

## In Vitro Synthesis of Anti-Mycobacterial Antibodies in Biopsies from Skin Lesions of Leprosy Patients

RUDY F. M. LAI A FAT,<sup>1</sup>† JIMMY CHAN PIN JIN,<sup>1</sup> RALPH VAN FURTH,<sup>2</sup> AND MORTEN HARBOE<sup>3</sup>\*

*Dermatological Service, Paramaribo, Suriname,<sup>1</sup> Department of Infectious Diseases, University Hospital, Leiden, The Netherlands,<sup>2</sup> and University of Oslo, Institute for Experimental Medical Research, Ullevaal Hospital, Oslo 1, Norway<sup>3</sup>\**

To demonstrate local synthesis of anti-*Mycobacterium leprae* antibodies, biopsies from skin lesions of leprosy patients were cultured in vitro in a medium containing <sup>14</sup>C-labeled lysine and isoleucine, and the culture fluids were analyzed by crossed immunoelectrophoresis with intermediate gel and autoradiography. The results show that anti-*M. leprae* antibodies were synthesized in vitro in the biopsies from the skin lesions of leprosy patients and that the specificity of the locally produced antibodies varied from patient to patient.

In a previous study it was demonstrated that immunoglobulins (mostly immunoglobulin G [IgG]) are produced locally in skin lesions of leprosy patients. The amount of locally synthesized IgG ranged from small in tuberculoid to distinct in borderline and large in lepromatous leprosy (6).

Antibodies against various mycobacterial antigens occur in sera from leprosy patients in amounts increasing from the tuberculoid to the lepromatous end of the spectrum (1, 5, 9, 13). The increasing amounts of anti-mycobacterial antibodies correlate to a certain extent with the increasing number of leprosy bacilli in the skin lesions. These observations indicate that at least a part of the locally produced IgG may represent anti-mycobacterial antibodies.

To investigate whether the locally synthesized immunoglobulins contain antibodies directed against *Mycobacterium leprae* antigens, lesional skin of leprosy patients was cultured in vitro in a medium containing [<sup>14</sup>C]lysine and [<sup>14</sup>C]isoleucine (7). The culture fluids were incorporated in the intermediate gel of crossed immunoelectrophoresis (CIE) plates with *M. leprae* antigen in the first dimension gel and a reference anti-*M. leprae* serum in the top gel. By autoradiography, it was found that some of the precipitin lines contained radioactivity. This shows that anti-mycobacterial antibodies were synthesized in vitro during the short time culture of lesional skin, confirming our initial hypothesis.

### MATERIALS AND METHODS

**Tissues.** Biopsy specimens were taken from seven untreated leprosy patients who were classified clinically

and histopathologically according to the Ridley and Jopling scale (10, 11). From each patient, six biopsy specimens with a diameter of 5 mm were taken without previous disinfection of the skin. One sample was used for histological examination, one was used for immunofluorescence investigation, and four were used for culture. Two of those used for culture were processed directly, and the other two were cultured after killing the cells by freezing (-20°C) and thawing (three times) to serve as controls.

**Sera.** Sera were obtained from venous blood at the same time as biopsy specimens were taken and stored at -20°C until used.

**Demonstration of immunoglobulin synthesis in vitro.** The method used for demonstration of immunoglobulin synthesis has been described in detail previously (7). Briefly, the biopsy specimen was minced in Hanks balanced salt solution after removal of the epidermis, and the tissue fragments were incubated for 48 h in 1 ml of modified Eagle medium containing 1 μCi of L-[<sup>14</sup>C]lysine (specific activity, 312 mCi/mmol; Schwarz Bio Research, Orangeburg, N.Y.) and 1 μCi of L-[<sup>14</sup>C]isoleucine (specific activity, 312 mCi/mmol; Schwarz Bio Research). To this medium, gentamicin (25 μg/ml) was added. After incubation, the cultures were frozen (-20°C) and, after being thawed, were dialyzed against phosphate buffer (0.015 M; pH 7.6) to remove excess radioactive amino acids. Next, the culture fluid was concentrated by lyophilization, dissolved in 0.1 ml of bidistilled water, and analyzed by micro-immunoelectrophoresis. Because concentrated culture fluids often contain too little protein to provide well-defined precipitation lines, an appropriate carrier serum was used, and the newly synthesized immunoglobulins were detected and identified by autoradiography. The plates were exposed to Kodak Royal-X pan film (1200 ASA) for 21 days and developed with 10% Rodinal solution (Agfa).

