In Vitro Synthesis of Antimycobacterial Antibodies with Different Specificities in Various Tissues of Leprosy Patients[1]

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Production of anti-*Mycobacterium leprae* antibodies has been shown to occur in skin lesions of leprosy patients ([13]). In that study the most frequently occurring antibodies were directed against *M. leprae* antigens 2 and 5. It was also found that the specificity of the locally synthesized antibodies varied from patient to patient. Furthermore, when antibodies of a particular specificity were synthesized in a skin lesion, antibodies of the same specificity were always found in the serum, but the reverse was not always the case. For instance, antibodies against *M. leprae* antigen 7 were found in the serum, but synthesis of antibodies against this antigen could not be demonstrated in the skin lesions.

To find out if, as this striking finding suggests, the specificity of the antibodies depends on the site of synthesis, we cultured samples of tissue from various sites from individual leprosy patients *in vitro* in a medium containing ^{14}C-labeled lysine and isoleucine. The culture fluids were incorporated into 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. Autoradiography was used to detect newly synthesized anti-*M. leprae* antibodies.

MATERIALS AND METHODS

Tissues. Biopsy specimens were taken from 14 untreated leprosy patients classified as: indeterminate = 1; tuberculoid (TT) = 4; borderline tuberculoid (BT) = 1; borderline (BB) = 1; subpolar lepromatous (LLs) = 4; and polar lepromatous (LLp) = 3.

The clinical and histopathological diagnoses were based on the criteria of Ridley and Jopling ([18]) and Ridley ([16, 17]). Biopsy specimens originated from different sites in different patients: samples of TT and BT lesions were taken along the edge (=active site) and from the center, which showed healing (=inactive center). In 3 patients (2 LLs and 1 LLp) tissue samples were taken from the larynx as well. In 2 LLp patients specimens were collected from skin lesions, nasal mucosa, and an inguinal lymph node; in 1 of these 2 LLp patients bone marrow was also sampled.

Serum. Serum was obtained from venous blood collected at the same time as the biopsies were performed, and was stored at −70°C until used.

Cultivation of tissues. The method used for the study of immunoglobulin (Ig) synthesis *in vitro* was originally described by Hochwald, *et al.* ([9]) and later in detail by Lai A Fat, *et al.* ([14]). Briefly, this procedure is as follows. After incubation of minced tissue or bone-marrow flakes for 48 hr in 1 ml modified Eagle's medium containing 1 μCi/ml ^{14}C L-lysine (specific activity, 312 mCi/mmol; Schwarz Bio Research, Orangeburg, New York, U.S.A.) and 1 μCi/ml ^{14}C L-isoleucine (specific activity 312 mCi/mmol; Schwarz), to which gentamycin 25 μg/ml had been added. The cultures were frozen (−20°C), thawed, and then dialyzed

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against phosphate buffer (0.015 M; pH 7.6) to remove the unincorporated radioactive amino acids. The culture fluid was concentrated by freeze-drying and dissolved in 0.1 ml bidistilled water. For the detection of Ig synthesis, the culture fluid was analyzed by micro-immunoelectrophoresis. The antisera used were horse anti-human serum, sheep anti-IgA, and sheep anti-IgM, all obtained from the Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam, The Netherlands. Because concentrated culture fluid contains too little protein to provide well-defined precipitation lines, an appropriate carrier serum was used. Newly synthesized Ig was detected and identified by autoradiography of the immunoelectrophoretic pattern of the culture fluid. The plates were exposed to Kodak Royal-X film (1200 ASA) for 21 days and developed with 10% Rodinal® solution (Agfa-Gevaert, Leverkusen, West Germany).

The intensity of the autoradiographic lines, indicating the amount of protein synthesized, is classified according to a scale ranging from − = negative; (+) = just visible; + = clearly visible; to a maximum of ++++, by comparison with a standard autoradiographic pattern of synthesized immunoglobulins ([3, 14]). All readings were made independently by two observers.

