

INTERNATIONAL JOURNAL OF LEPROSY

And Other Mycobacterial Diseases

VOLUME 38, NUMBER 2

APRIL-JUNE, 1970

Studies on the Antigenic Specificity of *Mycobacterium leprae*

I. Demonstration of Soluble Antigens in Leprosy Nodules by Immunodiffusion¹

Masahide Abe²

Antigenic analysis of mycobacteria by agar-diffusion, first reported by Parlett and Youmans (14), has been developed by many investigators (15, 16, 22) and applied to serologic identification and classification of mycobacteria. Recently, Fukui *et al.* (10) reported that an antigen of *M. lepraemurium* may be identical with the avian γ antigen. However, little is known regarding the antigenic structure of *M. leprae*, although several investigations (4, 11, 16, 18, 20) have applied immunodiffusion methods to studies of the serology of leprosy. For example, Burrell and Rheins (4), Pepys *et al.* (16) and Navalkar *et al.* (11) observed precipitation patterns in agar gel which indicated cross reactivity between lepromin and mycobacterial antigens. Similar findings were reported by Tuma and Silva (21) and Estrada Parra *et al.* (7) using antigens obtained from unheated leprosy nodules. Accordingly, there is no doubt that leprosy bacilli have common antigens with other mycobacteria.

However, the presence of any antigen specific to *M. leprae*, has not been demonstrated and no trial has been reported on the serologic identification of this bacillus. An antigenic analysis of *M. leprae* is very difficult unless enough of the purified bacillary body is obtained. On the other hand, some investigations (6, 13) of the lepromin reaction suggest that leprosy nodules contain a water-soluble antigen that is thought to be an extracellular protein of the leprosy bacilli and to cause a delayed-type skin hypersensitivity in leprosy (Fernández reaction). If this is true, separation of this antigen may be technically considerably easier than extraction of the antigen from the purified bacillary body.

The present paper reports results of immunodiffusion detection of antigen in water soluble extracts from unheated leprosy nodules.

MATERIALS AND METHODS

Leproma and other tissues. Lepromas were obtained from relapse cases that were currently under treatment at the National Leprosaria, Tama Zensho-en and Hoshizuka Keiai-en. Aseptically excised nodules were trimmed to remove unnecessary

¹ Received for publication 10 June 1969.

² M. Abe, M.D., Laboratory of Serology, National Institute for Leprosy Research, Higashimurayama-shi, Tokyo, Japan.

tissues, transported to the laboratory as soon as possible in an ice-jar and stored in a deepfreezer at -20°C until used. Human skin, used as control material, was collected from cadavers of leprosy patients who had died of noninfectious diseases in the cold winter season. Within three to four hours after death, intact skin was cut from the inner thigh and immediately deepfrozen at -20°C . Subcutaneous lymph nodes were excised from the inguinal, axillary and cervical regions of an autopsy case which had tuberculoid leprosy and died of cirrhosis of the liver.

Preparation of tissue extracts. All procedures were performed in a cold room. Pooled unheated lepromas were minced with scissors and homogenized with 15 to 20 volumes of 0.2 M sucrose solution, using a "Homo Blender" (type 500, Sakuma Works Co., Tokyo). This suspension was centrifuged at 1,000 rpm for 10 minutes, and the supernatant was further centrifuged at 10,000 rpm for 20 minutes to obtain a clear or slightly turbid solution. It was dialyzed against distilled water for 48 hours, concentrated with an ultrafiltration apparatus⁽¹⁹⁾, or by dialysis against a 20 per cent solution of polyvinylpyrrolidone. An insoluble precipitate occurred during concentration and was removed by centrifugation and thus a clear, yellowish-brown solution was obtained. This was designated as the "nodule-extract" (NE). Three specimens of nodule-extract (NE), NE-1, NE-2, and NE-3 were obtained from different pools of lepromas. The larger part of NE-1 was lyophilized and the others were kept at -20°C until used.

Human skin and the lymph nodes from the cadavers were treated respectively by the same method as above to obtain a skin-extract (SE) and a lymph node extract (LYE). The residue of the skin tissue was ground in a mortar by mixing it with an equal amount of quartz sand. The crushed tissue was washed with saline and subsequently with acetone, centrifuged, dried in a vacuum, powdered by grinding, and stored in a desiccator. This powder was used for absorption of antiserum.

Purification of leprosy bacilli. Leprosy bacilli (LB) precipitated from the original

suspension of lepromas with 0.2 M sucrose were purified according to the method described by Prabhakaran and Braganca⁽¹⁷⁾. However, some tissue remnants were still present with the bacillary mass after this procedure and these were removed by digestion with 1 per cent trypsin (1:250, Difco, pH 8.0) at 37°C for three hours and subsequently by repeated washing with distilled water, and by centrifugation at 10,000 rpm for 20 minutes. The final precipitate of the bacillary mass was almost free of tissue by microscopic examination. The purified bacilli were lyophilized and kept in a refrigerator.

Preparation of mycobacterial antigens. The mycobacterial strains used for the present experiment were as follows: *M. tuberculosis hominis* (H_2); *M. bovis* (BCG); *M. avium* (Flamingo); *M. microti* (vole bacillus); *M. fortuitum* (No. 266); and unclassified mycobacteria (P7, P16, P37). They were grown in Sauton's artificial media. *M. lepraemurium* (Hawaiian strain) was obtained from the subcutaneous nodules of mice by a conventional method. Disintegration of the bacterial cells and preparation of subcellular fractions were carried out according to the method described by Fukui *et al.*⁽⁹⁾. Most of the soluble proteins in the supernatant obtained by centrifugation at 105,000 g were precipitated by dissolving 56.1 gm. of solid ammonium sulfate per 100 ml. distilled water to give an 80 per cent saturated solution. The precipitate was collected by centrifugation, dissolved in a small amount of water and dialyzed against distilled water. This material was designated as protein fraction (PR). The supernatant was dialyzed against distilled water and concentrated by the method stated above. This fraction contained mainly polysaccharides (PS).