The antisera used were horse anti-human serum, sheep anti-IgA, and sheep anti-IgM obtained from the Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam, The Netherlands.

**CIE.** CIE with intermediate gel was carried out on

† Present address: Department of Dermatology, University Hospital, Paramaribo, Suriname.

glass plates (5 by 5 cm), and the plates were washed, pressed, and stained with Coomassie brilliant blue as described in detail elsewhere (2). The gels were made with 1% agarose, Litex type HSA with moderate electroendosmotic flow (Litex, Glostrup, Denmark). The top gel contained 150  $\mu$ l of rabbit anti-*M. leprae* antiserum; the intermediate gel contained 100  $\mu$ l of buffer, patient serum, or reconstituted culture fluid as specified below. The circular antigen well contained 10  $\mu$ l of an *M. leprae* sonic extract prepared as described previously (4).

By incorporation of patient serum in the intermediate gel of a CIE plate, its antibody content can be analyzed by direct comparison with a reference system defined by the *M. leprae* antigens in the circular well and the anti-*M. leprae* antibodies in the top gel. Antibodies against *M. leprae* antigens 2, 5, and 7 are the most frequently occurring in sera of lepromatous leprosy patients (4). Component no. 4 usually gives a very distinct line in CIE with rabbit anti-*M. leprae* serum against *M. leprae* sonic extract. Component no. 6 often gives a faint, rather diffuse precipitate in this system, and the height of this peak was less reproducible than for the other components analyzed.

The antiserum was prepared by pooling different rabbit anti-*M. leprae* antisera to obtain a pattern where components no. 2, 4, 5, and 7 gave distinct lines lying free of each other and thus providing optimal conditions for demonstration of the corresponding antibodies (Fig. 1A). An additional, very distinct component is seen between components 5 and 7; it has not yet been assigned a definite number and is denoted component X in this paper.

For demonstration of antibodies in sera, 100  $\mu$ l of patient serum was incorporated in the intermediate gel and the plates were analyzed for retention of antigen in the intermediate gel as described elsewhere (3, 4) (Fig. 1B).

For the study of culture fluids, 100  $\mu$ l of reconstituted culture fluid was incorporated in the intermediate gel. In some instances, the total antibody content in the culture fluid—the original antibody present in the intercellular fluid plus newly synthesized antibody—was sufficient to change the position of a precipitation line (cf. the extended legs of precipitin line 5 in Fig. 1C). The newly synthesized antibodies in the culture fluids were detected and identified by autoradiography of the CIE plates. Autoradiography was performed with X-ray film, the exposure time being 4 and 12 weeks. As a control for antibody synthesis during the 48-h culture period, CIE plates with intermediate gels in which culture fluid of dead tissue was incorporated were subjected to autoradiography in the same way.

## RESULTS

### Anti-*M. leprae* antibodies in patient sera.

For demonstration of anti-*M. leprae* antibodies in sera, 100  $\mu$ l of patient serum was incorporated in the intermediate gel of the CIE plate. The results were compared with the reference plate which contained buffer in the intermediate gel and evaluated as described by Harboe et al. (4) (Fig. 1A and B).

The results of these experiments are given in Table 1. The antibody content in serum varied from patient to patient, the number of antibody specificities and the amount of antibody decreased toward the tuberculoid end of the spectrum, and anti-*M. leprae* 2, 5, and 7 were the most frequently occurring specificities corresponding to previous findings (4).

**Immunoglobulin synthesis.** The analysis of immunoglobulin synthesis *in vitro* demonstrated that IgG was synthesized in different amounts in the lesional skin of the patients investigated. The amounts varied from large in polar and subpolar lepromatous to distinct in borderline and barely detectable or nondetectable in tuberculoid leprosy. Production of IgA and IgM could not be detected. These results confirm those published previously (6).

