Immunologic Analysis of *Mycobacterium leprae* Antigens by Means of Diffusion-in-Gel Methods[1, 2, 3]

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Previous studies on identification and characterization of antimycobacterial antibodies in sera from leprosy patients indicated the presence of two serologically distinct humoral antibodies when antigen preparations from various mycobacteria were used. One of the antibodies was found to be present in the majority of sera from lepromatous and dimorphous patients whereas the other was present in sera from all three types of patients. The latter was designated as *anti-delta* and the former as *anti-beta* precipitins ([6, 7, 8, 9]).

More recently, Ulrich *et al.* ([14]) have shown the presence of humoral antibodies in sera of leprosy patients using a number of mycobacterial antigens. Their work confirms our observations on the frequency of circulating antibodies in these sera.

This report concerns the study of the antigenic mosaic of *M. leprae* by the use of heterologous antigen-antibody systems derived from other mycobacteria and employing the homologous antiserum prepared in rabbits by immunizing the animals with disrupted *M. leprae* obtained from human lepromas. Recently, Estrada-Parra ([3]) presented studies on identification of a defined antigen of *M. leprae* which he designated as Poly I Nb. This antigen gave positive precipitin reaction with selected sera from patients with tuberculosis, leprosy and mycetoma. Estrada-Parra stated that *M. leprae* contain either Poly I Nb or a very similar polysaccharide with similar antigenic determinants.

MATERIALS AND METHODS

Antigens. Twenty times concentrated culture filtrates (CF) were prepared from *M. smegmatis* (NTCC 8159), *M. kansasii* (ATCC 12478) and *M. balnei* (Runyon 687) which was grown both at 30° and 37°C. In addition to these antigens, disrupted cellular extract of tissue-separated *M. leprae* was used in the analysis.

Reference antisera. Antisera were prepared in rabbits against the various antigens mentioned above.

Sera from patients. Sera from patients in various stages of leprosy infection were also used in the present study.

Preparation of culture filtrate (CF) antigens. The three strains mentioned above were grown on Sauton medium ([11]) for a period of four weeks for *M. smegmatis* and six weeks for *M. kansasii*, and for *M. balnei* (30°C and 37°C) from inocula grown two weeks to four weeks on the same medium. The surface pellicle was separated from the medium on a Buchner funnel and the cells were collected, washed in 0.15 sodium chloride and stored for future use.

The medium (CF) was passed through a sterile Seitz filter into presterilized dialysis bags which were subjected to pervaporation at room temperature overnight. Pervaporation was interrupted after 24 hours to permit dialysis against cold tap water for another 24 hours. Intermittant pervaporation and dialysis was carried out until the culture filtrates were concentrated to approximately twenty times less than their original volume. Upon completion of sterility tests, the concentrated culture filtrates, containing 1:10,000 merthiolate as preservative, were stored.

1 Received for publication 29 January 1971.
2 This investigation was supported by the United States-Japan Cooperative Medical Science Program administered by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health, Department of Health, Education and Welfare (grant no. AI-08647), Bethesda, Maryland 20014.
3 Part of this work was presented at the Ninth International Leprosy Congress, London, September 1968.
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Disrupted *M. leprae* antigen. Lepromas were obtained from India and stored at −20°C till sufficient quantity was available. All the lepromas were pooled and treated as follows.

The pooled tissues were first allowed to soak in sterile phosphate buffer (pH 7.0) and the epidermis removed by peeling. The tissues were then minced and suspended in fresh buffer. The suspension was homogenized in a Teflon grinder for a few minutes. Care was taken to maintain a low level of temperature by immersing the glass grinding chamber in an ice bath during homogenization. The resulting suspension was centrifuged at low speed to sediment the heavy particles and the supernate was collected in a sterile container. The sediment was resuspended in fresh buffer and homogenized again. This material was centrifuged as before and the supernate pooled with the first one. The residue was subjected to repeated homogenization and centrifugation until such time as the final supernate lacked turbidity. The pooled supernates were centrifuged once again, first at low speed to remove residual heavy particles and then at 30,000 rpm for 30 minutes to obtain a near tissue-free sediment of bacilli. This residue was smear stained for acid-fast bacilli to determine the extent of tissue contamination, which appeared to be relatively minimum.

