera showed wide cross-reactivity with other mycobacterial species.

Antibody formation in human leprosy against the antigenic components of *M. leprae* detected by rabbit antisera. To investigate whether the components detected in the *M. leprae* sonic extract by the rabbit antisera are involved in immune reactions in leprosy, CIE was carried out with serum 3204, tap 5 in

TABLE 3. *Reactions of various antisera with antigenic components of M. leprae*[a]

Antiserum	Component						
	1	2	3	4	5	6	7
Anti-BCG 60						↓[b]	↓↓[c]
738							
662							
Anti-MLM 13				↓↓			
Anti-BCG	↓		↓↓	↓↓	↓↓	↓↓	↓↓
Anti-*M. avium*		↓↓	↓	↓↓	↓↓	↓	↓↓
Anti-*M. duvalii*	↓	↓↓	↓		↓↓	↓↓	↓↓
Anti-*M. smegmatis*		↓↓	↓	↓↓	↓↓	↓	↓↓
Anti-MLM	↓	↓↓	↓↓	↓↓	↓↓	↓↓	↓↓
Anti-*N. asteroides*		↓	↓	↓↓	↓↓	↓	↓↓
Lepromatous pool	(↓)[d]	↓↓	↓	↓	↓↓	↓	↓↓

[a] Antisera were incorporated into the intermediate gel of CIE plates with anti-*M. leprae* (3204, tap 5) in the upper gel and *M. leprae* sonic extract (A/10) in the antigen well.

[b] ↓, Precipitate was lower than normal, with its feet extending down into the intermediate gel.

[c] ↓↓, Precipitate was located close to the bottom of the intermediate gel.

[d] (↓), Indicates a slight but definite change in the precipitin line (often "the inward-feet reaction") (4).

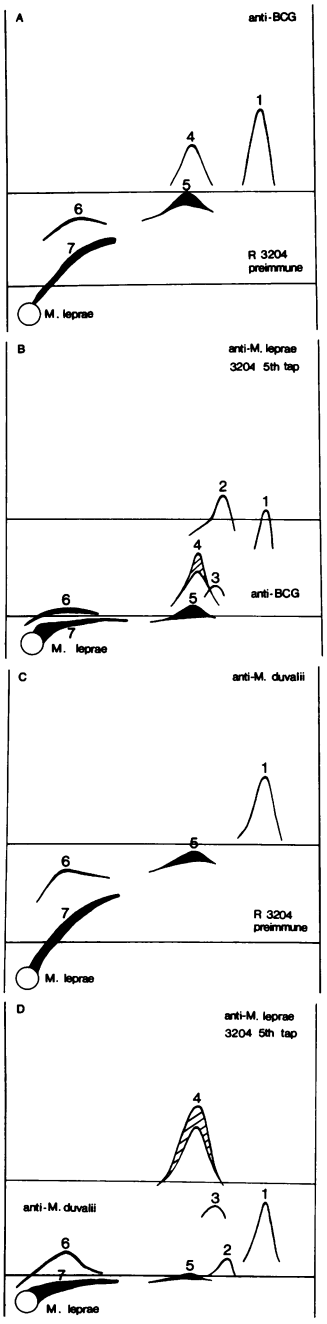

FIG. 5. *Cross-reaction between M. leprae, M. bovis (BCG), and M. duvalii, as tested by direct precipitation and intermediate gel analysis. For further explanation, see text.*

the upper gel, the A/10 *M. leprae* sonic extract in the antigen well, and a lepromatous serum pool and 20 individual sera from patients with lepromatous leprosy in the intermediate gel (4, 5). The findings are summarized in Table 4 and illustrated in Fig. 7. Two arrows indicate that the precipitin line is located far down into the intermediate gel. One arrow means that there is a distinct retention of the antigen with the precipitate extending far down into the intermediate gel. An arrow in parentheses means a slight but definite change in the precipitin line. Table 4 shows that the precipitin lines corresponding to all of these components were affected by incorporating pooled lepromatous serum in the intermediate gel, but some lines were more affected than others. This shows that all of the antigens detected are involved in the antibody response against *M. leprae* during lepromatous leprosy in man. The tests with the individual sera further illustrate that the specificity of the response varied from patient to patient. The antibody response in lepromatous leprosy is particularly strong with regard to components 2, 5, and 7. All of the sera investigated contained antibodies against component 2. Sixteen of the 20 sera contained antibodies against component 5, and 18 of them contained antibodies against component 7. Antibody activity was also demonstrated in individual sera against the other components, but less frequently.

