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Mycobacterial Antigens in Antibody Responses of Leprosy Patients [1]

G. Kronvall, G. Bjune, J. Stanford, S. Menzel and D. Samuel [2]

The regular occurrence of antimycobacterial antibodies in low-resistance forms of leprosy is well documented ([1, 2, 4, 6, 14-18, 26, 28]). In most of these studies cultivable mycobacterial strains have been used as the source of antigen, reflecting the difficulties in obtaining sufficient quantities of *M. leprae* bacilli for antigen preparation. In cases where *M. leprae* antigens have been used, the number of antigens detected was rather low ([1, 2, 6, 16, 18, 28]). When comparisons were made with other mycobacteria, the antigens could also be detected in such cultivable strains. Only Abe claims to have found an antigen of protein nature which is exclusively limited to *M. leprae* ([1, 2]). No comparisons have been made so far with a common reference system for the purpose of identifying the antigens detected in different laboratories.

Novel advances in immunoprecipitation technics ([3, 5, 10]) have recently been applied to mycobacterial analysis ([29, 30]) and to studies of antibodies in leprosy against BCG antigens ([4]). In the present investigations a similar reference system was set up for *M. smegmatis*. The studies were aimed at a definition of antibody activities in leprosy sera against antigens of *M. smegmatis* and the identification of cross reacting antigens in other cultivable mycobacteria. The availability of *M. leprae* bacilli grown *in vivo* in the armadillo also made a comparison with antigens of the causative organism in leprosy possible ([8, 25]). One antigen, here designated no. 21 and also found in other mycobacteria, was shown to carry antigenic determinants which were unique to *M. leprae*.

MATERIALS AND METHODS

Serum samples from patients and controls. Serum samples were obtained from leprosy patients seen at Princess Zenebework Hospital, ALERT. Samples from 91 patients were analyzed in crossed immunoelectrophoresis. Serum samples were also obtained from 25 leprosy patients for studies using a radioimmuno-assay. All patients were classified histopathologically according to the Ridley-Jopling scheme ([19, 20]) with modifications ([21]). Two pools of serum samples from clinically diagnosed lepromatous patients (BL and LL) were prepared. The lepromatous serum pools contained 25 (LS I) and

[1] Received for publication 23 August 1975.

[2] G. Kronvall, M.D., Director, AHRI, P.O. Box 1005, Addis Ababa, Ethiopia. Present Address: Institutionen for Medicinsk Mikrobiologi, Solvegatan 23, S-223 62 Lund, Sweden; G. Bjune, M.D., J. Stanford, Ph.D., S. Menzel, M.D. and D. Samuel, M.D., Armauer Hansen Research Institute, P.O. Box 1005, Addis Ababa, Ethiopia.

43 (LS II) individual sera, respectively. Serum samples obtained from five patients with active pulmonary tuberculosis were included in some experiments. These five patients were newly diagnosed cases verified by specific culture and x-ray. They had been on treatment for not more than two weeks. Control sera included five serum samples from normal, apparently healthy adult Ethiopians, as well as five serum samples from contacts of leprosy patients. A normal human serum pool was used as a control in immunoelectrophoresis. The serum samples included in this pool were taken from 15 apparently healthy Scandinavians working in Ethiopia. All serum samples were stored at −20°C until used.

Mycobacterial antigen preparations. Mycobacterial strains (Table 3) were grown on Sauton's medium solidified with 1.5% agar. After maximal growth had been obtained, the bacteria were harvested and washed three times in phosphate buffered saline (PBS, 0.12 M NaCl, 0.03 M phosphate, pH 7.4) containing 0.02% sodium azide (PBSA) and then suspended in 10 ml of PBSA per gm wet weight. The suspension was ultrasonicated for 30 minutes using a Branson Sonifier, B 12. The supernatant containing soluble antigens was recovered by centrifugation at 17,800 × g (Spinco Model L). This antigen extract was stored at −70°C until used.

M. leprae antigens were prepared from bacilli grown *in vivo*. *M. leprae* infected armadillo tissues were kindly supplied by Dr. G. Walsh.[3] Bacilli were purified from tissues using the method of Mudd ([13]) as described by Stanford ([22]) with modifications. Soluble material was first extracted from the homogenized tissues with saline. The remaining sediment containing bacilli and tissue residues was dehydrated and the bacilli were then extracted with an oil chloroform mixture (Shell Ricella 917, 8 vol. + chloroform, 2 vol.) Combined extracts were centrifuged and the pellet washed with acetone and ether. One milliliter of PBSA was added to every 20 mg of dry weight pellet and the suspension was ultrasonicated and centrifuged as above. Protein determinations on antigen preparations were performed using the modified Folin method ([11]).