Immunization of rabbits. White male rabbits weighing 2.5 to 3.0 kgm. were used for immunization. Anti-LB serum was obtained from five rabbits immunized with a mixture of one volume saline suspension of LB (2 mgm./ml.) and one volume of Freund's incomplete adjuvant, by the following procedures: an initial intramuscular injection of 1 ml. per animal; two weeks

later intracutaneous injection of 0.1 ml. per animal and an additional injection into each of the four foot pads (0.1 ml. per site) in three of five rabbits. Six weeks later a second intramuscular injection of 1 ml. of LB suspension (0.4 mgm./ml.) in incomplete adjuvant, and 10 weeks later a booster injection of 1 ml. of sonicated LB suspension in saline (1 mgm./ml.). One week after the last injection, the rabbits were sacrificed for total bleeding.

For immunization with NE, two rabbits were given an initial injection of a mixture of 0.4 ml. of NE solution (10 mgm./ml.) and 0.4 ml. of incomplete adjuvant, dividing the suspension into 0.2 ml. for each foot pad. One month later an intramuscular injection of 0.5 ml. of the same emulsion was given and the animals were exsanguinated two weeks later.

Antisera against the other mycobacteria were prepared from the blood of two or three rabbits for each bacterial strain. In the cases of BCG and *M. microti*, about 100 mgm. of wet bacilli were suspended in 1 ml. of saline and mixed with 1 ml. of incomplete adjuvant. For immunization with heat-killed *M. tuberculosis hominis* (H₂ strain) as well as with unheated *M. lepraemurium*, saline suspensions of the dried bacilli at a concentration of 20 mgm. per ml. of saline were emulsified with incomplete adjuvant. The immunization procedure with these emulsions was the same as that with NE.

A part of each antiserum was inactivated by heating at 56°C for 30 minutes, sodium azide was added to a concentration of 0.1 per cent, and the preparation was stored in a refrigerator. The remainder of each antiserum was preserved in a deepfreezer until used for other experiments.

The goat antiserum against human serum used in the experiments was a commercial product for immunoelectrophoresis (IEP serum, Hyland).

Immunodiffusions. Double diffusion tests were carried out on microscopic slide, glass plates covered with 1 per cent purified agar (Difco) in 0.01 M sodium phosphate-buffered saline (pH 7.0) containing 0.1 per cent sodium azide as a preservative. The well pattern was varied to suit the individ-

ual experiment. The concentration of the antigen applied to the well was usually adjusted to 1 per cent. The plates were placed in a moist chamber at room temperature and photographed at one, two and three days. Agar plates in Petri dishes, 6 cm. in diameter, were also prepared with the same agar solution as above for the tests with the NE fractions obtained by starch-block zone-electrophoresis as has been previously described (²). In these tests, the precipitation patterns were observed at one, three and seven days with incubation at 37°C.

Immunoelectrophoresis was performed by a method previously described (²). Precipitation patterns were observed and photographed at 24 and 48 hours after incubation in a moist chamber at room temperature, then dried and stained with amino black 10B.

Other serologic tests. The technics of complement fixation reaction with lepromin or Dharmendra's bacillary suspension were the same in principle as Ogata's method of serodiagnosis for syphilis (¹²), except that the volume of each reactant was reduced to 0.1 ml. Dilution of the antigen used for the titration of antiserum was 1:8, for lepromin and 1:2 for Dharmendra's antigen. The modified method of Middlebrook-Dubos' passive hemagglutination and hemolysis reactions have been reported elsewhere (¹). Formalinized, tanned cell agglutination tests were carried out by the method described by Daniel *et al.* (⁵). The optimal concentration of antigen for sensitization of red cells was 0.25 mgm./ml. for NE and about 1 mgm./ml. for sonicated LB. Agglutinin titers against human A, B and O type erythrocytes were measured by the conventional method for titrating blood group antibodies.³

RESULTS

Immunoelectrophoretic patterns of extracts from leprosy nodules and other tissues. Figure 1 shows the immunoelectrophoretic patterns of three specimens of nodule extracts (NE-1, NE-2, NE-3), three specimens of skin tissue extracts (SE-1,

³ The author is very grateful to Dr. T. Matsushashi and Miss M. Usui, of the Institute of Medical Science, University of Tokyo, for performing these tests.