DISCUSSION

By immunization of rabbits with leprosy bacilli purified from infected armadillo liver tissue, we have obtained antisera that define seven components in CIE. The precipitin lines were distinct and the immune response showed a striking similarity in the five rabbits during immunization. The CIE pattern was clear and could be used for analysis of cross-reactions between these antigenic components in *M. leprae* and other mycobacteria and for characterization of the antibody response in lepromatous leprosy. This pattern, with only seven distinct lines differs markedly from the patterns we have obtained in CIE of antigen preparations made from other mycobacteria and corresponding rabbit antibodies (10, 15, 16), in which a much larger number of components are detectable, usually above 40. The latter finding corresponds to previous reports in which more than 40 antigenic components have been demonstrated by CIE of *M. tuberculosis* (17, 38) and *M. smegmatis* (21). *M. leprae* preparations have previously been found to give a low number of precipitin lines in conventional double-diffusion tests in agar (1, 26, 33) and in CIE with pooled lepromatous serum as antibody reagent (22). In several instances, pooled lepromatous serum has given more precipitin lines against cross-reacting mycobacteria than against *M. leprae* antigen preparations (21).

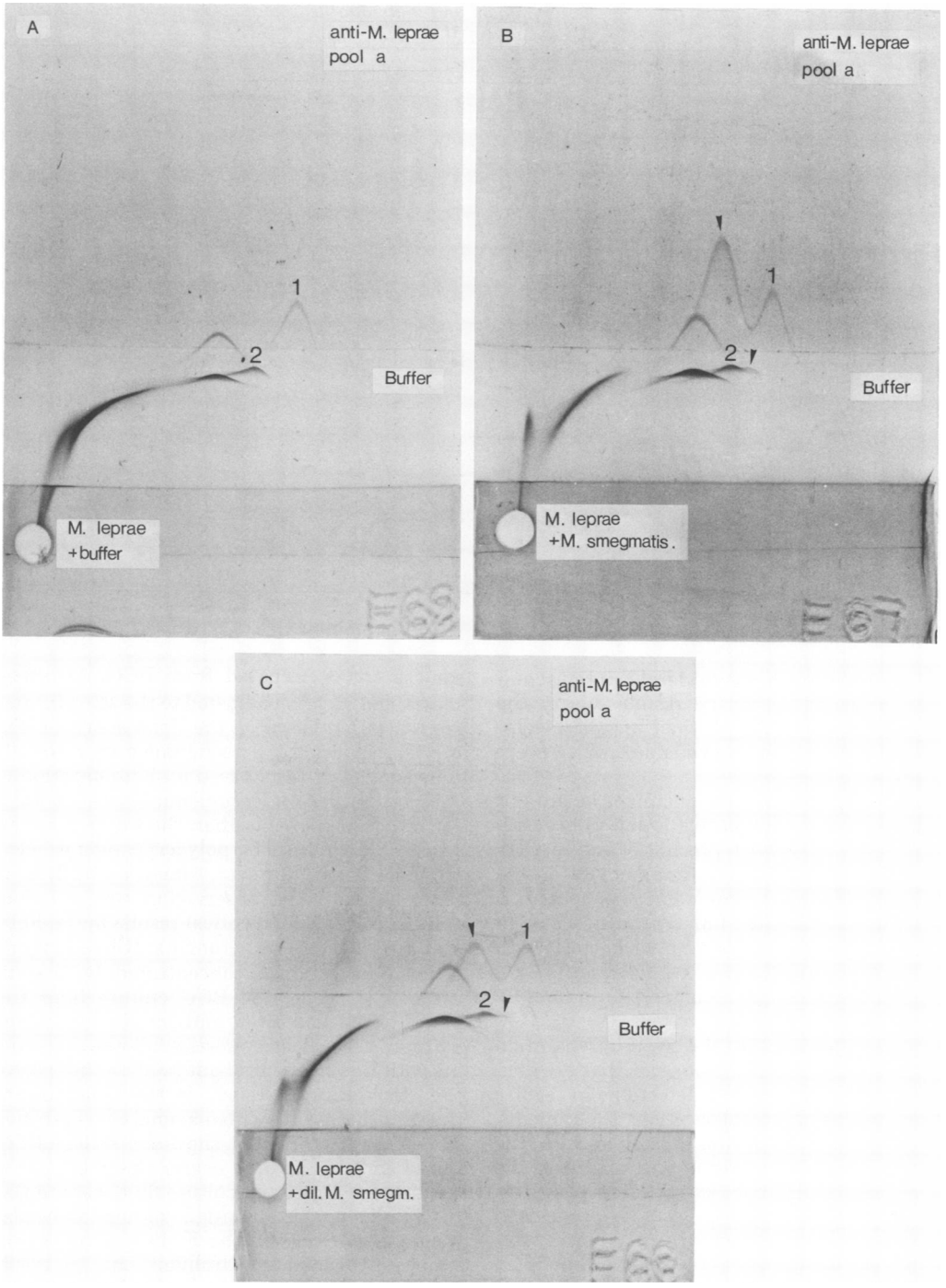

FIG. 6. *CIE of mixtures of M. leprae and M. smegmatis antigens against anti-M. leprae to demonstrate cross-reacting antigen in M. smegmatis. Note the additional precipitin line in frames B and C, which fuses with the line of M. leprae antigen 1. This shows that there is a cross-reacting antigen with slower electrophoretic mobility in M. smegmatis. Similar but less pronounced changes are also seen with regard to antigen 2.*