Rabbit antisera against mycobacterial antigens. Antisera against antigens of *M. smegmatis* and *M. duvalii* were induced in rabbits by subcutaneous injections every other week of 2 mg of antigen preparations emulsified with Freund's incomplete adjuvant. After three months the rabbits were bled twice a week for another month and suitable antiserum samples were pooled.

Crossed immunoelectrophoresis. The Laurell method of crossed immunoelectrophoresis ([10]) was performed according to the method of Axelsen ([3, 5]).

Gel filtration. Antigen preparations were separated on a Sephadex G-200 column (diameter, 25 mm; length, 950 mm) equilibrated with 0.2 M ammonium bicarbonate buffer, pH 8.0, and eluted at a rate of 13.5 ml per hour. Fractions were read at 280 mμ, then freeze-dried and dissolved in 1/10 volume and analyzed for presence of antigens by the rocket electrophoresis technic ([5, 10]).

Radioimmuno-assay. A modification of the staphylococcal radioimmuno-assay was used to measure antibodies against crude mycobacterial antigens ([7]). Serum samples (0.1 ml, dil. ½) were incubated for 15 minutes with 1 ml of 5% stabilized staphylococci in PBSA. The IgG coated staphylococci were then washed three times and incubated with ^{125}I-labelled, crude antigen from *M. avium* and from *M. duvalii* for five hours in a total volume of 0.3 ml. After three washings, the radioactivity of the pellet was measured in a Packard Gamma Counter. The amount of radioactivity in the pellet represents a measure of the antibody activity in the serum samples tested. Radio-labelling followed the procedure of McConahey and Dixon ([12]).

RESULTS

A reference system was set up for *Mycobacterium smegmatis* using the method of crossed immunoelectrophoresis. The reference system detected optimally more than 50 antigens, 38 of which were numbered as in Figure 1. Serum samples from 91 leprosy patients were analyzed for antibody activities using the intermediate gel technic in the *M. smegmatis* reference system ([3, 4]). The results are shown in Table 1. Patients

[3]Gulf South Research Institute, New Iberia, Louisiana, U.S.A.

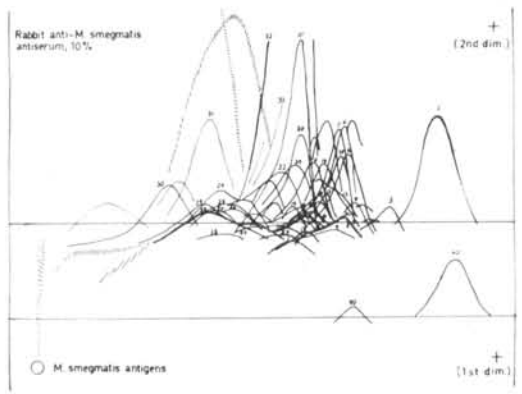

FIG. 1. Reference system for *M. smegmatis* antigens in crossed immunoelectrophoresis. Antigen nos. 40 and 41 were only detected by selected leprosy sera included in the intermediate gel.

were classified according to histopathologic criteria ([19, 20, 21]). The tuberculoid group (TT, TT/BT, BT) all had quite low titers of anti-*M. smegmatis* activity against few antigens and differed markedly from the lepromatous groups (BL, LL) which showed high titers of antibody activities with several antigens being involved. (Table 1). The mean numbers of precipitin lines in the tuberculoid groups were: TT 0.83, TT/BT 0.69, and BT 1.0; whereas in the lepromatous groups the figures were: BL 3.54, and LL 4.30. Only five individual *M. smegmatis* antigens were involved in the few antibody activities found in tuberculoid cases. Among borderline lepromatous (BL) cases more than 9 antigens and in the polar lepromatous (LL) at least 14 antigens were involved. Antigen nos. 40 and 41 (shown in Figure 1) were not correlated to precipitin lines in the reference system. Antibodies against these two antigens were detected in three and twelve lepromatous cases, respectively. One individual precipitin line in a serum sample from a BL patient could not be identified in the reference system.