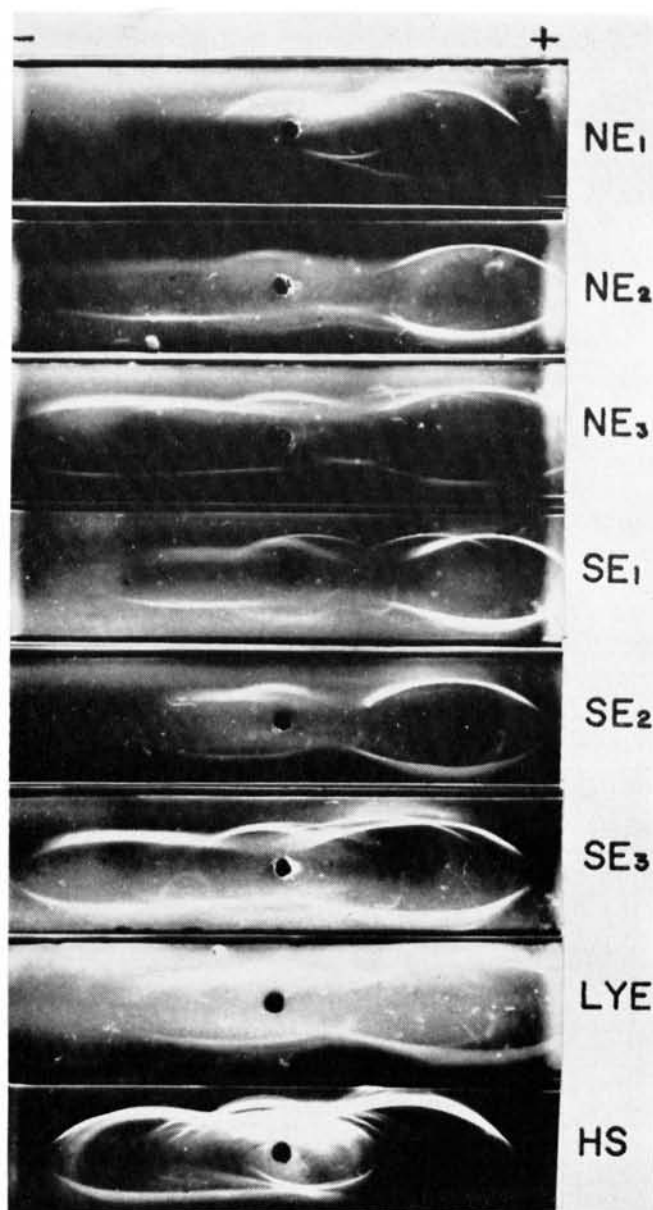


FIG. 1. Immunoelectrophoretic patterns of leprosy nodule extracts (NE-1, NE-2, NE-3), skin extracts (SE-1, SE-2, SE-3), lymph node extract (LYE) and normal human serum (HS). The upper trough of each plate is filled with goat anti-HS serum and the lower with rabbit anti-NE serum.

SE-2, SE-3), a lymph node extract (LYE) and a normal human serum (HS) as a control, against goat antiserum to human whole serum (anti-HS) as well as against antinodule extract rabbit serum (anti-NE). Although the precipitation lines of the respective specimens were variable in their shape, number and position, a common finding was that the precipitation lines of

HS, SE and LYE against anti-NE were partial reflections of the lines formed against anti-HS. This means that these materials consist of antigens which are common to serum proteins. In contrast, each NE specimen showed another precipitation line on the anodal side against anti-NE, but not against anti-HS.

In order to clarify whether this peculiar

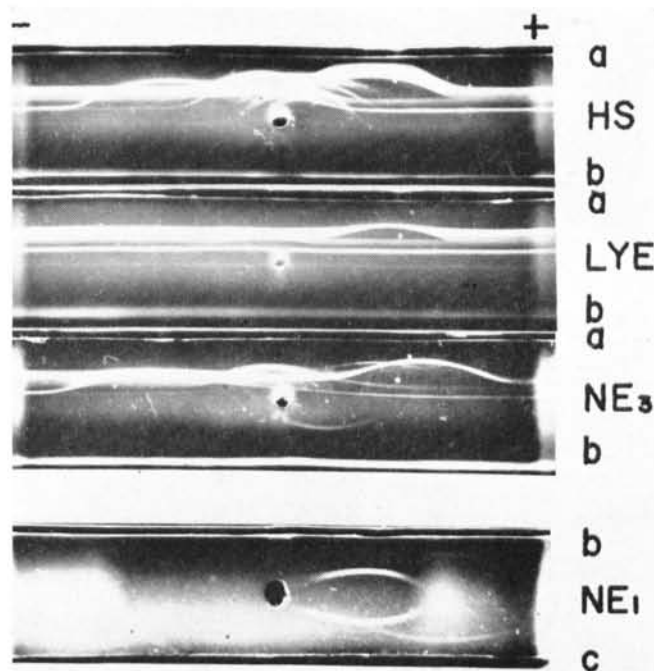


FIG. 2. Immunoelectrophoretic patterns of human serum (HS), lymph node extract (LYE) and leprosy nodule extract (NE-3) against anti-HS serum (trough a) and anti-NE serum absorbed with HS (trough b) or skin tissue (trough c).

precipitation line could have been due to the presence of an antigen different from serum proteins or normal skin proteins, absorption tests were carried out using anti-NE serum which had previously been mixed with an equal volume of human serum or acetone treated powder of normal skin tissue. The results are shown in Figure 2. It is apparent that the anti-NE serum after absorption still produces one precipitation line with NE, while HS and LYE show no precipitation against absorbed anti-NE.

Effect of heat on the antigenicity of the nodule-extract. A small amount of NE was sealed into a capillary tube and heated in an autoclave at 120°C for 20 minutes. After cooling, the heated product (NE') was used for immunoelectrophoresis in parallel with the unheated antigen (NE). The results are shown in Figure 3. The uppermost part of this figure indicates that NE' produced no precipitation against anti-NE itself nor the same absorbed with human serum, in contrast to the fairly well developed patterns of NE against these antisera. This means that these antigens, including the antigen peculiar to NE, are

heat-labile. On the other hand, anti-LB serum produced one precipitation line with NE on the cathodal side only. A faint precipitation corresponding to this position was still observed in the case of NE'. In other words, the anti-LB serum contained an antibody which reacted with the heat-stable antigen in NE. Furthermore, NE' produced a precipitation line with the anti-vole bacillus serum at the same position as that of anti-LB. These observations were reconfirmed by the microimmunodiffusion test as shown in Figure 4. The anti-LB and antivole bacillus sera exhibited a positive reaction against NE whether it was heated or not, while anti-NE serum reacted with the unheated antigen only. Accordingly it is apparent that NE contained at least two antigens, one of which was heat-stable and another which was heat-labile.