TABLE 4. *Reactions of the lepromatous serum pool and individual lepromatous sera with antigenic components of M. leprae*[a]

Serum	Component						
	1	2	3	4	5	6	7
Lepromatous pool	(↓)	↓↓	↓	↓	↓↓	↓	↓↓
30/75	(↓)	↓↓	↓		↓↓		↓↓
31/75		↓↓			↓		↓↓
45/75		↓↓			↓↓		↓↓
46/75		↓↓					
49/75	↓	↓↓	↓		↓↓		↓↓
52/75		↓↓	↓	↓↓	↓↓		↓↓
55/75		↓↓	↓	↓↓	↓↓		↓↓
62/75		↓					↓
63/75		↓				↓	
65/75		↓↓	↓↓	↓↓	↓↓	(↓)	↓↓
71/75		↓↓	↓		↓↓	↓	↓↓
69/75		↓					↓↓
72/75		↓			↓		(↓)
73/75	(↓)	↓↓	↓		↓↓	↓	↓↓
74/75	↓	↓↓	↓↓	↓↓	↓↓	↓↓	↓↓
75/75		↓↓	↓↓		↓↓		↓↓
77/75		↓↓	↓↓	↓	↓↓		↓
78/75		↓↓	↓		↓↓		↓↓
79/75		↓↓	?	(↓)	↓↓	↓	↓↓
80/75		↓↓	(↓)	(↓)	↓		↓

[a] Experimental setup and recording as in Table 3.

These findings are of considerable interest and pose the question: are the results due to the presence of only a few distinct antigenic components in the currently available antigen preparations of *M. leprae*?

Some antigenic components might be present in the A/10 preparation in too low concentrations to give a distinct precipitin line in CIE, whereas they might act as immunogens in the rabbits. Evidence on this point was sought in CIE by using mixtures of *M. leprae* antigen and antigen from a series of other mycobacteria with anti-*M. leprae* in the top gel (cf. Fig. 6). We have detected two additional precipitin lines by this technique.

The possibility that the A/10 preparation might contain several components that are nonprecipitating and thus not detectable in regular CIE was tested by absorption experiments. When anti-BCG that was absorbed with the A/10 *M. leprae* preparation was placed in the top gel and BCG sonic extract was placed in the antigen well, the position of five precipitin lines differed from that on corresponding control plates with unabsorbed anti-BCG antibody in the top gel. These lines were those due to BCG components cross-reacting with *M. leprae* antigens 1, 4, 5, 6, and 7. Additional non-precipitating components were thus not detected.