Crossed immunoelectrophoresis (CIE). CIE with intermediate gel was performed according to Axelsen, *et al.* ([2]). Briefly, CIE was performed on glass plates (5 × 5 cm) in 1% agarose, Litex type human serum albumin (HSA), with moderate electro-endosmotic flow (Litex, Glostrup, Denmark). The top gel contained 150 µl of rabbit anti-*M. leprae* anti-serum, and the intermediate gel 100 µl of buffer or patient serum or reconstituted culture fluid. The circular antigen well contained 10 µl of an *M. leprae* sonicate prepared according to Harboe, *et al.* ([7]).

When patient serum is incorporated into the intermediate gel of a CIE plate, its antibody content can be analyzed by comparison with a reference system that is defined by the *M. leprae* antigens in the circular well and the anti-*M. leprae* antibodies in the top gel.

The antiserum we used was pooled rabbit anti-*M. leprae* antisera. This pooled antiserum led to a pattern in which components 2, 4, 5, and 7 gave distinct and separate lines, thus providing optimal conditions for the demonstration of the corresponding antibodies.

For the demonstration of the anti-*M. leprae* antibodies in sera, 100 µl of patient serum was incorporated into the intermediate gel, and the plates were analyzed for retention of antigen in the intermediate gel according to Axelsen ([1]) and Harboe, *et al.* ([7]).

For the demonstration of newly synthesized anti-*M. leprae* antibodies in the culture fluids, 100 µl of reconstituted culture fluid was incorporated into the intermediate gel. 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, and the exposure times were 4 and 16 weeks.

RESULTS

Anti-*M. leprae* antibodies in patient sera. The results of this study show variation in serum antibody content from patient to patient, as well as in the number of antibody specificities, and a decrease in the amount of antibody from the lepromatous to the tuberculoid end of the leprosy spectrum (Table 1). Antibodies to *M. leprae* antigens 2, 5, and 7 were the most frequently occurring specificities in this study.

Synthesis of immunoglobulins. The amounts of synthesized IgG varied from large in polar lepromatous (LLp), distinct in subpolar lepromatous (LLs), and small in borderline (BB), borderline-tuberculoid (BT), and polar tuberculoid (TT) leprosy (Table 2). Synthesis of IgG was found not only in the active area (= edge) of the tuberculoid lesion but also in the inactive part (= center). The same amounts of IgG were synthesized in the peripheral and central parts of the lesions. None of the skin cultures showed synthesis of IgA or IgM.

The cultures of mucous membranes (larynx and nasal mucosa) synthesized IgG and IgA but not IgM. The lymph-node cultures produced IgG in large amounts and IgA in small amounts; the bone-marrow culture produced very large amounts of IgG and distinct amounts of IgA. IgM production did not occur in the cultures of lymph nodes or bone marrow.

TABLE 1. *Reaction of patient sera with antigenic components of* Mycobacterium leprae.

Patient	Diagnosis[a]	Reaction with *M. leprae* antigens[b]						
		1	2	4	5	6	7	11[c]
1	LLp	↓↓	↓↓	↓↓	↓↓	↓	↓↓	↓
2	LLp	—	↓↓	↓	↓↓	↓↓	↓↓	↓
3	LLp	—	↓↓	↓	↓↓	↓	↓↓	↓
4	LLs	—	↓↓	—	↓↓	↓	↓↓	—
5	LLs	↓	↓↓	↓↓	↓↓	↓	↓↓	↓
6	LLs	—	↓↓	↓	↓	↓	↓	↓
7	LLs	?	↓↓	?	↓↓	↓	↓	?
8	BB	—	↓↓	—	↓↓	↓	↓↓	—
9	BT	—	↓	(↓)	↓	↓	↓	—
10	TT	?	↓↓	—	↓	?	↓	—
11	TT	?	↓	—	↓	↓	↓↓	—
12	TT	—	↓	—	(↓)	—	↓	—
13	TT	—	↓	—	↓	—	↓	—
14	I	—	↓	—	(↓)	↓	↓	—

[a] LLp = polar lepromatous, LLs = subpolar lepromatous, BB = borderline, BT = borderline tuberculoid, TT = tuberculoid, I = indeterminate.