Fractionation of soluble antigens by starch-block zone-electrophoresis. In order to separate the two antigens, about 120 mgm. of NE dissolved in 5 ml. of veronal buffer was applied to starch-block zone-electrophoresis. After electrophoresis for 24 hours, separated proteins were extracted

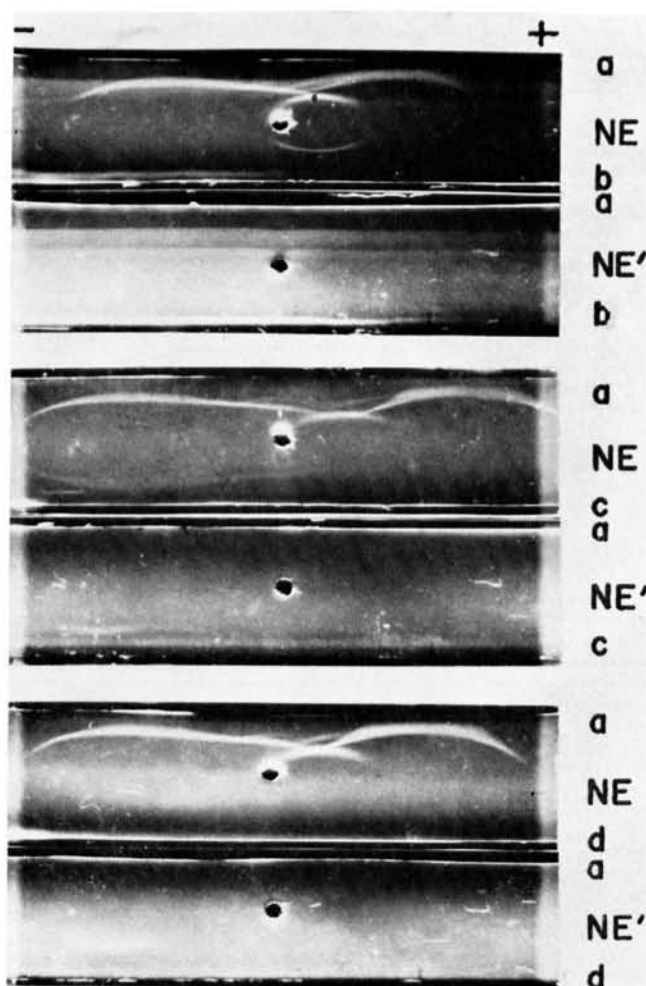


FIG. 3. Immunoelectrophoretic patterns of leprosy nodule extract (NE) and its product after autoclaving (NE'). Troughs a,b,c and d are filled with the following antisera respectively; (a) anti-NE, (b) anti-NE absorbed with human serum, (c) anti-LB and (d) antivole bacillus.

from the starch block cut into 1 cm. wide segments and analyzed for their content by the Folin method as well as for reactivity with anti-NE serum by the immunodiffusion test. The results are shown in Figure 5. Although each protein peak was not tentatively obtained according to the precipitation line formed between each eluate and anti-NE serum. One of these fractions must have contained the antigen in question, because it gave a precipitation line against anti-NE absorbed with human serum.

Identification of the antigens in these four fractions was attempted by double diffusion in agar gel using various antisera. The results are shown in Figure 6. It is

evident that F1 and F2 contained merely the antigen common with serum protein, as shown by comparison of the precipitation patterns produced by anti-HS (Fig. 6a) and by anti-NE (Fig. 6b). F3 and F4 contained such antigens also, this fact indicating their broad distribution in the field of zone-electrophoresis. However, F3 showed one precipitation line independent of the others and this line was not seen in the other fractions. The pattern obtained by anti-LB serum in the central well (Fig. 6c) was composed of two precipitation lines; one fused among NE, F3 and F4, and another fused between NE and F4. Since these lines did not reach to the well containing HS, it was considered that they

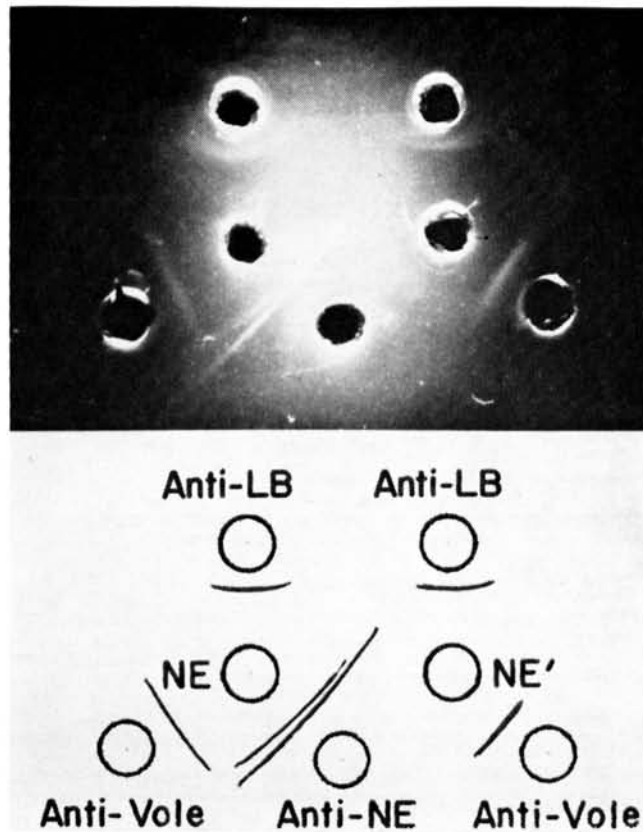


FIG. 4. A micro-immunodiffusion pattern of leprosy nodule extract (NE) and its product after autoclaving (NE').

were produced by antigens different from serum proteins. Furthermore, as shown in Figure 6d, anti-vole bacillus serum produced two precipitation lines against NE and F4, this fact indicating the presence of a common antigen between this bacillus and NE.

The precipitation pattern between F3 in a central well and various antisera in peripheral wells is shown in Figure 6e. This fraction produced two precipitation lines with anti-NE serum, another line with anti-LB, but did not react at all with the antisera against other mycobacteria. On the contrary, as shown in Figure 6f, F4 produced two lines which were common against anti-NE and anti-LB. Moreover, one of them fused with the line formed against anti-vole bacillus as well as anti-BCG sera.