Antibody activity against antigen no. 1 of the *M. smegmatis* reference system was detected in 50% of tuberculoid cases and in 100% of lepromatous cases (Table 1). Antibody titers against this antigen were semiquantified using a four point scale referring to the degree of dislocation of the precipitin line towards the antigen containing gel. The results are shown in Table 2. There was a marked difference between tuberculoid and lepromatous cases also in respect to levels of antibody activities. In the lepromatous cases 41 of 47 had titers of three or four plus. In contrast, only 2 of 44 tuberculoid cases had such titers.

The overall similiarity of BL and LL serum samples, apparent in Tables 1 and 2, justified the pooling of such sera for use as antibody reagents in comparative studies of mycobacterial antigens. Two such pools (LS I and LS II) were set up in crossed immunoelectrophoresis experiments against antigen preparations from four slow growing mycobacterial species, 14 fast growing mycobacteria, and also against an antigen preparation from *M. leprae* purified from infected armadillos (Table 3). The number of precipitin lines seen between the two lepromatous pools and the antigen preparations varied between 2 and 12 and were, in most cases, on the order of 4 to 5. The number of lines was not related to slow growing or fast growing species and did not correlate with protein concentrations of the antigen

TABLE 1. *Antibodies against* M. smegmatis *antigens in serum samples of leprosy patients.*

Histopathologic classification	No. of Patients	Serum samples with precipitins against *M. smegmatis* antigens	Antibody activity against individual *M. smegmatis* antigens													
			1	3	5	8	13	14	15	17	19	21	28	30	40	41
TT	6	3 (50%)	3	1									1			
TT/BT	16	10 (63%)	8			1				1			1			
BT	22	12 (55%)	11	3						2			6			
BL	24	24 (100%)	24	10	1	2				10	8	4	19		1	5
LL	23	23 (100%)	23	13	1	4	1	1	1	10	6	9	20	1	2	7
Total	91	72 (79%)	69	27	2	7	1	1	1	23	14	13	47	1	3	12

preparations. The overall picture is rather uniform without definite suggestions as to antigenic relationships with any specific mycobacterial strain. The uniform pattern pointed to similarity of the antigens detected in the experiments. Antigenic comparisons were therefore made in crossed immunoelectrophoresis using the so called "tandem technic" ([14]). The nos. 1, 21, and 28 antigens of *M. smegmatis* were first identified by adding increasing amounts of the lepromatous serum pools in the intermediate gel of the *M. smegmatis* reference system. Subsequent studies by the tandem technic showed that these three antigens were present in most antigen preparations tested, including the one from *M. leprae* (Table 3).

TABLE 2. *Antibody titers in leprosy sera against* M. smegmatis *antigen no. 1.*

Anti-1 titers	Histopathologic classification[a]				
	TT	TT/BT	BT	BL	LL
Neg.	3	8	11	0	0
+	1	1	3	1	0
++	1	6	8	2	3
+++	1	1	0	9	9
++++	0	0	0	12	11

[a] Number of patients responding.

The antigen designated no. 1 was studied further in order to determine its apparent molecular weight. *M. duvalii* antigens were separated on a calibrated Sephadex G-200 column and antigens were detected in concentrated eluate fractions using a lepromatous serum pool as the antibody reagent in rocket immunoelectrophoresis ([10]). Antigen no. 1 of *M. duvalii* was detected as a homogenous peak corresponding to a molecular weight of about 290,000. All efforts failed to iodinate purified preparations of this antigen, using both the lactoperoxidase and the chloramine-T method, suggesting a nonprotein nature of the antigen. A nonprotein nature of antigen no. 1 of *M. duvalii* is also supported by pepsin digestion experiments which showed that antigen no. 1 is resistant to the action of the proteolytic enzyme. Antigen no. 1 has been compared by Dr. Navalkar with antigens of his reference system, identifying it as the β-antigen described by him ([16]), and also by

TABLE 3. *Precipitins against mycobacterial antigens in two lepromatous serum pools.*