[b] Symbols: — = no antibody activity; ? = barely detectable antibody; (↓) = slight but definite change in the precipitin line; ↓ = precipitate lower than normal, with feet extending down into intermediate gel; ↓↓ = precipitin line close to bottom of intermediate gel (7).

[c] This antigen was formerly designated x (13) and was later assigned the number 11 (6).

Synthesis of anti-*M. leprae* antibodies at different sites. For the detection of anti-*M. leprae* antibodies synthesized *in vitro,* culture fluids of tissue originating from various sites were analyzed by CIE with intermediate gel and autoradiography. The results show that anti-*M. leprae* antibodies with different specificities were produced *in vitro* in cultures of lesional skin from the patients (Table 2) and also in the cultures of other tissues, i.e., from the larynx, nasal mucosa, and lymph nodes.

The findings in patient 1, who suffered from polar lepromatous leprosy, are shown in The Figure and Table 2. Antibodies against *M. leprae* antigens 2 and 5 were produced in the lesional skin, and autoradiographically the intensity of *M. leprae* antigen 2 was greater than that of *M. leprae* antigen 5. The nasal mucosa produced antibodies against *M. leprae* antigen 5, and the lymph-node culture showed antibodies against *M. leprae* antigen 2. From these findings it was concluded that in this patient anti-*M. leprae* antibodies were produced locally in the skin lesions and also in the nasal mucosa and lymph-node tissue, and that the specificity of the synthesized antibodies varied among the various sites. Synthesis of anti-*M. leprae* antibodies in the bone-marrow culture could not be evaluated, because the intermediate gel was completely dark due to the strong synthesis of other proteins which were not washed out by the procedure that was adequate for other tissues. The results of the bone-marrow culture are indicated as negative in Table 2.

In another patient (no. 2) with polar lepromatous leprosy, antibodies against *M. leprae* antigens 6 and 7 were synthesized in the skin lesions, and synthesis of antibodies against *M. leprae* antigen 5 occurred in the nasal mucosa and lymph-node cultures. From these results, it may be concluded that this patient also produced anti-*M. leprae* antibodies with different specificities in the different tissues.

Patient 7, who suffered from subpolar lepromatous leprosy, produced antibodies with the same specificity at two different sites, i.e., skin lesion and larynx. The intensity of *M. leprae* antigen 5 was stronger than that of *M. leprae* antigen 2 in the autoradiograph of the skin culture.

Patient 11, with polar tuberculoid leprosy, synthesized antibodies against *M. leprae* antigens 2, 6, and 7 in the active part of the skin lesion (= edge) as well as in the quiet part (= center). The intensity of the individual labeled lines in the autoradio-

TABLE 2. *Synthesis of immunoglobulins and antibodies in various types of leprosy patients.*

Patients	Diagnosis[a]	Tissue	Immunoglobulins[b]		Synthesis of antibodies against *M. leprae* antigens[c]			
			IgG	IgA	2	5	6	7
1	LLp	Skin lesion	++	−	+	+	−	−
		Nasal mucosa	++	++	−	+	−	−
		Lymph node	++	(+)	+	−	−	−
		Bone marrow	++++	+	−[d]	−	−	−
2	LLp	Skin lesion	++	−	−	−	+	+
		Nasal mucosa	++	−	−	+	−	−
		Lymph node	++	(+)	−	+	−	−
3	LLp	Skin lesion	+	−	−	+	−	−
		Larynx	++	++	−	−	−	−
4	LLs	Skin lesion	+	−	+?	+?	+	+
5	LLs	Skin lesion	−	−	−	−	−	−
		Lymph node	++	−	−	−	−	−
6	LLs	Larynx	(+)	++	−	+	−	−
7	LLs	Skin lesion	+	−	+	+	−	−
		Larynx	(+)	+	+	+	−	−
8	BB	Skin lesion	(+)	−	+	−	−	−
9	BT	Skin lesion						
		Edge	(+)	−	−	−	+	+
		Centre	(+)	−	−	−	+	+
10	TT	Skin lesion						
		Edge	+	−	−	−	+	+
		Centre	(+)	−	−	−	+	+
11	TT	Skin lesion						
		Edge	(+)	−	+	−	+	+
		Centre	(+)	−	+	−	+	+
12	TT	Skin lesion						
		Edge	ND[e]	ND	−	−	−	−
		Centre	ND	ND	−	−	−	−
13	TT	Skin lesion						
		Edge	(+)	−	−	−	−	−
		Centre	(+)	−	−	−	−	−
14	I	Skin lesion	+	−	−	−	−	−