The presence of at least two antigens in F3 and F4 was clearly demonstrated in the precipitation pattern in agar gel containing

human serum (Fig. 6g and 6h). In this case, the antigens which cross-reacted with serum proteins were not concerned in the formation of the precipitate. The strong precipitation line seen between F3 and anti-NE was quite independent of the other faint line formed by F4 and anti-LB. F4 produced another precipitation line which fused with that formed by the antivole bacillus serum.

Cross-reaction with mycobacterial antigens. Figure 7 shows the precipitation tests with anti-LB and anti-NE sera against mycobacterial antigens. PS was a polysaccharide fraction and PR contained mainly proteins of bacterial cells. Anti-LB produced a common precipitation line against every antigen, while anti-NE did not react at all with these antigens.

Other serologic reactivity of anti-LB and anti-NE sera. Results of various serologic tests with rabbit antisera against LB and NE are summarized in Table 1. Anti-LB

TABLE 1. Various serologic reactions of rabbit antisera.

Serologic test	Antigen used	Antibody titers of	
		Anti-LB	Anti-NE
Complement-fixation	Lepromin	320	(-)
	Dharmendra antigen	320	(-)
Middlebrook-Dubos' hemagglutination	Polysaccharide of <i>M. tuberculosis</i>	2,560	320
" " hemolysis	" "	5,120	160
Formalinized tanned cell agglutination	Nodule-extract	10,240	204,800
	Sonicated LB	320	2,560
Human red blood cell agglutination	A cells	8	128
	B cells	8	512
	O cells	4	128

showed high complement-fixation titers with lepromin or Dharmendra's antigen, Middlebrook-Dubos' hemagglutination and hemolysis tests and agglutination of formalinized tanned cells sensitized with NE, but a relatively low titer in the last test using sonicated LB suspension. Titers of blood group antibodies in this antiserum

were very low, this fact indicating complete removal of blood group antigens from LB. On the contrary, anti-NE serum showed low complement-fixation titers and Middlebrook-Dubos' tests, but very high agglutination titers of erythrocytes sensitized with NE and LB and also high titers of blood group antibodies.

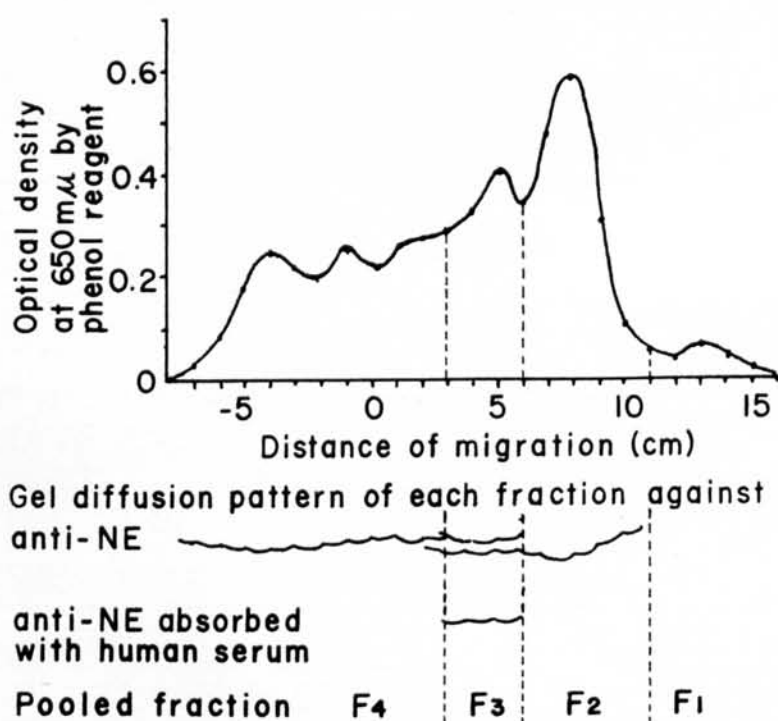


FIG. 5. Fractionation of leprosy nodule extract by starch-block zone-electrophoresis.

