Mycobacterial Antigens in Antibody Responses of Leprosy Patients

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

The regular occurrence of antimycobacterial antibodies in low-resistance forms of leprosy is well documented (1, 2, 4, 6, 8, 16, 18, 26). 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, 26). 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 techniques (3, 5, 10) have recently been applied to mycobacterial analysis (7, 8) 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 (5, 7). 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 radioimmunoassay. All patients were classified histopathologically according to the Ridley-Jopling scheme (10, 16) with modifications (12). Two pools of serum samples from clinically diagnosed lepromatous patients (BL and LL) were prepared. The lepromatous serum pools contained 25 (LS I) and
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 gram 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. Bacilli were purified from tissues using the method of Mudd (13) as described by Stanford (12) 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 (14).

Rabbit antisera against mycobacterial antigens. Antisera against antigens of *M. smegmatis* and *M. davanii* 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.

Coomassie blue stained gels were visualized for clear bands that were considered antigenic. Antigenic bands were cut out and eluted in a total volume of 0.3 ml. After three washings, the radioactivity of the pellet was measured in a Packard Gamma Counter. The radioactivity in the pellet represents a measure of the antibody activity in the serum samples tested.

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 (14). The results are shown in Table 1. Patients...
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 I) 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 similarity 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 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.

![Figure 1. Reference system for \( M.\) smegmatis antigens in crossed immunoelectrophoresis.](image)

**Table 1. Antibodies against \( M.\) smegmatis antigens in serum samples of leprosy patients.**

<table>
<thead>
<tr>
<th>Histopathologic classification</th>
<th>Serum samples with precipitins against ( M.) smegmatis antigens</th>
<th>Antibody activity against individual ( M.) smegmatis antigens</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT</td>
<td>6 (35%)</td>
<td>3 1 1 11 10 5 3 1 1</td>
</tr>
<tr>
<td>TT/ BT</td>
<td>16 (63%)</td>
<td>8 1 1 1 1 1 1</td>
</tr>
<tr>
<td>BT</td>
<td>12 (55%)</td>
<td>14 3 1 1 1 1</td>
</tr>
<tr>
<td>BL</td>
<td>24 (100%)</td>
<td>24 10 1 2 10 8 4 19 1 5</td>
</tr>
<tr>
<td>LL</td>
<td>23 (100%)</td>
<td>23 13 1 1 1 1 10 6 9 20 1 7</td>
</tr>
<tr>
<td>Total</td>
<td>91 (79%)</td>
<td>69 27 2 1 1 1 23 14 13 47 1 3 12</td>
</tr>
</tbody>
</table>
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).

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 α-antigen described by him (16), and also by

**Table 2. Antibody titers in leprosy sera against _M. smegmatis_ antigen no. 1.**

<table>
<thead>
<tr>
<th>Antibody titer</th>
<th>Histopathologic classification</th>
<th>Number of patients responding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TT/TT/TT/BL/LL</td>
<td></td>
</tr>
<tr>
<td>Neg.</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>++</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>+++</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Mycobacterial strain*</th>
<th>Protein conc. of antigen extract, mg ml</th>
<th>Number of precipitins with serum pool detected</th>
<th>Antigens detected†</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. avium</em>, NCTC 8551</td>
<td>1.9</td>
<td></td>
<td>1-21-28</td>
</tr>
<tr>
<td>Slow growing mycobacteria</td>
<td>1.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>_M. avium intracell., ATCC 25153</td>
<td>2.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. kansasi</em>, N.C. no. 81</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. gordonae</em>, NCTC 80267</td>
<td>2.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. smegmatis</em>, NCTC 333</td>
<td>2.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. phlei</em>, NCTC 8156</td>
<td>1.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. vaccae</em>, ATCC 15483</td>
<td>2.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. dierkhoferi</em>, ATCC 19340</td>
<td>2.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. flavescens</em>, NCTC 20271</td>
<td>2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fast growing mycobacteria</td>
<td>2.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. chelonae</em>, NCTC 946</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. gilvum</em>, NCTC 1042</td>
<td>1.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. chelonae</em>, ATCC 358</td>
<td>1.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. enshelkia</em>, J.S. no. 831</td>
<td>1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. gilvum</em>, ATCC 27024</td>
<td>2.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. chelonae</em>, NCTC 10445</td>
<td>2.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. thermoresistible</em>, NCTC 10409</td>
<td>0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. leprae</em></td>
<td>4.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

†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* (15).