[a] See Table 1 for abbreviations.

[b] Grading of intensity of the autoradiographic lines: − = negative, (+) = just visible, + = clearly visible up to a maximum of ++++. IgM was not synthesized in any culture and is not included in table.

[c] − = no line on the autoradiograph; + = line present on the autoradiograph; ? = dubious. Antibodies against antigens 1, 4 and 11 were not synthesized in any culture and are not included in table.

[d] Intermediate gel of the antibody assay was completely black, and individual precipitation lines were indistinguishable due to strong background.

[e] ND = not done.

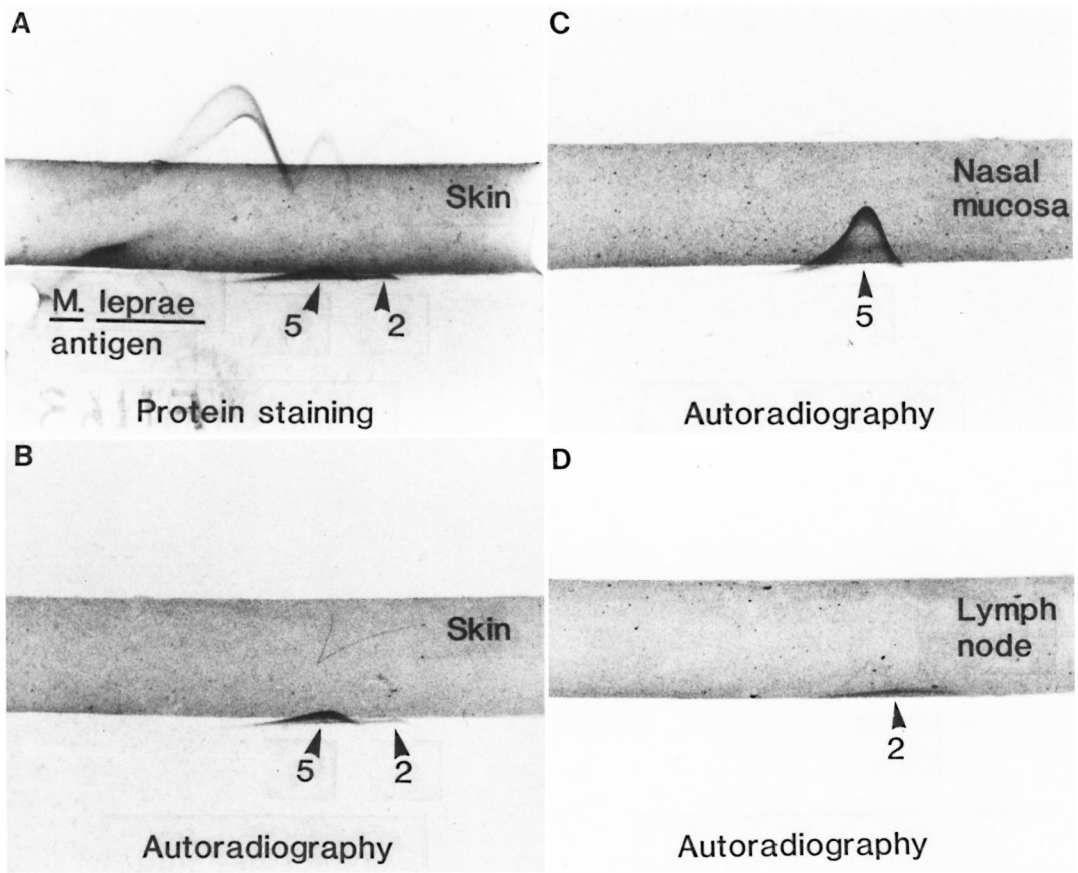
graph of the CIE pattern of the culture fluid was the same for the active and quiet parts of the lesion.