**Demonstration of anti-*M. leprae* antibodies in culture fluids.** For demonstration of *in vitro*-synthesized anti-*M. leprae* antibodies, culture fluids of biopsies of skin lesions were analyzed by CIE with intermediate gel and autoradiography (Table 2, Fig. 2 and 3).

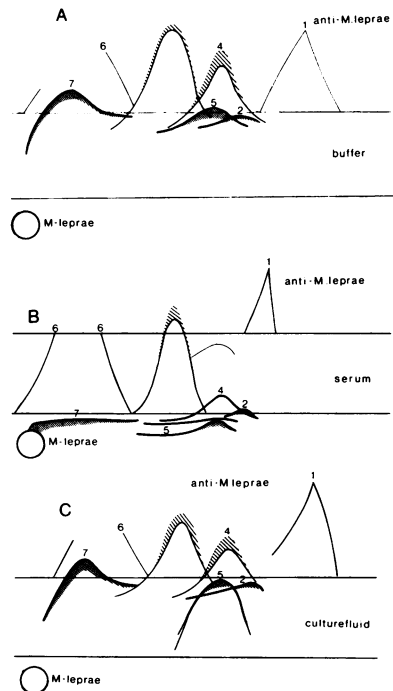


FIG. 1. Drawings of the original plates. (A) Reference pattern. (B) Strong antibody activity against *M. leprae* antigens 2, 4, 5, and 7 and distinct antibody activity against X in the serum of a borderline patient (patient no. 4). (C) The antibodies against *M. leprae* antigen 5 present in intercellular fluid (patient no. 4) plus the *in vitro*-synthesized antibodies are sufficient to change the position of precipitin line 5.

TABLE 1. Reaction of patient sera with antigenic components of *Mycobacterium leprae*<sup>a</sup>

Serum <sup>b</sup>	Reaction with the following component:						
	1	2	4	5	X	6	7
1; LLp	—	↓↓	↓	↓↓	↓	↓↓	↓↓
2; LLs	?	↓↓	?	↓↓	?	↓	(↓)
3; LLs	(↓)	↓	↓↓	↓	?	↓	↓
4; BB	—	↓↓	↓↓	↓↓	↓	?	↓↓
5; BT	—	↓	(↓)	↓	—	(↓)	?
6; BT	—	↓	—	↓	—	—	(↓)
7; TT	—	(↓)	—	—	—	—	—

<sup>a</sup> The results are indicated as described by Harboe et al. (4). —, No antibody activity detected; ?, barely detectable antibody activity; (↓) slight but definite change in the precipitin line; ↓, precipitate lower than normal, with its feet extending down into the intermediate gel; ↓↓, precipitate located close to the bottom of the intermediate gel.

<sup>b</sup> Patient number and classification.

TABLE 2. *In vitro*-synthesized antibodies in biopsies of skin lesions from leprosy patients<sup>a</sup>

Biopsy	Synthesis of antibodies against <i>M. leprae</i> antigen						
	1	2	4	5	X	6	7
1; LLp <sup>b</sup>	—	—	—	+	—	—	—
2; LLs	—	+	—	+	—	—	—
3; LLs	+	+	+	+	—	+	—
4; BB	—	+	+	+	—	—	—
5; BT	—	—	—	—	—	+	—
6; BT	—	—	—	—	—	—	—
7; TT	—	—	—	—	—	—	—

<sup>a</sup> —, No line on the autoradiograph; +, line on the autoradiograph.

<sup>b</sup> Patient number and classification.

Figure 2 shows the patterns of the autoradiographs in three patients. Local synthesis of antibodies against one, two, or three components of *M. leprae* was demonstrated in these individuals.