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 BT, 6 BL, 8 TT, BT, and 2 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 tuberculoid 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 technique 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-I 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.

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 arnoldi-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 (14-16). Reported frequencies for antimycobacterial antibodies in lepromatous patients are regularly high, varying from 74% (14), 75% (16), 89% (15), 95% (14), to 100% (16), 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 immunoassays.
nuclelectrophoresis. 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 techniques. Also, tuberculoid 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 Turcot 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 (23). 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 (39). 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 280,000, 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 inmunoelectroforesis cruzada, para estudiar las actividades de anticuerpo en muestras de suero de 91 pacientes
con lepra. Todos los pacientes polares y border-
line- lepromatosos fueron positivos. Los números
propios de 14 antígenos de M. smegmatis in-
cluidos, fueron de 4.3 y 3.5 respectivamente. Las
precipitinas contra el antígeno N° 1 se observa-
ron en todos los casos lepromatosos. Se detec-
taron anticuerpos contra este antígeno en 50%
 de los casos tuberculoides (polares, sub-polares y
borderline). El antígeno N° 21 no se detectaron
anticuerpos contra antígenos de M. avium y M. davauli, utili-
izando un radioimmunoensayo establecido.
Los casos borderline y lepromatosos polares mos-
traron niveles elevados. Se hicieron comparas-
iones antigenicas entre cuatro microbacterias de
crecimiento lento, catorce microbacterias de cre-
cimiento rápido y el bacilo de la lepra, utilizando
mezclas de sueros lepromatosos como antígenos
reactivos. Cuatro de los antígenos observados en
el M. leprae se encontraron también en una espe-
cie de crecimiento lento y en una de crecimiento
rápido, indicando que se encuentran general-
mente en los microbacterias. El antígeno N° 1 de
M. davauli, que aparentemente tiene un peso
molecular de 290,000, mostró características no-
protéticas. Estudios más completos del antígeno
N° 21, utilizando mezclas de sueros lepromatosos
como anticuerpos reactivos, indicaron la exis-
tencia de por lo menos dos grupos de determinantes
antigénicos. Además de los determinantes com-
tenidos en las microbacterias, hallan estruc-
turas antigenicas aparentemente exclusivas del
M. leprae.

RÉSUMÉ

Un système de réferences pour les antigènes de
M. smegmatis dans des réactions croisées
immunoélectrophorétiques a été utilisé pour étudier
l'activité en anticorps du sérum de 91
malades de la lépre. Tous les malades présen-
tant une forme lépromateuse polaire, ou de type bor-
derlineer, étaient positifs. Parmi 14 antigènes à M.
smegmatis, le nombre moyen d'antigènes im-
pliqués dans ces réactions était respectivement
de 4.3 et de 3.5. Des précipitines contre l'antigène n°
1 ont été observées chez tous les malades lépro-
maux, y compris des patients borderline. Des anti-
corps actifs contre les antigènes de M. avium et
M. davauli ont été également détectés, en ayant
recours à une méthode d'évaluation radio-
immunoélectrophorétique des staphylocoques. Les cas
lépromateux polaires et borderline ont montré
moindres titres. Des comparaisons antigeniques
ont été menées entre 4 souches de mycobactéries
à pousses lentes, 14 mycobactéries à croissance
rapide, et le bacille de la lépre, 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

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