The residue was then weighed and resuspended in a known amount of phosphate buffer at pH 7.2 so as to obtain a concentration of 1.2 mg/ml. This suspension was subjected to fractionation in the refrigerated cell fractionator (Sorvall) at 40,000 psi. The resulting cell extract was used to prepare anti-serum in rabbits.

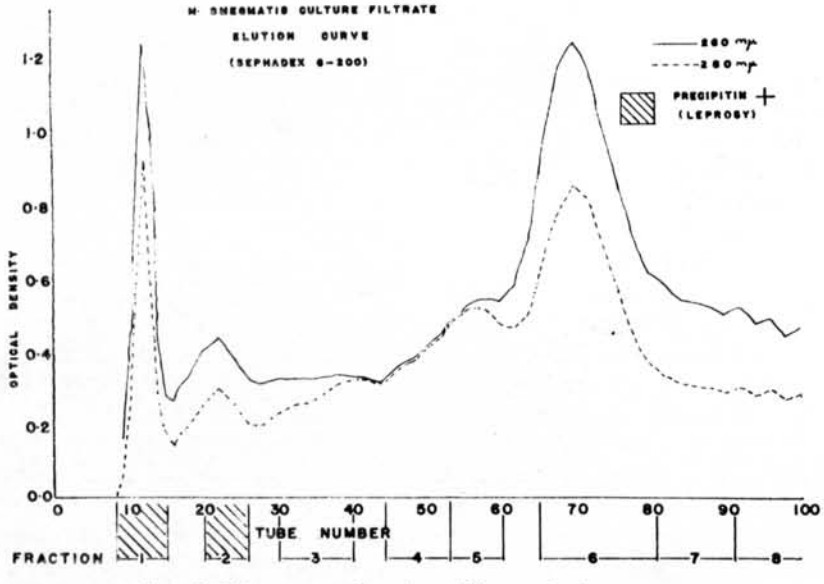

FIG. 1. *M. smegmatis* culture filtrate elution curve.

Separation of *M. smegmatis* culture filtrate on dextran column. The *M. smegmatis* culture filtrate was charged on a column packed with Sephadex G-200 and eluted with 0.15 M sodium chloride. A series of such fractionations were carred out to accumulate sufficient amounts of the various leprosy active fractions in order to facilitate analytical study of the antigens of *M. leprae*. Figure 1 is a typical elution curve of the fractionation. The shaded areas indicate the fractions that were active when tested against sera from leprosy patients.

Preparation of reference antisera. White, male rabbits, weighing 1.5 to 2.0 kg were bled prior to sensitization with the respective antigenic preparations described

above. The sensitizing dose was 1 ml of 1:1 mixture of the individual antigen and Freund's incomplete adjuvant, given subcutaneously in the hind quarter weekly for six weeks. One week after the last injection, the animals were ear-bled from the marginal vein and the antiserum was tested for antibody response. A week later, the animals were exsanguinated and sera from those rabbits that gave a uniform antibody response were pooled for each system. A 1:10,000 concentration of merthiolate was added as a preservative to each of the antisera prior to storage.

Preparation of antiserum against *M. leprae* extract. The protocol described above was followed for the preparation of the homologous antiserum, except that the sensitization schedule was extended to eight weeks and the animals were exsanguinated at 10 weeks after the first injection.

Establishment of reference systems. Reference systems were established according to the method of Lind ([5]) and as described in previous publications ([6, 8, 9]).

Analysis of the *M. leprae* systems. The comparative immunodiffusion (ID) technic of Ouchterlony ([10]) as modified by Hanson ([4]) and the immunoelectrophoretic (IEP) technic of Wadsworth and Hanson ([15]) were used in the analysis of the *M. leprae* system employing the various reference system described above.

RESULTS

Comparative immunodiffusion analysis of the *M. leprae* system indicated that when it was used against the *M. smegmatis* reference system, the organism shared at least two antigens with *M. smegmatis*, one of which has been identified by us as the *beta* antigen, an antibody to which can also be demonstrated in the majority of bacillary positive leprosy sera ([6, 8, 9]). Figure 2 shows the results of this analysis. The *beta*-anti-*beta* precipitate is seen here hooking into the center well which contains the anti-*M. leprae* serum. This is known as the deviation phenomenon and it occurs when the concentration of the antibody is at a level lower than that required to form a complete reaction of identity. The top left well contains the antiserum against the *M. smegmatis* culture filtrate and the top right well contains saline. The lower wells contain the *M. smegmatis* culture filtrate and the leprosy bacillus extract. In addition to the two immunoprecipitates seen between the smegmatis system and the leprosy system, there are at least three additional precipitates seen with the homologous leprosy system,. indicating that the leprosy bacillus has, in all, approximately five antigens, two of which it shares with *M. smegmatis*. When the leprosy system was compared with the *M. kansasii* system a similar situation was observed. It therefore seems that the other antigen that the leprosy bacillus shares with both the *M. smegmatis* and the *M. kansasii* strains, could be the *delta* antigen. An antibody to this antigen has also been shown to be present in the sera from leprosy patients.