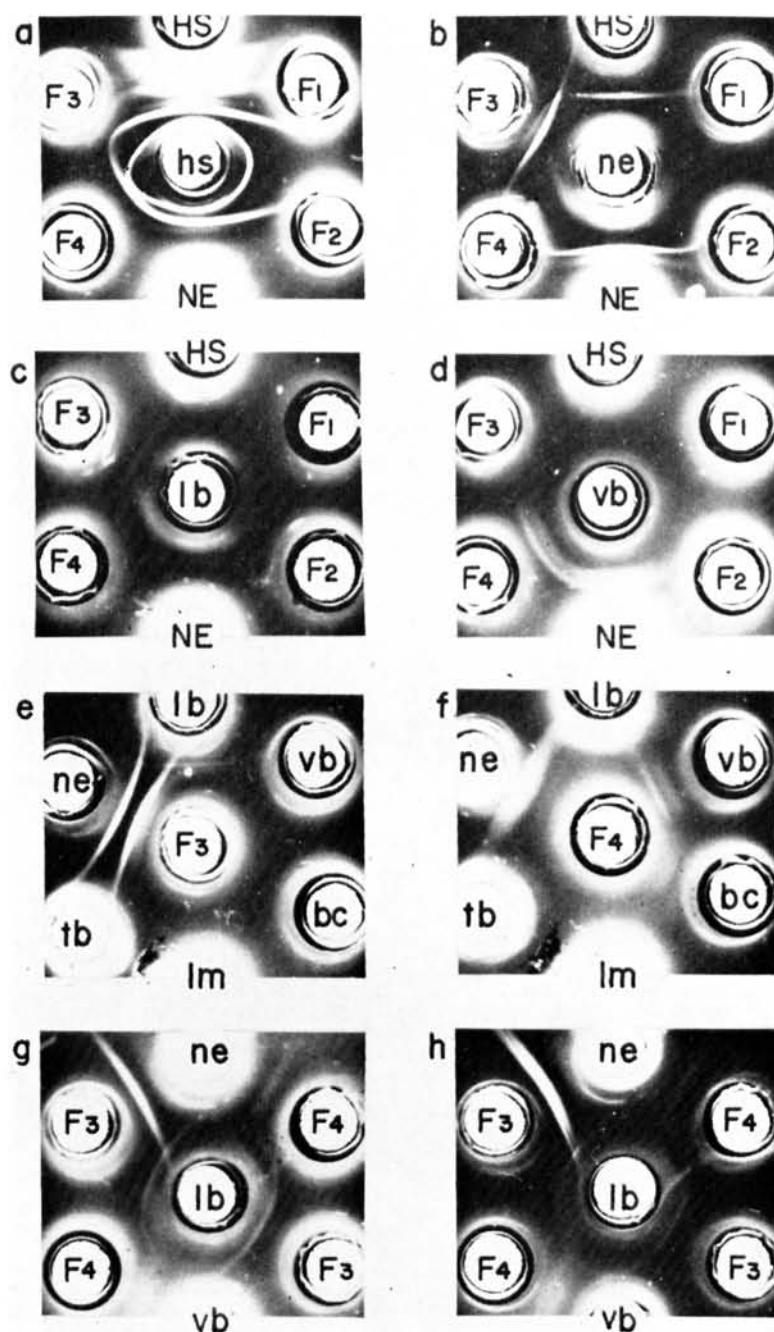


FIG. 6. Immunodiffusion patterns of leprosy nodule extract (NE) and its fraction (F1-F2) against various antisera: hs, antihuman serum (HS); ne, anti-NE; lb, anti-leprosy bacillus (LB); bc, anti-BCG; vb, antivole bacillus; lm, antimurine leprosy bacillus; and tb, antitubercle bacillus. Patterns a to f and h were photographed at seven days, while pattern g was at three days.

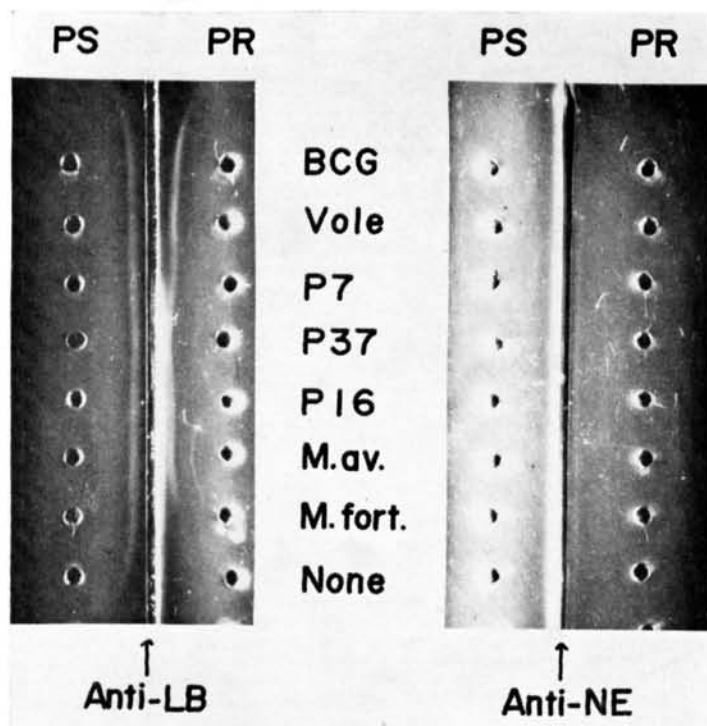


FIG. 7. Cross reaction between anti-LB, anti-NE sera and soluble components of mycobacteria.

DISCUSSION

From the described experiments it is evident that in the water-soluble extract of leprosy nodules (NE) there exists at least two antigens which are not found in normal human skin and lymph node tissues. One of these antigens is heat-stable and has the slower electrophoretic mobility. It appears to be a polysaccharide of *M. leprae* which has a common antigenicity with the other mycobacteria, because it causes a precipitation reaction not only with rabbit antiserum against purified leprosy bacilli (LB) but also with rabbit antisera against BCG and vole bacillus. This finding is in accord with the results of immunodiffusion tests, using lepromin and serum from leprosy patients, as described by Burrell and Rheins (⁴), Pepys *et al.* (¹⁶) and Navalkar *et al.* (¹¹). Besides, the presence of a common antigen in leprosy bacilli is also supported by the facts that anti-LB serum produced a positive precipitate with the polysaccharide fraction from various strains of mycobacteria and high antibody-titers in Middlebrook-Dubos' hemagglutination and hemolysis reactions.

More interesting and important findings are concerned with the other antigen which was demonstrated by anti-NE serum. This antigen was inactivated by autoclaving. Therefore, this heat-labile antigen, or the antibody responding to it, could not be detected by the immunodiffusion tests using lepromin as antigen. This antigen in NE may be a protein having an electrophoretic mobility similar to α_2 - or β_1 -globulin, but it differs from serum proteins or a protein in normal skin because it caused a precipitation reaction even with anti-NE serum absorbed with human serum or skin tissue. The high agglutination titer of anti-NE serum against human red blood cells of type A and type B suggests the existence of blood group substances in NE. However, the above-mentioned antigen is not related to these substances, because its precipitation line with anti-NE serum was not influenced by the absorption of this antiserum with either A or B red blood cells.