	Mycobacterial strain[a]	Protein conc. of antigen extract, mg/ml	Number of precipitins with serum pool		Antigens detected[b]		
			I	II	1	21	28
	M. avium, NCTC 8551	1.9	7	8	+	+	+
Slow growing	*M. avium intracell.*, ATCC 25153	1.6	12	8	+	+	+
mycobacteria	*M. kansasii*, J.S. no. 8	2.3	3	2	+		+
	M. gordonae, NCTC 10267	0.5	3	3	+	+	+
	M. smegmatis, NCTC 333	2.2	10	6	+	+	+
	M. phlei, NCTC 8156	2.1	5	4	+		
	M. vaccae, ATCC 15483	1.4	5	4	+	+	+
	M. diernhoferi, ATCC 19340	2.7	6	5	+	+	+
	M. flavescens, NCTC 20271	2.2	2	3	+		+
Fast growing	*M. ranae*, NCTC 2891	2.0	6	5	+	+	+
mycobacteria	*M. chelonei*, NCTC 946	2.3	5	4		+	+
	M. gilvum, NCTC 10742	0.5	4	4	+		+
	M. duvalii, NCTC 358	1.4	4	4	+		+
	M. engbaekii, J.S. no. 831	1.1	3	4	+	+	+
	M. gadium, J.S. no. 920	1.3	2	3	+		
	M. rhodesiae, ATCC 27024	2.5	4	4	+		+
	M. chitae, CNCTC 10485	2.4	2	3	+		+
	M. thermoresistibile, NCTC 10409	0.8	2	3	+	+	+
	M. leprae	4.6	7	7	+	+	+

[a] Strain numbers refer to National Collection of Type Cultures (NCTC), American Type Culture Collection (ATCC), or to a collection held by Dr. John Stanford (J.S.).

[b] Numbers refer to the *M. smegmatis* reference system, Figure 1.

Dr. B. Myrvang who identified it as the same as the d-antigen of *M. duvalii* (14).

The common nature of the mycobacterial antigens involved in antibody responses in leprosy patients was illustrated by radioimmuno-assays using radio-labeled, crude antigen preparations from *M. avium* and *M. duvalii* (Fig. 2). The patients were histopathologically classified as follows: 5 LL, 5 BL, 6 BT, 8 TT/BT, and 1 TT patient. The results are shown in Figure 2. The two lepromatous groups, BL and LL, showed significantly higher uptake of labeled antigen from both mycobacterial species as compared to the tuberculoid groups. Serum samples from tuberculoid patients did not differ significantly from normal human serum samples. Interestingly, five serum samples from tuberculosis patients gave values ranging from high values as seen in the lepromatous groups down to the low values of the tuberculoid groups (Fig. 2). These sera were analyzed in crossed immunoelectrophoresis in direct precipitin tests against *M. leprae* antigens and also by using the intermediate gel technic in an *M. duvalii* reference system detecting 32 antigens. One of the serum samples showed direct precipitation against one *M. leprae* antigen as well as anti-1 activity in intermediate gel analysis. Another serum sample was negative in both tests. The two positive sera showed the highest antibody levels in the radioimmuno-assay.

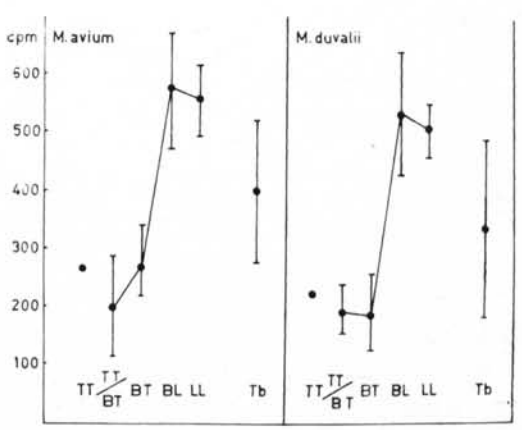

FIG. 2. Antibody activities against *M. avium* and *M. duvalii* antigens as measured by staphylococcal radioimmuno-assay using radiolabeled, crude antigen preparations. Groups TT—LL refer to histopathologic classification of leprosy patients, and group Tb to tuberculosis.

Three of seven antigens of *M. leprae* could be detected in both slow growing and fast growing mycobacterial species (Table 3: nos. 1, 21 and 28). Using individual leprosy sera as reagents, antigen no. 41 could also be detected in three different mycobacteria tested: *M. smegmatis,* a fast grower; *M. avium intracellulare*, a slow grower; and *M. leprae*. The results suggest a common occurrence of these four antigens studied. Further studies of antigen no. 21 indicate the existence of *M. leprae* specific antigenic determinants. When comparisons were made in tandem crossed immunoelectrophoresis using lepromatous serum pools as antibody reagents between antigen no. 21 from *M. leprae*, and the same antigen in *M. avium intracellulare* and *M. smegmatis*, there was always a spurring by the *M. leprae* antigen over the other ones. These results indicate that antigen no. 21 of *M. leprae* carries antigenic determinants which are unique to this species in addition to antigenic structures common to antigen no. 21 in probably all mycobacterial species. The antibody sources capable of detecting these species specific determinants were two different pools of lepromatous sera. These findings therefore also reflect the identity of armadillo-grown *M. leprae* with the immunizing agent in lepromatous leprosy.