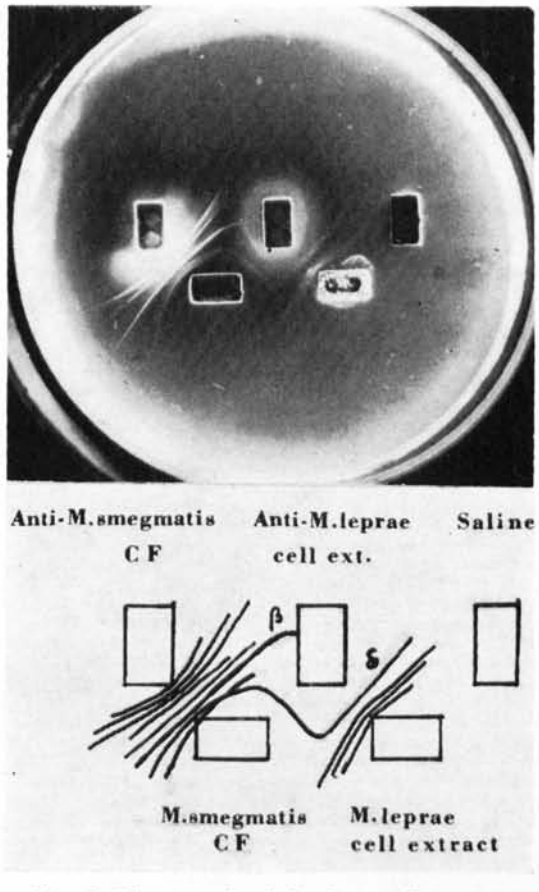

FIG. 2. Photograph of the five-well Ouchterlony plate and a schematic representation of the immunoprecipitates between the reactants.

A much better representation of the sharing of antigens by *M. leprae* with *M. smegmatis* and *M. kansasii* is shown in Figure 3. A set of three antigens, *M. smegmatis* (top left well), *M. kansasii* (center) and *M. balnei* (right), were analyzed against the leprosy bacillus antiserum. A reaction of identity is seen between the precipitates formed with the *M. smegmatis* and the *M. kansasii* CF antigens and the leprosy bacillus antiserum. No precipitate is seen with the *M. balnei* antigen. However, when the *M. balnei* antigens were used by themselves against the antiserum, a reaction was observed between precipitates formed with the antiserum and the two antigenic preparations, viz., the 30°C and 37°C grown *M. balnei* sets, thus indicating that the leprosy bacillus also shares with *M. balnei* at least one antigen.

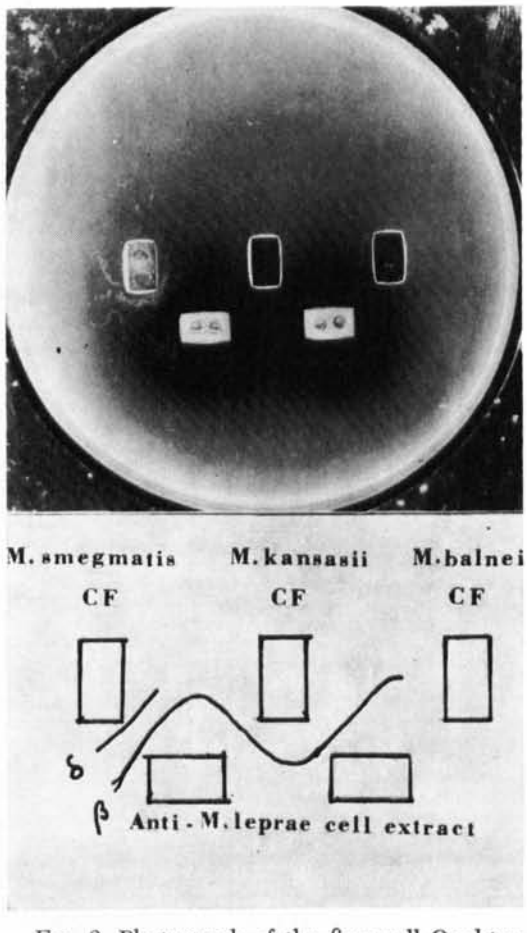

FIG. 3. Photograph of the five-well Ouchterlony plate and a schematic representation of the immunoprecipitates between the reactants.

