Enzyme-Linked Immunosorbent Assay for Distinguishing Serological Responses of Lepromatous and Tuberculoid Leprosies to the 29/33-Kilodalton Doublet and 64-Kilodalton Antigens of Mycobacterium tuberculosis

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Immunoblot assays for the antibodies to Mycobacterium tuberculosis sonic extracts showed that all serum specimens of 40 lepromatous and of 28 tuberculoid leprosy patients reacted in a significant manner to 29/33-kilodalton (kDa) doublet and 64-kDa antigens, respectively. By using an enzyme-linked immunosorbent assay, we observed a significantly high immunoglobulin G antibody titer to the purified M. tuberculosis 29/33-kDa doublet and 64-kDa antigens in lepromatous and tuberculoid leprosy patients, respectively, as compared with normal subjects and tuberculosis patients. This enzyme-linked immunosorbent assay serology may be useful for distinguishing two polar types of leprosy and for diagnosing leprosy in general.

In mycobacterial infections such as leprosy and tuberculosis, antibody response plays a minor role in the overall effector mechanism (3, 15) but is considered to be important in progressive pathology (13). Despite the effort over the past few years to develop methods for measuring serum antibodies to species-specific mycobacterial antigens (16, 22), it is still difficult to define a mycobacterial protein antigen for determination of a disease-specific antibody response (4).

The major proteins of the mycobacterial protein and glycoprotein antigens are present in Mycobacterium bovis BCG, Mycobacterium tuberculosis, and M. leprae (10, 23). The cause of variation in the immune response of individual hosts to M. leprae infection is not yet clarified. However, the importance of the responses of hosts to immunologically cross-reactive antigenic components of mycobacteria (mycobacterial ImCRAC) has been emphasized for the spectral pathogenesis of leprosy (24).

In this regard, we proposed that the spectral pathology of leprosy may be due to the varied abilities of the hosts to discriminate among different mycobacterial antigens (6). We showed that sera from lepromatous leprosy (L-lep) and tuberculoid leprosy (T-lep) patients reacted to different antigens of M. bovis BCG subcellular extracts.

Recently, several investigators demonstrated that sera from the L-lep polar form of the disease reacted preferentially with certain protein antigens of M. tuberculosis, M. leprae, and M. bovis BCG (1, 2, 11, 17, 20). Such results were not surprising, because mycobacteria share many antigens (12, 14). We also reported similar findings and stated that the sera of the population at large contain antibody reactivities to different mycobacterial ImCRAC and that such antibody specificities appeared to be disease related, particularly for leprosy (P. K. Das, M. Halperin, J. G. Baas, and J. Thole, J. Invest. Dermatol. 87:134, 1986; P. K. Das, J. Invest. Dermatol., abstr. no. 163, 91:404, 1988).

However, we have been cautious in publishing our results in detail and are still busy studying larger numbers of samples from patients with a wider range of diseases, since mycobacterial etiology had often been implicated in diseases other than mycobacterioses (21).

Our findings and those of previous authors (1, 2, 20) are interesting, because in endemic areas, both leprosy and tuberculosis coexist with sufficient frequency (11). Therefore, separation of mycobacterial ImCRAC from M. tuberculosis in distinguishing the serum responses of these two diseases will be useful for developing a differential but objective immunodiagnostic method.

(The preliminary results of this research were presented at the 4th International Colloquium on Mycobacteria [7].)

The purpose of this report is to confirm the pilot results on the identification of mycobacterial ImCRAC for an enzyme-linked immunosorbent assay (ELISA) which, on the one hand, discriminates between L-lep and T-lep patients and, on the other, diagnoses the leprosy patients specifically from the population at large which has been exposed to mycobacteria.

We have investigated serum specimens from 40 L-lep, 28 T-lep, and 20 tuberculosis (TB) patients, 15 healthy leprosy contacts, and 12 normal Spanish and 15 normal Dutch individuals. The sera of leprosy patients and those with close contact, together with some normal subjects, were collected in the Fontilles Leprosarium, Alicante, Spain, as well as in our Lepra clinic, whereas sera of TB patients and normal Dutch donors were supplied by various hospitals in The Netherlands. None of the leprosy patients had concurrent TB at the time of sample collection. However, at least 70% of the patients and the healthy individuals were tuberculin skin test positive.