The *M. leprae* sonic extract gave far fewer lines after polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue than sonic extracts prepared from BCG, *M. smegmatis*, and *M. phlei* adjusted to the same protein concentration based on the Folin assay (cf. Fig. 4). This shows, by a technique that is totally independent of tests based on immunization of rabbits, that the *M. leprae* sonic extract contains far fewer components than the other sonic extracts. The findings by polyacrylamide gel electrophoresis are, however, somewhat difficult to reconcile with the observation that the four sonic extracts gave identical results in the Folin test. In separate experiments (Closs, unpublished observations), concentrated BCG sonic extracts, concentrated BCG culture fluid and partially purified BCG antigen 60 were tested by agarose gel electrophoresis followed by staining with Coomassie brilliant blue. In these tests, the gels were not stained in the area close to the application sites with maximal concentration of BCG antigen 60, but showed a series of distinct bands in the area nearer the anode when total sonic extract and culture fluid were tested. BCG antigen 60 is an antigen of high molecular weight that reacts in the Folin test but is not stained by Coomassie brilliant blue; it reacts with concanavalin A and is probably a glycoprotein (16). It is localized in the BCG cell wall, cross-reacts markedly with other mycobacteria, and corresponds to *M. leprae* antigen 7 (cf. Tables 1 and 3). The observations made by polyacrylamide gel electrophoresis and the Folin test are thus compatible with each other if *M. leprae*

antigen 7 or similarly reacting components are major constituents of the A/10 preparation.

It is well established that the concentrations of different antigenic constituents may vary in a given bacterial species, e.g., depending on the conditions during culture (3). Recent studies by Turcotte (35) have shown that tubercle bacilli grown in vivo were antigenically deficient compared with tubercle bacilli grown in vitro. The leprosy bacilli used in this study were grown in vivo in the armadillo, but our pool of rabbit antisera against MLM grown in vivo in mice gave a pattern with 42 distinct antigenic components in CIE (10). Growth in vivo thus does not appear to be the essential feature per se. Different viability of the mycobacteria may be an important feature. In mouse leprosy, MLM was purified from heavily infected mice. The bacilli had a higher morphological index than is usually found in *M. leprae* preparations, indicating that a larger proportion of the bacilli have an intact structure. Limited information is available on leprosy bacilli harvested from infected armadillos. The bacilli have been obtained from the animals much later after inoculation, the infection has been slowly progressing, and, in some animals, the morphological index is low, indicating that many of the bacilli are dying or dead (8, 18, 20). Antigenic loss in vivo under such conditions is not unexpected. Loss of antigens during purification must also be considered, since collagenase and proteinase from *Streptomyces griseus* are used in Draper's procedure for purification of *M. leprae* from infected armadillo liver tisse (11). No significant antigenic loss was detected by CIE in MLM purified from mouse liver by Draper's procedure as compared with MLM bacilli purified by conventional procedures without use of enzymes (P. Draper, personal communication). Antigenic loss during purification of *M. leprae* from armadillo liver may, however, be significantly higher, since bacilli with a low morphological index may be particularly prone to leakage and degradation by proteolytic enzymes.

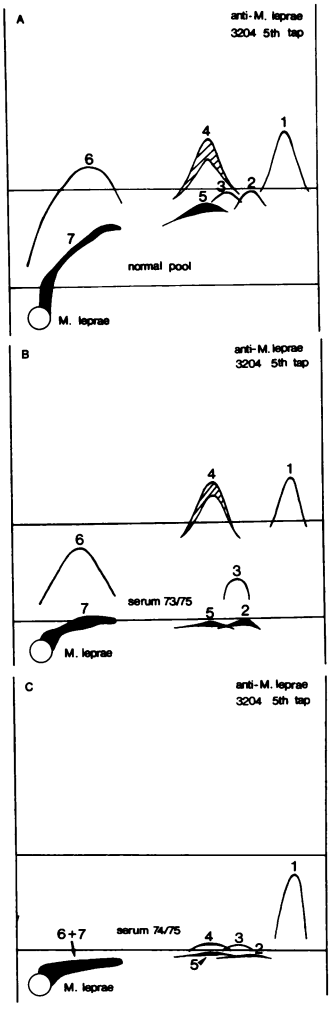

FIG. 7. *Demonstration of antibody activity in lepromatous sera by CIE with intermediate gel. 7A shows the reference pattern; 7B and 7C show the result with individual lepromatous sera in the intermediate gel. The effect of these two sera demonstrates antibody activity against all of the components of the reference pattern, but the antibody specificity is different in the two cases.*

For immunological studies, it is essential to obtain sufficient amounts of leprosy bacilli with an antigenic constitution corresponding to that during active growth of the organism. Such a preparation has been difficult to obtain from nodules of patients with lepromatous leprosy, and it may also be difficult to obtain from infected armadillos. In the armadillo, further attempts should be made to develop conditions for inoculation favoring rapid growth of the bacilli. The time of harvest may also be a critical feature. Relatively early harvest may provide a greater fraction of viable bacilli in active growth, but this will be at the expense of a seriously reduced yield. For some purposes, studies of the presently detected antigens are sufficient, and it would then appear to be important to work under conditions where a maximal number of bacilli are present in the tissues so that they can be purified relatively easily.