In another patient (no. 10) with polar tuberculoid leprosy, antibodies against *M. leprae* antigens 6 and 7 were formed in both the active and the quiet parts of the lesion.

Four out of five cultures of mucous membrane (two from the larynx and two from the nasal mucosa) showed synthesis of antibodies against *M. leprae* antigen 5. One of the two larynx cultures also produced antibodies against *M. leprae* antigen 2. Of the three lymph-node cultures, two synthesized anti-*M. leprae* antibodies with different specificities, one of them against *M. leprae* antigen 2 and the other against *M. leprae* antigen 5. Antibodies against antigens 1, 4, and 11 could not be demonstrated in any of the cultures.

DISCUSSION

The results of this study show that synthesis of anti-*M. leprae* antibodies occurs locally in skin lesions and other tissues of leprosy patients, i.e., the larynx, nasal mu-



THE FIGURE. Anti-*M. leprae* antibodies at various sites in patient LG who suffered from polar lepromatous leprosy: **A** = CIE pattern: intermediate gel contained fluid from a skin-lesion culture; **B** = Autoradiograph of CIE pattern in **A**, showing *in vitro* synthesis of antibodies against *M. leprae* antigens 2 and 5; **C** = Autoradiograph of CIE pattern of culture fluid of nasal mucosa, showing *in vitro* synthesis of antibodies against *M. leprae* antigen 5; **D** = Autoradiograph of CIE pattern of culture fluid of lymph node, showing *in vitro* synthesis of antibodies against *M. leprae* antigen 2.

cosa, and lymph nodes, and that the specificities of the antibodies produced at the different sites varied in individual patients.

In previous studies, the labeling of immunoglobulin lines was found to be specific and not due to nonspecific adsorption of radioactive amino acids to carrier proteins ([3, 14]). In all probability, labeling of *M. leprae* antigen lines is due to a reaction between *M. leprae* antigens and antibodies made radioactive by the incorporation of labeled amino acids during synthesis *in vitro* ([13]). The present results concerning immunoglobulin synthesis *in vitro* in skin lesions are consistent with our earlier findings ([12, 13]).

In contrast with our findings in normal tissue ([4, 14]), we could not demonstrate synthesis of IgM in cultures of the nasal mucosa, lymph nodes, or bone marrow of leprosy patients. Synthesis of IgG seemed to be normal, but no conclusions could be drawn about IgA synthesis because of the small number of patients.

Comparison of the data on the synthesis of Ig and the production of anti-*M. leprae* antibodies (Table 2) showed that they occurred together except in the larynx culture of patient 3, the skin cultures of two patients (patient 13 with tuberculoid leprosy and patient 14 with indeterminate leprosy), and the lymph-node culture of patient 5.

Our earlier finding of synthesis of anti-*M. leprae* antibodies in skin lesions ([13]) was confirmed by the present results with re-

spect to the synthesis of antibodies against *M. leprae* antigens 2, 5, and 6. In addition, synthesis of antibodies against *M. leprae* antigen 7 was found in some of the skin cultures in the present series. This is an interesting finding, because it is well known that high levels of antibodies against antigen 7 occur in the serum of leprosy patients ([15, 19]) and serve as a valuable indicator of a systemic infection in the armadillo after experimental inoculation with *M. leprae* ([5, 8]).

In tuberculoid lesions, we found synthesis of IgG and anti-*M. leprae* antibodies in the active part (= edge) as well as in the quiet part (= center) of the lesion. In general, there was no difference in the amounts of IgG and anti-*M. leprae* antibodies synthesized in the two parts of the tuberculoid lesions. However, histopathological examination of biopsy specimens from the edge and center of the skin lesions showed that the epithelioid cell granulomas were less pronounced in the center than those at the edge of the lesion.