Aoki and Fujinami (³) reported tissue-specific soluble antigens in human skin by immunodiffusion tests. The major component of these antigens migrated to the

cathodal side of agar gel in immunoelectrophoresis. Accordingly, this substance also seems to be different from the antigen in NE. As lepromas consist of tissue cells and fibers modified from those in normal skin, it is reasonable to consider that the antigen in NE is not found in normal tissues, even assuming its source to be from tissue components. In other words, this antigen may be one of abnormal proteins such as CRP which could localize in the nodular areas. Another hypothesis as to the origin of this antigen is that it may be a protein of *M. leprae*. These problems were discussed in serial reports in which the skin reactivity of this antigen and immunofluorescence using anti-NE serum were examined.⁴ In any case, it is probable that this antigen is very specific to the leprosy nodule, because anti-NE serum absorbed with human serum does not react at all with normal tissue extracts nor with soluble components of cultivatable mycobacteria.

The heat-stable antigen in NE could not be detected by anti-NE serum in immunodiffusion tests, although Middlebrook-Dubos' hemagglutination and hemolysis reactions were weakly positive with this antiserum. These facts can be explained by a low level of antibody production in the rabbit when immunized with NE containing no bacillary body. On the other hand, the fact that anti-LB serum produced no precipitation reaction with the heat-labile antigen in NE does not mean the absence of this antigen in the cell wall or cytoplasm of *M. leprae*, but rather may indicate that the greater part of the soluble antigen in this bacillus has been lost by the severity of purification procedures such as trypsin digestion and repeated centrifugal washing.

In the immunoelectrophoresis of NE, anti-LB serum produced a precipitation line on the cathodal side, the line resembling that formed by human γ -globulin and anti-globulin antibody. Absorption of anti-LB serum with purified γ -globulin revealed that this antiserum actually contained the antibody against this globulin. Rao *et al.* (18) have also reported that immunization

with *M. leprae* treated with trypsin gives rise to antibodies in rabbits which react with components of human serum. These facts suggest that it is very difficult to completely remove serum proteins, especially γ -globulin, from *M. leprae*. This seems to be inconsistent with the explanation concerning the facile loss of soluble antigens from *M. leprae* as described above. A probable interpretation of this may be either the mere localization of γ -globulin in the nodular area or that *M. leprae* could be combined with antibodies *in vivo* or in the process of homogenization of lepromas. If the latter explanation is true, the formation of such a complex may render the greater part of the peptide chain in the antibody molecule insoluble in spite of the drastic trypsin digestion.

SUMMARY

Immunodiffusion tests with a water-soluble extract from unheated leprosy nodules were carried out, in order to determine whether any antigens of *M. leprae* may be present in the water-soluble components of leprosy nodules. It was found that the leprosy nodule extract contained at least two antigens which were easily differentiated from human serum proteins and not present in normal human skin and lymph nodes. One of these antigens appeared to be a heat-stable polysaccharide of *M. leprae*, because it caused a precipitation reaction not only with the rabbit antiserum against purified leprosy bacilli but also with the rabbit antisera against BCG and *M. microti*. Another antigen was a heat-labile protein and produced a single precipitation line with the rabbit antileprosy nodule extract serum which had been absorbed with human serum or skin tissue, but did not react at all with the antisera against mycobacteria. The former antiserum showed no cross reaction with the soluble components of various cultivatable mycobacteria.

RESUMEN

Se hicieron pruebas de inmunodifusión con un extracto soluble en agua de nódulos de lepra, no calentados, para determinar si hay antígenos del *M. leprae* presentes en los componentes solubles en agua de nódulos de lepra.

⁴ These studies were reported at the leprosy symposium of U.S.-Japan Cooperative Medical Science Program held in August 1968; the abstract appeared in THE JOURNAL 36 (1968) 514-515.

Se encontró que el extracto de nódulos de lepra contenía por lo menos dos antígenos que se diferenciaban fácilmente de las proteínas séricas humanas y que no se encontraban en piel humana normal y ganglios linfáticos. Uno de estos antígenos parecía ser un polisacárido termoestable del *M. leprae*, ya que producía líneas de precipitación no sólo con el antisuero de conejo contra bacilos de lepra purificados, sino también con antisuero de conejo contra BCG y *M. microti*. El otro antígeno era una proteína termo-lábil y producía una sola línea de precipitación con el suero de conejo anti-extracto de nódulo de lepra, que se había absorbido previamente con suero humano o con piel humana, no reaccionando en absoluto con los antisueros contra micobacterias. El antisuero anterior no mostró reacciones cruzadas con los componentes solubles de varias micobacterias cultivables.

RÉSUMÉ

On a pratiqué des épreuves d'immuno-diffusion avec un extrait hydrosoluble, préparé à partir de nodules lépreux non chauffés, en vue de déterminer la présence éventuelle d'antigènes de *M. leprae* dans les constituants hydrosolubles des nodules de la lèpre. On a trouvé que l'extrait de nodules lépreux contenait au moins deux antigènes qui pouvaient être facilement distingués des protéines sériques humaines, et qui n'étaient pas présents dans la peau humaine normale et dans les ganglions lymphatiques. L'un de ces antigènes s'est révélé être un polysaccharide thermo-stable de *M. leprae*, car il produisait une réaction de précipitation non seulement avec l'anti-sérum du lapin contre des bacilles de la lèpre purifiés, mais encore avec des anti-séras de lapins préparés contre le BCG et contre *M. microti*. On a trouvé un autre antigène, qui était une protéine thermo-labile et qui produisait une bande unique de précipitation avec le sérum de lapins préparé contre l'extrait nodulaire lépreux et absorbé au préalable par du sérum ou par du tissu cutané; cet antigène ne réagissait pas à tous les anti-séras anti-mycobactériens. Le premier anti-sérum signalé ne témoignait pas de réaction croisée avec les constituants solubles des diverses mycobactéries qui peuvent être cultivées.

Acknowledgement. This study was supported in part by a grant from the U.S.-Japan Cooperative Medical Science Program, Geographic Medicine Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md. 20014.