Based on double-diffusion studies in gel, Stanford (31) has described four distinct types of mycobacterial antigens: the first common to all species of mycobacteria, the second common to fast growers, the third common to slow growers, and the fourth specific for a given mycobacterial species. A crucial question when antigen prepa-

rations, specific for a given mycobacterial species, are to be made is whether these antigens occur on different molecules that may be separated from each other, or whether they are distinct antigenic determinants that partly occur on the same molecules. Kronvall et al. (22) have reported on detailed studies of the antigenic determinants on component 21 of *M. leprae*. Three types of antigenic determinants were detected on this component: one type, called 21A, was shared with 12 mycobacterial species; another, called 21B, was limited to antigen 21 of *M. leprae*; and a third, called 21C, was present in the 11 other species tested but not in *M. leprae*.

All of the seven components currently detected in *M. leprae* antigen by means of rabbit antisera showed extensive cross-reactions with other species of mycobacteria and *N. asteroides*. It remains to be shown to what extent these components contain antigenic determinants that are specific for *M. leprae*. Such determinants were detected by Kronvall et al. (22) on antigen 21 of *M. leprae*, but could not be demonstrated on the antigen termed no. 1 by them. A further search for such antigenic determinants should have high priority. The possibility that *M. leprae* antigens currently available from the armadillo can be used to develop a specific skin test reagent for leprosy, as distinct from other mycobacterial infections, would appear to be critically dependent on the amount of *M. leprae*-specific antigenic determinants that are present on the main components of the preparation. Fractionation of the current antigen preparation, with removal of strongly cross-reacting antigenic components, appears to be difficult, as judged from the present investigations. If all cross-reacting components should be removed from the A/10 preparation to make a specific skin test reagent, all of the presently defined components would have to be removed. With the currently available material, it might thus be better to develop a partially specific antigen based on components that are partly cross-reacting but characterized by a maximal content of *M. leprae*-specific antigenic determinants.

By incorporation of individual lepromatous sera in the intermediate gel of CIE plates, it was established that antibodies are produced against all of the currently demonstrated antigenic components of *M. leprae* during clinical disease in man (cf. Table 4). Our CIE pattern with rabbit antiserum is strikingly similar to the patterns obtained by Kronvall et al. (22), using pooled lepromatous sera as antibody reagent. An essential question is whether this is convincing evidence that these seven components are involved specifically in the immune reactions of leprosy. By tandem CIE with pooled lepromatous serum in the top gel, *M. leprae*-specific antigenic determinants were detected by Kronvall et al. on one of the components and termed 21B (22). More recently, we have developed a sensitive radioimmunoassay for demonstration of antibodies against *M. leprae*-specific antigenic determinants on cross-reacting components. Application of this assay on lepromatous sera showed that a large fraction of their anti-mycobacterial antibodies are directed against *M. leprae*-specific determinants. The titer of *M. leprae*-specific antibodies in a lepromatous serum pool was 10^5, whereas control sera from African patients with active pulmonary tuberculosis did not contain *M. leprae*-specific antibodies detectable by the assay (Harboe, Closs, Bjune, Kronvall, and Axelsen, unpublished observations). We consider this as strong evidence that the currently defined components of *M. leprae* are specifically involved in induction of antibody formation in leprosy. Another important question on which there is only little evidence is whether these components are also involved in cell-mediated immune reactions that are considered to be of primary importance for protective immunity in leprosy. It seems improbable that, during hyperimmunization, the rabbit should only detect antigenic constituents of *M. leprae* that are involved in a postulated selective antibody response in man, but our present findings stress that it is essential to obtain more information of the development of cell-mediated immune reactions and of antibody formation against individual and defined antigenic components of the leprosy bacillus. This approach is currently under development, based on the use of specific immunoadsorbent procedures to remove individual antigenic components from the *M. leprae* antigen preparations and subsequent in vitro and in vivo tests for cell-mediated immunity.

ACKNOWLEDGMENTS

This work was supported by grants from Anders Jahre's Fund for the Promotion of Science, The Norwegian Research Council for Science and the Humanities, and the Danish Medical Research Council (grant 512-5404). The work was also supported by the World Health Organization through its Immunology of Leprosy (IMMLEP) program.