To confirm that the antigen that is shared is in fact the *beta* antigen, we used the leprosy active fractions from *M. smegmatis* CF which were obtained by Sephadex fractionation (Fig. 1), one of which was known to possess the *beta* antigen through our previous work on leprosy sera.

In Figure 4, the upper wells contain saline (left), cell extract of *M. leprae* (center) and Fraction II of the *M. smegmatis* culture filtrate (right) obtained by Sephadex separation. The two lower wells contain the antiserum against the leprosy bacillus extract. It can be seen that there is a reaction between Fraction II and one of the antibodies in the *M. leprae* system. This fraction is known to have the *beta* antigen in it. The other fraction showed no reaction between the reacting substances, except with the homologous system.

DISCUSSION

In the past, investigations carried out by various workers have shown the presence of antimycobacterial antibodies in sera from leprosy patients ([1, 2, 3, 6, 8, 9, 12, 13]). Our investigations have indicated that not only can such antibodies be demonstrated in these sera but that the antigens related to these antibodies can be identified and characterized by employing already established reference systems prepared from most of the mycobacteria. This indirect means of suggesting that the reacting antigens may be shared by the leprosy bacillus has now been supplemented by the use of the direct method of employing a homologous system prepared from the leprosy bacillus itself.

A very recent report by Abe ([1]) on studies on antigenic specificity of *M. leprae* indicates that one of the antigens that the organism possesses has been found to be shared by BCG and *M. microti*. Estrada-Parra ([3]) has also reported the presence of a defined antigen of *M. leprae* which he has designated as Poly I Nb. This antigen reacts with sera from patients with tuberculosis and mycetoma. Both these reports tend to suggest that the leprosy bacillus does share some of its antigenic constituents with other mycobacteria.

Ulrich *et al.* ([14]), however, state that their efforts to induce antibodies to tissue separated *M. leprae* did not meet with success, despite an immunization schedule extending over a period of one year. They have, however, confirmed our observations on the presence of antibodies against other mycobacteria in the majority of sera from leprosy patients they have tested. Their failure to elicit antibody formation to the lepra bacilli is in contradiction to the observations of Abe ([1]) and Estrada-Parra ([3]) and also our work. We have not only shown that antibodies to *M. leprae* can be produced in rabbits by the use of the antigenic preparation described above, but also that with the help of this antiserum we can identify and characterize some, if not all, of the antigens that the organism seems to possess. We have shown that the leprosy bacillus does share the *beta* antigen with the strains tested, *viz., M. smegmatis, M. kansasii* and *M. balnei.* More definitive evidence is presented by Figure 4 which shows the coalescence of the precipitates formed between the reacting substances, *viz*, Fraction II of the *M. smegmatis* culture filtrate, *M. leprae* antigen and the antiserum to the leprosy bacillus. As stated earlier, Fraction II contains the *beta* antigen. The evidence thus presented indicates that the *beta* antigen forms a part of the possible antigenic make-up of *M. leprae.*