REFERENCES

1. ABE, M. Serological relationship of leprosy, tuberculosis and syphilis. II. *In vitro* antigenicity of the lipid recovered from leprous nodule. *La Lepro* **26-28** (1960) 59-65. (Selected article)
2. ABE, M., CHINONE, S. and HIRAKO, T. Rheumatoid-factor-like substance and antistreptolysin O antibody in leprosy serum. Significance in erythema nodosum leprosum. *Internat. J. Leprosy* **35** (1967) 336-344.
3. AOKI, T., and FUJINAMI, T. Demonstration of tissue-specific soluble antigens in human skin by immunodiffusion. *J. Immunol.* **98** (1967) 39-45.
4. BURRELL, R. G. and RHEINS, M. S. Antigenic analysis of lepromin by agar-diffusion. *Internat. J. Leprosy* **25** (1957) 223-230.
5. DANIEL, T. M., WEYAND, JR., J. G. M. and STAVITSKY, A. B. Micro-methods for the study of proteins and antibodies. IV. Factors involved in the preparation and use of a stable preparation of formalinized, tannic acid-treated, protein-sensitized erythrocytes for detection of antigen and antibody. *J. Immunol.* **90** (1963) 741-750.
6. DHARMENDRA, The lepromin test—a review. *Leprosy Rev.* **18** (1947) 92-126.
7. ESTRADA-PARRA, S., CALDERON-MANES, S., SALAZAR-MALLEN, M. and AMEZCUA, M. E. Isolation of a group-specific polysaccharide from tissues infected with *Mycobacterium leprae*. *Internat. J. Leprosy* **34** (1966) 294-297.
8. FUKUI, Y. and YONEDA, M. Extracellular proteins of tubercle bacilli. III. Column chromatographical purification of the proteins of a virulent strain (H₃₇Rv) of *Mycobacterium tuberculosis*. *Biken's J.* **4** (1961) 187-196.
9. FUKUI, Y., HIRAI, T., UCHIDA, T. and YONEDA, M. Extracellular proteins of tubercle bacilli. IV. α and β antigens as major extracellular protein products and as cellular components of a strain (H₃₇Rv) of *Mycobacterium tuberculosis*. *Biken's J.* **8** (1965) 189-199.
10. FUKUI, Y., MORI, T., KOHSAKA, K., NISHIMURA, S. and YONEDA, M. Antigenic structure of *Mycobacterium lepraemurium*. I. Presence of avian type α antigen in strains of *M. lepraemurium*. *Biken's J.* **9** (1966) 63-75.
11. NAVALKAR, R. G., NORLIN, M. and OUCH-

- TERLONY, O. Characterization of leprosy sera with various mycobacterial antigens using double diffusion-in-gel analysis. *Internat. Arch. Allergy* **25** (1964) 105-113; **28** (1965) 250-260.
12. OGATA, T. Fundamental principles applicable to highly sensitive serological tests for syphilis. *Japanese J. Microbiol.* **1** (1957) 423-431.
13. OLMOS CASTRO, N. and ARCURI, P. B. Attempts to obtain an antigen (LPT) suitable for study of hypersensitivity in leprosy. *Internat. J. Leprosy* **26** (1958) 51-56.
14. PARLETT, R. and YOUMANS, G. P. Antigenic relationship between mycobacteria as determined by agar diffusion precipitin techniques. *American Rev. Tuberc. & Pulmon. Dis.* **73** (1956) 637-649.
15. PARLETT, R. C. and YOUMANS, G. P. Antigenic relationship between ninety-eight strains of mycobacteria using gel-diffusion precipitation techniques. *American Rev. Tuberc. & Pulmon. Dis.* **77** (1958) 450-461.
16. PEPYS, J., AUGUSTIN, R. and PATTERSON, A. B. Common antigenic components of mycobacterial extracts. *Tubercle* **40** (1959) 163-172.
17. PRABHAKARAN, K. and BRAGANCA, B. M. Glutamic acid decarboxylase activity of *M. leprae* and occurrence of α -amino-butyric acid in skin lesions of leprosy. *Nature (London)* **196** (1962) 589-590.
18. RAO, S. S., NADKORNI, J. S. and KHANOLKAR, V. R. Immunologic studies with *Mycobacterium leprae* and an acid-fast mycobacterium cultivated from human leprosy nodules. *Internat. J. Leprosy* **32** (1964) 103-116.
19. SOBER, H. A., CUTTER, F. J., WYCKOFF, M. M. and PETERSON, E. A. Chromatography of proteins. II. Fractionation of serum protein on anion-exchange cellulose. *J. American Chem. Soc.* **78** (1956) 756-763.
20. SUSHIDA, K. and HIRANO, N. The detection of antibodies against "atypical acid-fast bacilli" in the serum of leprosy patient by the Ouchterlony method. *La Lepro* **30** (1961) 81-88 (in Japanese).
21. TUMA, M. and SILVA, C. Antigenic relationship between the Hansen bacillus and other mycobacteria. *Internat. J. Leprosy* **30** (1962) 71-76.
22. YONEDA, M. and FUKUI, Y. Extracellular proteins of tubercle bacilli. I. Zone-electrophoretic purification of the proteins of a virulent strain ($H_{37}Rv$) of *Mycobacterium tuberculosis*. *Biken's J.* **4** (1961) 25-40.
23. YONEDA, M. and FUKUI, Y. Extracellular proteins of tubercle bacilli. II. Some serological properties of purified extra-cellular proteins of a virulent strain ($H_{37}Rv$) of *Mycobacterium tuberculosis*. *Biken's J.* **4** (1961) 121-130.
24. YONEDA, M., FUKUI, Y. and YAMANOUCHI, T. Extracellular proteins of tubercle bacilli. V. Distribution of α and β antigens in various mycobacteria. *Biken's J.* **8** (1965) 201-223.