The findings with patient no. 3 are shown more completely in Fig. 3. Autoradiography with an exposure time of 4 weeks revealed production of antibodies against *M. leprae* antigens 2, 4, and 5 (Fig. 3C). The autoradiograph developed after a prolonged exposure time of 12 weeks showed labeling of two additional lines, namely, corresponding to antigens no. 1 and 6 (Fig. 3E). In the culture fluids of dead tissues, labeled anti-*M. leprae* antibodies could not be demonstrated (Fig. 3G).

From these results it was concluded that anti-*M. leprae* antibodies were produced *in vitro* during the short time culture of lesional skin and that the specificity of the locally synthesized antibodies varies from patient to patient.

The autoradiographs developed after an extended exposure time of 12 weeks showed in only one case (patient no. 3) labeling of 2 additional precipitin lines. In the other cases there was no difference between exposure times of 4 and 12 weeks.

The demonstration of *in vitro*-synthesized IgG and anti-*M. leprae* antibodies in culture fluids of lesional skin make it very conceivable that the anti-*M. leprae* antibodies demonstrated belong to the IgG class. To confirm this, culture fluids from three lepromatous leprosy patients (two LLs and one LLp) were pooled, and the IgG fraction was purified by diethylaminoethyl-Sephadex chromatography. Next, the IgG fraction was incorporated in the intermediate gel of a CIE plate and analyzed as described above. The autoradiograph showed that the isolated IgG fraction contained the *in vitro*-synthesized antibodies against *M. leprae* antigens.

DISCUSSION

The analysis of the culture fluids by CIE with intermediate gel and autoradiography showed several labeled *M. leprae* antigen lines. Labeling of these lines would result from reaction between radioactive antibodies synthesized during *in vitro* culture and *M. leprae* antigens. The possibility that labeled products from the cultured tissue are sticky and nonspecifically may cause labeling of precipitin lines was also considered. Biopsies from patients with tuberculoid leprosy were included as controls, representing living

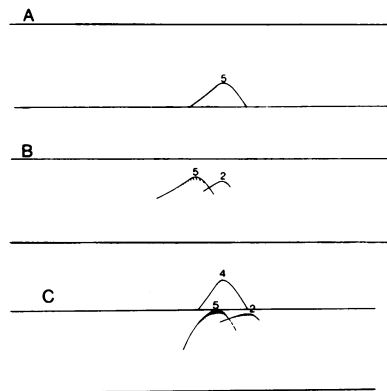


FIG. 2. Drawings of autoradiographs of the CIE plates, demonstrating antibody production *in vitro* during the short time cultures. (A) Patient no. 1; (B) patient no. 2; (C) patient no. 4. The top gel had the same size in all original plates but has been cut to save space. In Fig. 2 the two horizontal lines in A, B, and C show the limits of the intermediate gel of the corresponding plates. In Fig. 3 similar cuts have been made, but the complete intermediate gel is shown in A to F.