We thank R. J. W. Rees and P. Draper, National Institute for Medical Research, London, for purified *M. leprae*; K. Bunch-Christensen and M. Magnusson, Statens Seruminstitut, Copenhagen, and J. Stanford, Middlesex Hospital, London, for other mycobacteria; B. G. Johansson, Clinical Chemistry Laboratory, University Hospital, Lund, Sweden, for polyacrylamide gel electrophoresis; and Anne-Lise Gamst, Inga Henriksen, and Kirsten Svindahl for excellent technical assistance.

LITERATURE CITED

1. **Abe, M.** 1970. Studies on the antigenic specificity of *Mycobacterium leprae*. I. Demonstration of soluble antigens in leprosy nodules by immunodiffusion. Int. J. Lepr. **38**:113-125.
2. **Abe, M., F. Minagawa, Y. Yoshino, and K. Okamura.** 1972. Studies on the antigenic specificity of *Mycobacterium leprae*. II. Purification and immunological characterization of the soluble antigen in leprosy nodules. Int. J. Lepr. **40**:107-117.
3. **Arvidson, S., T. Holme, and T. Wadström.** 1971. Influence of cultivation conditions on the production of extracellular proteins by *Staphylococcus aureus*. Acta. Pathol Microbiol. Scand. Sect. B **79**:399-405.
4. **Axelsen, N. H.** 1973. Intermediate gel in crossed and in fused rocket immunoelectrophoresis. Scand. J. Immunol. **2**:(Suppl. 1):71-77.
5. **Axelsen, N. H., M. Harboe, O. Closs, and T. Godal.** 1974. BCG antibody profiles in tuberculoid and lepromatous leprosy. Infect. Immun. **9**:952-958.
6. **Axelsen, N. H., J. Kröll, and B. Weeke (ed).** 1973. *A Manual of Quantitative Immunoelectrophoresis. Methods and Applications.* Scand. J. Immunol. Vol. 2, Suppl. 1.
7. **Barnetson, R. St. C., G. Bjune, J. M. H. Pearson, and G. Kronvall.** 1975. Antigenic heterogeneity in patients with reactions in borderline leprosy. Br. Med. J. **4**:435-437.
8. **Binford, C. H., E. E. Storrs, and G. P. Walsh.** 1976. Disseminated infection in the nine-banded armadillo (*Dasypus novemcinctus*) resulting from inoculation with *M. leprae*. Observations made on 15 animals studied at autopsy. Int. J. Lepr. **44**:80-83.
9. **Bjune, G., R. St. C. Barneston, D. S. Ridley, and G. Kronvall.** 1976. Lymphocyte transformation test in leprosy; correlation of the response with inflammation of lesions. Clin. Exp. Immunol. **25**:85-94.
10. **Closs, O., M. Harboe, and A. M. Wassum.** 1975. Cross-reactions between mycobacteria. I. Crossed immunoelectrophoresis of soluble antigens of *Mycobacterium lepraemurium* and comparison with BCG. Scand. J. Immunol. **4**(Suppl. 2):173-185.
11. **Draper, P.** 1976. Cell walls of *Mycobacterium leprae*. Int. J. Lepr. **44**:95-98.
12. **Estrada-Parra, S.** 1972. Immunochemical identification of a defined antigen of *Mycobacterium leprae*. Infect. Immun. **5**:258-259.
13. **Estrada-Parra, S., S. Calderón-Manes, M. Salazar-Mallén, and M.-E. Amezcua.** 1966. Isolation of a group-specific polysaccharide from tissues infected with *Mycobacterium leprae*. Int. J. Lepr. **34**:294-297.
14. **Harboe, M.** 1976. Importance of immune reactions in leprosy. Ethiopian Med. J. **14**:205-212.
15. **Harboe, M., O. Closs, and J. Deverill.** 1976. Production of monospecific antisera against antigenic components of *Mycobacterium bovis* (BCG). Scand. J. Immunol. **5**:861-866.
16. **Harboe, M., O. Closs, K. Svindahl, and J. Deverill.** 1977. Production and assay of antibodies against one antigenic component of *Mycobacterium bovis* BCG. Infect. Immun. **16**:662-672.
17. **Janicki, B. W., G. L. Wright, Jr., R. C. Good, and S. D. Chaparas.** 1976. Comparison of antigens in sonic and pressure cell extracts of *Mycobacterium tuberculosis*. Infect. Immun. **13**:425-437.