Analyses of culture fluids and sera showed that when antibodies of particular specificities were synthesized during the culture period, antibodies of the same specificities were always present in the serum. The reverse was not always the case, however; that is to say, the number of antibodies produced locally was lower than the number occurring in the serum.

In lepromatous leprosy, which is a systemic disease, we observed synthesis of antibodies differing in specificities between sites in individual patients. This divergence is probably due to the influence of local factors involved in the induction of an immune response.

The stimulus for the production of anti-*M. leprae* antibodies in skin lesions, mucous membranes, and lymph nodes is probably a local release of *M. leprae* antigens due to the disintegration of macrophages containing ingested *M. leprae.* It is striking that in the two positive cultures of lymph-node tissue we found antibodies directed against only one antigen, although not the same one.

The biological functions of the antibodies produced at various sites in leprosy patients have not been studied. In all probability these are not protective, since lepromatous leprosy patients show high titers of *M. leprae*-specific antibodies but still have an abundance of bacilli in their tissues. It is conceivable that the antibodies serve to localize the antigens, e.g., by precipitating soluble antigens or by aggregating leprosy bacilli. Another possibility is that they are opsonizing antibodies, which would enhance the phagocytosis of *M. leprae* by macrophages. It would be of interest to investigate the culture fluids to find out if they contain antibodies against *M. leprae*-specific antigens such as phenolic glycolipid-I ([10]) or antibodies that recognize an epitope of the 36 kDa protein antigen of *M. leprae* ([11]).

SUMMARY

For the detection of the synthesis *in vitro* of anti-*Mycobacterium leprae* antibodies in various tissues of leprosy patients, biopsy specimens of skin lesions, nasal mucosa, larynx, lymph nodes, and bone marrow were cultured in a medium containing ^{14}C-labeled lysine and isoleucine. The culture fluids were analyzed by crossed immunoelectrophoresis with intermediate gel and autoradiography. The results show that synthesis of anti-*M. leprae* antibodies occurs at the investigated sites of leprosy patients and that the specificities of the synthesized antibodies differ between sites in individual patients. It is conceivable that these antibodies play a role in the local defense against *M. leprae.*

RESUMEN

Se cultivaron fragmentos de biopsias de lesiones dérmicas, mucosa nasal, laríngе, ganglios linfáticos y médula ósea de pacientes con lepra, en un medio con lisina e isoleucina marcadas con ^{14}C para buscar la síntesis *in vitro* de anticuerpos anti-*Mycobacterium leprae.* Los sobrenadantes de los cultivos se analizaron por inmunoelectroforésis cruzada con gel intermedio y autorradiografía. Los resultados muestran que la síntesis de anticuerpos anti-*M. leprae* ocurre en los sitios investigados y que las especificidades de los anticuerpos sintetizados difieren en los diferentes sitios de un mismo individuo. Es posible que estos anticuerpos juegen algún papel en la defensa local contra el *M. leprae.*

RÉSUMÉ

En vue de mettre en évidence la synthèse in vitro d'anticorps contre *Mycobacterium leprae,* dans différents tissus prélevés chez des malades de la lèpre, des biopsies ont été pratiquées dans des lésions cutanées, la muqueuse nasale, le larynx, les ganglions lymphatiques, et la moëlle osseuse. Ces biopsies ont été cultivées dans un milieu contenant de la lysine marquée au

C14, et de l'isoleucine. Les liquides de culture ont été analysés par des méthodes d'immunoélectrophorèse croisée sur gel intermédiaire et par l'autoradiographie. Les résultats ont montré qu'un synthèse d'anticorps contre *M. leprae* a lieu chez le malade de la lèpre au niveau des tissus étudiés et que les spécificités des anticorps ainsi synthétisés varient chez les individus d'endroit à endroit. On peut dès lors concevoir que ces anticorps ont un rôle dans les défenses locales contre *M. leprae.*

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