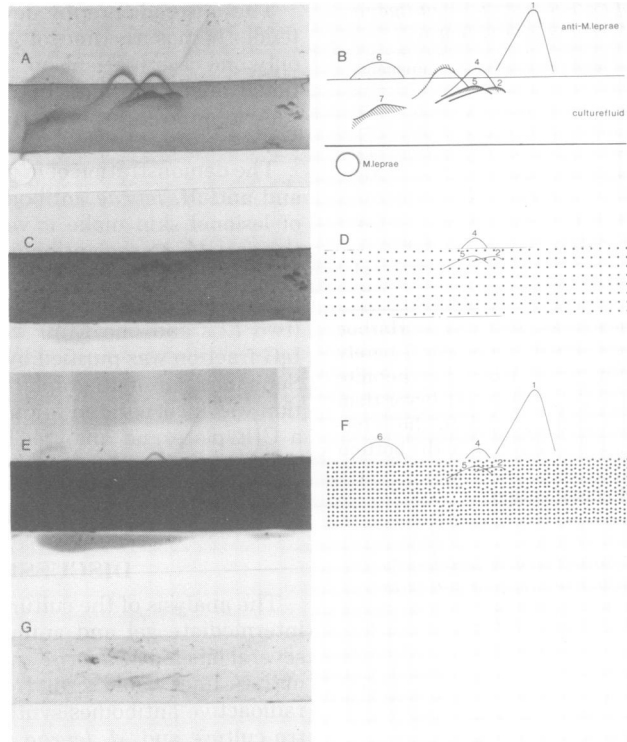


FIG. 3. Demonstration of anti-*M. leprae* antibodies in culture fluid of a patient with LLs leprosy (patient no. 3). (A) CIE pattern; the intermediate gel contained culture fluid. (C) Autoradiograph of the CIE plate after 4 weeks of exposure, demonstrating *in vitro* synthesis of antibodies against *M. leprae* antigens 2, 4, and 5. (E) Autoradiograph after 12 weeks of exposure. The intermediate gel is almost black, but the top gel shows two more antibody activities, i.e., antibodies against *M. leprae* antigens 1 and 6. (B, D, and F) Drawings of A, C, and E, respectively. The control experiment in which culture fluid of dead tissue was incorporated in the intermediate gel shows no antibody activities (G).

cultures where synthesis of antibodies is expected to be smaller due to a lower antigenic stimulus. In the culture of BT patient no. 5, a single line was labeled, whereas culture material from the two other patients with tuberculoid leprosy was completely negative. Labeling of lines might also be explained by reaction between radioactive antigens of *M. leprae* formed during the culture period and antibody present in the tissues. It is unlikely that a sufficient amount of radioactivity is incorporated in newly synthesized *M. leprae* antigen during the short culture period to be detectable by CIE and autoradiography. Liberation of antigen from the bacilli is also expected to be poor by the method used for processing of biopsied tissue. The different patterns observed in different patients is, in itself, also a control strongly supporting the view that labeling of lines is due to a reaction between *M. leprae* antigens and antibodies that are radioactive due to incorporation of labeled amino acids during synthesis *in vitro*.

In a previous study, Lai A Fat et al. (7) demonstrated synthesis of IgG in pathological skin of patients suffering from certain skin diseases such as eczema, lichen planus, mycosis fungoides, and immunological skin diseases. The function of the locally synthesized IgG was not studied, since the cause of these diseases is not known. In leprosy it is now shown that the IgG produced in the skin lesions contains antibodies directed against *M. leprae* antigens.

To our knowledge this is the first time that synthesis of specific antibodies has been demonstrated in lesional skin *in vitro*. This finding is not unexpected, however, since the granuloma in the skin lesions of borderline lepromatous leprosy contain lymphocytes, most probably B-lymphocytes, and variable numbers of plasma cells (11, 12). Moreover, by applying the direct immunofluorescence technique, Ig-positive cells which were morphologically typical lymphocytes and plasma cells could be demonstrated in the lesional skin of some of the presently re-

ported borderline lepromatous and lepromatous leprosy patients (J. Chan Pin Jin, Ph.D. thesis, University of Suriname, Paramaribo, Suriname, 1978). Other skin diseases which would also be of interest to study in this respect include leishmaniasis, syphilis, and tuberculosis.

The stimulus for the production of anti-mycobacterial antibodies in the skin lesions is probably local release of mycobacterial antigens from macrophages containing bacilli. The biological significance of the locally synthesized antibodies is not yet clear. They are probably not responsible for protective immunity, since lepromatous leprosy patients have high titers of *M. leprae*-specific antibodies and still numerous bacilli in their tissues. It is possible, however, that the antibodies may serve to localize the antigens, e.g., by precipitating soluble antigens or by agglutinating leprosy bacilli. The antibodies may also have an opsonizing effect resulting in enhanced phagocytosis.

When the results of tests on culture fluids and sera were compared, it was noted that when antibodies of a particular specificity were synthesized during culture (e.g., anti-*M. leprae* 5), antibodies of the same specificity were always found in serum. The opposite was, however, not the case, and the specificity of the locally synthesized antibodies was more restricted than in the serum. The findings with regard to anti-*M. leprae* 7 are particularly striking. Antibodies against this antigen occur very frequently in leprosy (Table 1) (4, 8, 13), whereas this line did not show radioactivity in any of the cases studied at present. This may be due to the peculiar shape of this precipitin line with lesser sensitivity for detection of the corresponding antibodies. Another and attractive hypothesis is that the specificity of the antibodies may vary and depend on the site of synthesis. Experiments are in progress to elucidate this problem by culturing tissue obtained from different sites in individual patients.