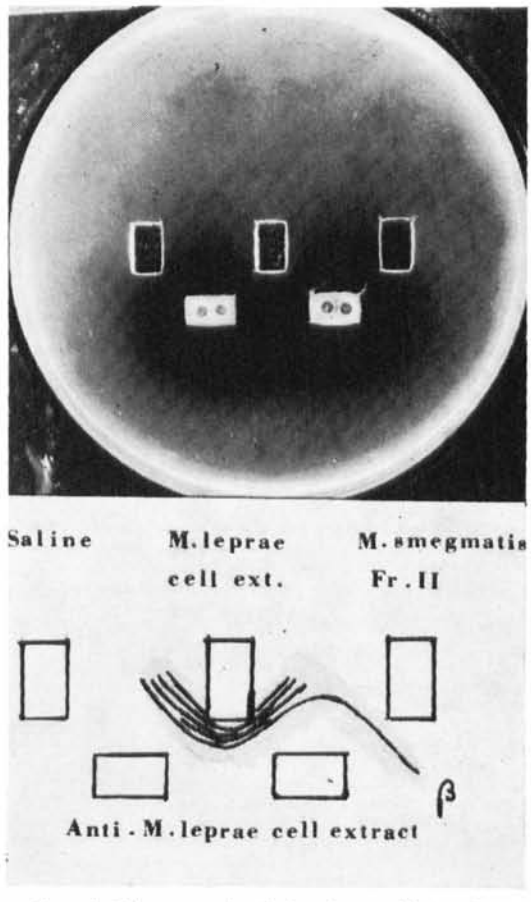

FIG. 4. Photograph of the five-well Ouchterlony plate and a schematic representation of the immunoprecipitates between the reactants.

Efforts to demonstrate antibodies in sera of leprosy patients employing the *M. leprae* antigen as well as the *M. leprae* system were not highly successful, although in some sera from advanced lepromatous cases an immunoprecipitate appeared. However, when the five-well comparative immunodiffusion technic was employed, using either the *M. smegmatis* or the *M. kansasii* reference systems together with the *M. leprae* system and a patient's serum, a reaction of identity with the common antigen could not be established. The *M. smegmatis* and the *M. kansasii* reference systems respectively did show the presence of the antibodies in these sera against the *beta* and the *delta* antigens as has always been observed before. The reason for this discrepancy may be that the concentration factor has played a role in the formation of a precipitate with the weaker system which in this case was the homologous one. Similar observations have been noted quite often when two unbalanced systems are used in the same plate to do comparative analysis of unknown sera. Apparently the stronger system will deplete the reacting materials to a degree whereby the weaker system is unable to react.

When the *M. smegmatis* system was employed in the five-well comparative analysis together with the *M. leprae* system, a reaction of identity was seen with an additional antigen which is apparently shared by the two organisms, besides the *beta* antigen. A similar phenomenon was also noticed when the comparative analysis was done with the *M. kansasii* system. Apparently this antigen is the *delta* antigen

which has been shown to have an antibody against it in the various sera of leprosy patients studied earlier. Thus at least two of the five antigens that *M. leprae* possesses can be identified on the basis of the analysis carried out with the two reference systems.

It is very likely that of the remaining three antigens, either one or more could be derived from the residual tissue that contaminated the bacillary suspension at the time of separation. However, when a normal tissue preparation was used against the leprosy bacillus system, no immunoprecipitates occurred between the reactants. It is possible that either the technics employed were not sensitive enough to elicit the reaction or that the antibody to the tissue element was at a level so low that no reaction between the tissue antigen and its corresponding antibody in the *M. leprae* system could be established.

The other possibility with regard to these three antigens is that they may be specific for *M. leprae*. Separation of the individual antigens by means of gel filtration would make it possible to study each of the antigens by itself. Paucity of material, however, has not permitted this. Additional material is being obtained and this study will be pursued. Failure to obtain very definitive reactions between the sera from patients and the leprosy bacillus extract is also a factor that has further limited the identification and characterization of these antigens.

A parallel study employing the immunoelectrophoresis technic and using all the materials that were used in the ID studies was carried out to determine whether the results would be more definitive than those seen in the ID studies. No changes that were specific in terms of the results obtained by the IEP method were found. An effort was made to carry out IEP studies using the patients' sera in the wells and the antigens in the troughs to determine whether the precipitates thus formed could be identified by the location and mobility of the immunoglobulin region. If the precipitate formed by the interaction of the leprosy sera and the *M. leprae* antigen was located at a different site than that formed by the interaction of the sera and the *M. smegmatis* antigen, some evidence of a different antigen other than the *beta* or the *delta* antigens might have been obtained. This would indicate differences between the antigens that constitute the antigenic mosaic of *M. leprae*. Although the *M. smegmatis* antigen reacted with the sera, there was no visible precipitate formed between the leprosy bacillus extract and the patients' sera. Here, too, the sensitivity of the technic coupled with the concentration factor of the reacting materials could have played a dominant role.

In view of the fact that positive results regarding the detection of homologous antibodies in the sera of leprosy patients when the ID and the IEP technics were employed were not obtained, the hemagglutination technic was employed to study the activity of the leprosy bacillus extract. The results of this study are at this time rather preliminary. Agglutination of the antigen coated RBC occurring at a titer of 1:160 and above in some sera, has been observed whereas some of the other sera were negative for agglutination.