DISCUSSION

The relatively uninhibited growth of *Mycobacterium leprae* in lepromatous leprosy patients seems to provide a strong antigenic stimulus for antibody formation (1, 14-17, 28). Reported frequencies for antimycobacterial antibodies in lepromatous patients are regularly high, varying from 74% (14), 75% (28), 89% (1), 95% (16) to 100% (4, 9), depending on the antigens used and on the methods employed. In the present studies all borderline and polar lepromatous cases showed antibody activities against *M. smegmatis* antigens. The frequencies were particularly high for antibodies against antigen no. 1. This antigen seems to be present in all mycobacterial species including *M. leprae*. Even in tuberculoid cases a rather high frequency of antibody activity against antigen no. 1 was detected. Other immunologic methods, such as hemagglutination or radioimmuno-assays, are more sensitive in detecting antibody activity than crossed immu-

noelectrophoresis. It is therefore possible that antibodies against antigen no. 1 or other defined mycobacterial antigens might be detected more often in the polar tuberculoid end of the spectrum as well, using such sensitive technics. Also, tuberculosis cases or patients with other mycobacterial infections might show similar increases in frequencies using methods of increased sensitivity to measure antibodies against these antigens common to all mycobacteria. Only seven precipitin lines were obtained when testing lepromatous serum pools against an *M. leprae* antigen preparation. This is far below the number of lines seen in the *M. smegmatis* or *M. duvalii* reference systems. The average number of antigens detected in other mycobacteria with the same serum pools was not significantly different. The four most prominent antigens of *M. leprae* and, therefore, the most easily studied ones were all detected in slow growing as well as fast growing mycobacteria. Judging from the very high titers obtained against some antigens, notably no. 1, lepromatous sera ought to be equivalent to hyperimmune anti-*M. leprae* sera. The rather few antigens detected in *M. leprae* antigen preparations therefore may not be explained on the basis of a lack of immunization by *M. leprae* bacilli in the patients. Recent studies by Turcotte have shown that *in vivo* grown tubercle bacilli were antigenically deficient as compared to *in vitro* grown bacilli ([27]). Our results may have a similar explanation. The *M. leprae* bacilli used to prepare antigen were grown in armadillos, whereas all other strains used in the present studies were grown on artificial media. Also, *M. leprae* growing in humans, by analogy, would stimulate the production of antibodies against a restricted number of antigens only.

One aim of the present studies was to seek antigens unique to *M. leprae*. Abe has reported the existence of one such antigen ([2, 3]). In our studies, no complete antigen unique to *M. leprae* was found. However, antigen no. 21 seemed to carry some determinants which were detected on the *M. leprae* antigen only. This was shown as a prominent spurring over other no. 21 antigens due to the presence of antibodies in lepromatous sera with specificity towards these species specific antigenic determinants on molecules of antigen no. 21 from *M. leprae*. This finding also indicates that *M. leprae* recovered from armadillos are identical with the main immunizing agent in leprosy. Similar data have also been obtained by Stanford ([24]). Although the low number of antigens detected in antigen preparations of leprosy bacilli seems to exclude conventional immunologic investigations of the taxonomy of *M. leprae*, the detection of antigenic heterogeneity on a submolecular level might offer an approach to such studies using lepromatous serum pools as antibody reagents ([23]).