18. **Kirchheimer, W. F., and R. M. Sanchez.** 1976. Quantitative aspects of leprosy in armadillos. Int. J. Lepr. **44**:84-87.
19. **Kirchheimer, W. F., and E. E. Storss.** 1971. Attempts to establish the armadillo (*Dasypus novemcinctus*, Linn) as a model for the study of leprosy. I. Report of lepromatoid leprosy in an experimentally infected armadillo. Int. J. Lepr. **39**:693-702.
20. **Kirchheimer, W. F., E. E. Storss, and C. H. Binford.** 1972. Attempts to establish the armadillo (*Dasypus novemcinctus*, Linn) as a model for the study of leprosy. II. Histopathologic and bacteriologic postmortem findings in lepromatoid leprosy in the armadillo. Int. J. Lepr. **40**:229-242.
21. **Kronvall, G., G. Bjune, J. Stanford, S. Menzel, and D. Samuel.** 1975. Mycobacterial antigens in antibody responses of leprosy patients. Int. J. Lepr. **43**:299-306.
22. **Kronvall, G., J. L. Stanford, and G. P. Walsh.** 1976. Studies of mycobacterial antigens with special reference to *Mycobacterium leprae*. Infect. Immun. **13**:1132-1138.
23. **Kwapinski, J. B. G., J. O. de Almeida, and E. H. Kwapinski.** 1972. Immunological determination of *Mycobacterium leprae* by means of cytoplasmic antigens. Bull. W. H. O. **46**:509-513.
24. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
25. **Myrvang, B., T. Godal, D. S. Ridley, S. S. Fröland, and Y. K. Song.** 1973. Immune responsiveness to *Mycobacterium leprae* and other mycobacterial antigens throughout the clinical and histopathological spectrum of leprosy. Clin. Exp. Immunol. **14**:541-553.
26. **Navalkar, R. G.** 1971. Immunologic analysis of *Mycobacterium leprae* antigens by means of diffusion-in-gel methods. Int. J. Lepr. **39**:105-112.
27. **Rees, R. J. W., and A. G. M. Weddell.** 1968. Experimental models for studying leprosy. Ann. N. Y. Acad. Sci. **154**:214-236.
28. **Ridley, D. S.** 1976. Hypersensitivity and immunity. Reactions and classification. Lepr. Rev. **47**:171-174.
29. **Ridley, D. S., and W. H. Jopling.** 1966. Classification of leprosy according to immunity. A five-group system. Int. J. Lepr. **34**:255-273.
30. **Ridley, D. S., and M. F. R. Waters.** 1969. Significance of variations within the lepromatous group. Lepr. Rev. **40**:143-152.
31. **Stanford, J. L.** 1973. Immunodiffusion analysis—a rational basis for the taxonomy of mycobacteria. Ann. Soc. Belge Med. Trop. **53**:321-330.
32. **Stanford, J. L., and G. A. W. Rook.** 1976. Taxonomic studies on the leprosy bacillus. Int. J. Lepr. **44**:216-221.
33. **Stanford, J. L., G. A. W. Rook, J. Convit, T. Godal, G. Kronvall, R. J. W. Rees, and G. P. Walsh.** 1976. Preliminary taxonomic studies on the leprosy bacillus. Br. J. Exp. Pathol. **56**:579-585.
34. **Tuma, M., and C. Silva.** 1962. Antigenic relationship between the Hansen bacillus and other mycobacteria. Int. J. Lepr. **30**:71-76.
35. **Turcotte, R.** 1973. Antigenic comparison between *in vivo* and *in vitro* grown tubercle bacilli. Can. J. Microbiol. **19**:925-930.
36. **Turk, J. L., and A. D. M. Bryceson.** 1971. Immunological phenomena in leprosy and related diseases. Adv. Immunol. **13**:209-266.
37. **Turk, J. L., and M. F. R. Waters.** 1971. Immunological significance of changes in lymph nodes across the leprosy spectrum. Clin. Exp. Immunol. **8**:363-376.
38. **Wright, G. L., Jr., and D. B. Roberts.** 1974. Two-dimensional immunoelectrophoresis of mycobacterial antigens. Comparison with a reference system. Am. Rev. Respir. Dis. **109**:306-310.