We have previously reported that both the *beta* and the *delta* antigens are polysaccharide in nature. Thus at least two of the components of the total antigenic mosaic of the leprosy bacillus are polysaccharides. The positive hemagglutination reactions with some of the sera from leprosy patients tend to indicate that the leprosy bacillus may also possess antigens of protein nature. Abe ([1]) reports the finding of two antigens in a water soluble extract of *M. leprae*, one of which appeared to be polysaccharide and the other a protein. This protein antigen did not react with the antisera against other mycobacteria. Estrada-Parra ([3]) has shown the presence of a polysaccharide antigen of the leprosy bacillus which reacted with various mycobacterial antisera and also was active against sera from leprosy and tuberculosis patients. Our observations are in agreement with those of Abe and Estrada-Parra.

SUMMARY

Precipitating antibodies against two antigens, *beta* and *delta*, common to many

mycobacterial species have been demonstrated in an extract prepared from tissue separated *M. leprae* and its homologous antiserum which was prepared in rabbits. In addition to these two antigens, the leprosy bacillus appears to possess three additional antigens which could not be identified. Efforts to determine whether one or more of these antigens could be of tissue origin did not meet with success.

Preliminary studies employing the hemagglutination technic suggested the possibility of the leprosy bacillus possessing a protein antigen. The *beta* and the *delta* antigens have been shown in earlier studies to be polysaccharide.

No significant differences were noted between the immunodiffusion and the immunoelectrophoretic technics in terms of sensitivity as related to the detection and identification of the five antigens, nor were they highly effective in detecting homologous antibodies in sera from leprosy patients in various stages of the disease.

Acknowledgments. The author would like to thank the Superintendent and Staff of the Acworth Leprosy Home, Bombay, India for supplying the sera and lepromas used in the study. Participation of R. P. Warick is sincerely appreciated.

RESUMEN

Se han demostrado anticuerpos precipitantes contra dos antígenos, *beta* y *delta*, comunes a muchas especies de micobacterias, en un extracto preparado con *M. leprae* obtenido de tejido y su antisuero homólogo que se preparó en conejos. Además de estos dos antígenos, el bacilo de la lepra parece poseer tres antígenos adicionales que no pudieron ser identificados. Los intentos que se hicieron para determinar si uno o más de estos antígenos pudiera ser de origen tisular no tuvieron éxito.

Estudios preliminares en los cuales se utilizó la técnica de hemaglutinación sugirieron la posibilidad de que el bacilo de la lepra posea un antígeno protéico. En estudios anteriores se ha demostrado que los antígenos *beta* y *delta* son polisacáridos.

No se encontraron diferencias significativas entre las técnicas de inmunodifusión y de inmunoelectroforésis en términos de sensibilidad en relación a la detección e identificación de los cinco antígenos, ni tampoco fueron especialmente efectivas para detectar anticuerpos homólogos en el suero de pacientes con lepra en diferentes etapas de la enfermedad.

RÉSUMÉ

Dans un extrait préparé à partir de *M. leprae* séparés de tissus et dans l'anti-sérum homologue qui avait été préparé chez les lapins, on a pu démontrer la présence d'anticorps de précipitation contre deux antigènes, *beta* et *delta*, communs à de nombreuses espèces de mycobactéries. Outre ces deux antigènes, il est apparu que le bacille de la lèpre possédaient trois antigènes supplémentaires qui n'ont pu être identifiés. Les efforts menés pour déterminer si l'un ou plusieurs de ces antigènes pourraient être d'origine tissulaire, n'ont pas réussi.

Les études préliminaires, utilisant une technique d'hemagglutination, ont suggéré que le bacille de la lèpre pourrait peut-être posséder un antigène protéinique. Des études antérieures ont montré que les antigènes *beta* et *delta* étaient de nature polysaccharidique.

Aucune différence significative n'a été observée entre la technique d'immunodiffusion et la technique d'immunoélectrophorèse, en ce qui concerne leur sensibilité respective pour la détection et l'identification des cinq antigènes mentionnés. Ces techniques n'étaient pas non plus fort efficaces pour déceler des anticorps homologues dans le sérum obetnu chez des malades de la lèpre à divers stades de la maladie.

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