#### ACKNOWLEDGMENTS

This work was supported by grants from Anders Jahre's Fund for the Promotion of Science and the Norwegian Research Council for Science and the Humanities. The work was also supported by the Immunology of Leprosy component of the United Nations Development Programme/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases, the Pan American Health Organization, and Hoffmann-La Roche B.V.

The cooperation of the Medical Staff of the Dermatological Service in Suriname in obtaining untreated leprosy patients is gratefully acknowledged. We also thank I. Oemrawsingh, Head of the Department of Medical Chemistry, for making it possible for us to process the biopsy samples and incubate the tissue fragments in his laboratory. We also thank Brit Sundsten and Martina Dieselhoff-den Hulk for their excellent technical assistance.

#### LITERATURE CITED

1. Abe, M., S. Izumi, T. Saito, and S. K. Mathur. 1976. Early serodiagnosis of leprosy by indirect immunofluorescence. *Lepr. India* 48:272-276.
2. Axelsen, N. H., J. Krøll, and B. Weeke (ed.). 1973. A manual of quantitative immunoelectrophoresis. Methods and applications. *Scand. J. Immunol.* 2(Suppl. 1): 15-35.
3. Axelsen, N. H. 1973. Intermediate gel in crossed and in fused rocket immunoelectrophoresis. *Scand. J. Immunol.* 2(Suppl. 1):71-77.
4. Harboe, M., O. Closs, B. Bjorvatn, G. Kronvall, and N. H. Axelsen. 1977. Antibody response in rabbits to immunization with *Mycobacterium leprae*. *Infect. Immun.* 18:792-805.
5. Harboe, M., O. Closs, G. Bjune, G. Kronvall, and N. H. Axelsen. 1978. *Mycobacterium leprae* specific antibodies detected by radioimmunoassay. *Scand. J. Immunol.* 7:111-120.
6. Lai A Fat, R. F. M., J. Chan Pin Jin, M. Diesselhoffden Hulk, and R. van Furth. 1979. In vitro synthesis of humoral factors (immunoglobulins and complement) in lesional skin of leprosy patients. *Infect. Immun.* 25: 891-895.
7. Lai A Fat, R. F. M., D. Suurmond, and R. van Furth. 1973. In vitro synthesis of immunoglobulins, secretory component and complement in normal and pathological skin and the adjacent mucous membranes. *Clin. Exp. Immunol.* 14:377-395.
8. Melsom, R., B. Naafs, M. Harboe, and O. Closs. 1978. Antibody activity against *Mycobacterium leprae* antigen 7 during the first year of DDS treatment in lepromatous (BL-LL) leprosy. *Lepr. Rev.* 49:17-29.
9. Myrvang, B., G. M. Feek, and T. Godal. 1974. Antimycobacterial antibodies in sera from patients throughout the clinicopathological disease spectrum of leprosy. *Acta Pathol. Microbiol. Scand. Sect. B* 82:701-706.
10. Ridley, D. S. 1974. Histological classification and the immunological spectrum of leprosy. *Bull. W. H. O* 51: 451-465.
11. Ridley, D. S. 1977. Skin biopsy in leprosy. Histological interpretation and clinical application. Documenta Geigy, Ciba-Geigy Ltd., Basel, Switzerland.
12. Ridley, M. J., D. S. Ridley, and J. Turk. 1978. Surface markers on lymphocytes and cells of the mononuclear phagocyte series in skin sections in leprosy. *J. Pathol.* 125:91-98.
13. Yoder, L., B. Naafs, M. Harboe, and G. Bjune. 1979. Antibody activity against *Mycobacterium leprae* antigen 7 in leprosy: studies on variation in antibody content throughout the spectrum and on the effect of DDS treatment and relapse in BT leprosy. *Lepr. Rev.* 50: 113-121.