SUMMARY

A reference system for *M. smegmatis* antigens in crossed immunoelectrophoresis was used to study antibody activities in serum samples of 91 leprosy patients. All polar and borderline lepromatous patients were positive. Mean numbers out of 14 *M. smegmatis* antigens involved were 4.3 and 3.5, respectively. Precipitins against antigen no. 1 were seen in all lepromatous cases. Antibodies against this antigen were detected in 50% of tuberculoid (polar, subpolar and borderline) cases. Antibody activity against *M. avium* and *M. duvalii* antigens was also detected using a staphylococcal radioimmuno-assay. Borderline and polar lepromatous cases showed elevated levels. Antigenic comparisons were made between four slow growing mycobacteria, fourteen fast growing mycobacteria and the leprosy bacillus using lepromatous serum pools as antibody reagents. Four of the antigens detected in *M. leprae* were also found in slow growing as well as fast growing species indicating a common occurrence among mycobacteria. Antigen no. 1 of *M. duvalii*, with an apparent molecular weight of 290,000, showed nonprotein characteristics. Further analysis of antigen no. 21, using lepromatous serum pools as antibody reagents, indicated the existence of at least two groups of antigenic determinants. In addition to determinants shared by all mycobacteria, there were antigenic structures apparently unique to *M. leprae*.

RESUMEN

Se utilizó un sistema de referencia para antígenos de *M. smegmatis* in inmunoelectroforesis cruzada, para estudiar las actividades de anticuerpo en muestras de suero de 91 pacientes

con lepra. Todos los pacientes polares y borderline-lepromatosos fueron positivos. Los números promedio de 14 antígenos de *M. smegmatis* incluídos, fueron de 4.3 y 3.5 respectivamente. Las precipitinas contra el antígeno N° 1 se observaron en todos los casos lepromatosos. Se detectaron anticuerpos contra este antígeno en 50% de los casos tuberculoides (polares, sub-polares y borderline). También se detectaron anticuerpos contra antígenos de *M. avium* y *M. duvalii*, utilizando un radioinmunoensayo estafilocócecico. Los casos borderline y lepromatosos polares mostraron niveles elevados. Se hicieron comparaciones antigénicas entre cuarto micobacterias de crecimiento lento, catorce micobacterias de crecimiento rápido y el bacilo de la lepra, utilizando mezclas de sueros lepromatosos como anticuerpos reactivos. Cuatro de los antígenos observados en el *M. leprae* se encontraron también en una especie de crecimiento lento y en una de crecimiento rápido, indicando que se encuentran generalmente en las micobacterias. El antígeno N° 1 de *M. duvalii*, que aparentement tiene un peso molecular de 290.000, mostró características no-proteícas. Estudios más completos del antígeno N° 21, utilizando mezclas de sueros lepromatosos como anticuerpos reactivos, indicaron la existencia de por lo menos dos grupos de determinantes antigénicos. Además de los determinantes comunes a todas las micobacterias, habían estructuras antigénicas aparentemente exclusivas del *M. leprae*.

RÉSUMÉ

Un système de références pour les antigènes de *M. smegmatis* dans des réactions croisées d'immunoélectrophorèse a été utilisé pour étudier l'activité en anticorps du sérum de 91 malades de la lèpre. Tous les malades présentant une forme lépromateuse polaire, ou de type borderline, étaient positifs. Parmi 14 antigènes à *M. smegmatis*, le nombre moyen d'antigènes impliqué dans ces réactions était respectivement de 4.3 et de 3.5. Des précipitines contre l'antigène n° 1 ont e'té observées chez tous les malades lépromateux. Des anticorps contre cet antigène ont été détectés chez 50 pour cent des malades tuberculoïdes (polaires, sub-polaire et borderline). Des anticorps actifs contre les antigènes de *M. avium* et *M. duvalii* ont été également détectés, en ayant recours à une méthode d'évaluation radio-immunologique des staphylocoques. Les cas lépromateux polaires et borderline ont montré des taux élévé. Des comparaisons antigéniques ont été menées entre 4 souches de mycobactéries à pousses lentes, 14 mycobactéries à croissance rapide, et le bacille de la lèpre, en utilisant des pools de sérum lépromateux comme anticorps de réaction. Quatre des antigènes détectés chez *M. leprae* ont également été relevés dans les souches à pousses lentes, de même que chez les espèces à croissance rapide, ce qui indique une coexistence de ces antigènes dans plusieurs espèces de mycobactéries. L'antigène n° 1 de *M. duvalii*, avec un poids moléculaire apparent de 290.000, a montré des caractéristiques qui ne sont pas celles des protéines. Une analyse plus poussée de l'antigène n° 21, avec des pools de sérum lépromateux comme anticorps de réactions, a révélé l'existence d'au moins deux groupes de déterminants antigéniques. En plus de ces déterminants qui sont partagés par toutes les espèces de mycobactéries, on a mis en évidence des structures antigéniques qui semblent spécifiques de *M. leprae*.

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