Sonicated mycobacterial extracts. Mycobacterial sonic extracts were prepared by breaking the bacteria in a French press and sonicating them at 4°C. M. tuberculosis BCG and Mycobacterium avium were RIVM-strain 7114, RIVM-P3, and RIVM-Myc 9155, Bilthoven, whereas M. leprosy was...
purified from armadillo liver (8), and sonic extracts were similarly prepared.

ImBA. Twenty-microgram samples of different mycobacterial sonic extracts were electrophoretically separated in separate lanes of sodium dodecyl sulfate–12% polyacrylamide gels (19). Subsequent immunoblot assays (ImBA) for serum antibodies to sodium dodecyl sulfate-polyacrylamide gel electrophoresis-separated mycobacterial antigens were performed as previously described (9) by using diluted sera (1:100) from various individuals. The immunoreactions of L-lep and T-lep pool sera with M. bovis BCG, M. tuberculosis, M. lepra, and M. avium sonic extracts are shown in Fig. 1A and B as representative samples. When the ImBA reactivities of all the sera to M. tuberculosis sonic extracts were evaluated, we observed that 100% of the 40 L-lep and 28 T-lep patients reacted with significant intensities to 29/33-kDa doublet and 64-kilodalton (kDa) antigens, respectively, as compared with that of other groups (Table 1). In this respect, it should be noted that many of the L-lep sera reacted to 35-, 33-, 31-, and 29-kDa antigens of M. avium sonic extract. However, cross-reactivity of the sera with specific mycobacterial sonic extracts and then by ImBA on other mycobacterial preparations indicated that the 29/33-kDa doublet and 64-kDa antigens of M. tuberculosis were similar to those present in the other three mycobacteria. Therefore, these antigens are mycobacterial ImCRAC. In many cases, including healthy individuals and TB patients, seroreactivities were also detected against many mycobacterial antigens. Nevertheless, sera from L-lep and T-lep patients (irrespective of their country of origin) typically reacted with strong (+ + + + + ) intensity to 29/33-kDa doublet and 64-kDa antigens of all four mycobacteria, respectively. Although most sera reacted to 64- or 65-kDa mycobacterial antigens, T-lep sera were strongly reactive to this antigen. On the other hand, some sera from individuals other than L-lep also reacted to antigens in the regions of 31 to 33, but none reacted to the distinct 29/33-kDa doublet, except one of the leprosy contacts. It should be emphasized that the reactivity patterns were disease specific and were not correlated with the status of the skin reactivities of the individuals to tuberculin.

ELISA serology with 29/33-kDa doublet and 64-kDa antigens of M. tuberculosis. A M. tuberculosis sonicated preparation was applied to a preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel electrophoresis slab gel (5 mg of electrophoresed material per 810 mm2 per run). Slices, 2 mm, were cut from the gel in the area of 29/33 kDa and 64 kDa. Bound proteins were eluted by diffusion in distilled water and then grinding the gel into dispersion. The gels were removed by centrifugation (12,000 × g for 15 min at 8°C). The resulting pellet was reextracted with distilled water, and the combined eluates were extensively dialyzed against phosphate-buffered saline (PBS), pH 7.4, and concentrated by vacuum dialysis to 800 μg of protein per ml. The homogeneity of the antigen preparations was reinvestigated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and ImBA by using L-lep pool sera for the 29/33-kDa doublet and using monoclonal antibody F-47-10-1 (18) for the 64-kDa antigen. The purified M. tuberculosis 29/33-kDa doublet and 64-kDa antigens were used in a direct ELISA to measure immunoglobulin G (IgG) and IgM antibodies in the sera of L-lep, T-lep, and TB patients and 45 healthy individuals, including leprosy contacts.

ELISA serology was performed by coating the microdilution plates (Greiner, Denmark) with a preevaluated optimal amount of 0.5 μg of protein of either 29/33-kDa doublet or 64-kDa M. tuberculosis antigen per well in 0.067 M PBS, pH 7.4, by incubating for 2 h at 37°C. The plates were washed thoroughly with PBS containing 0.05% Tween 20; this wash was followed by further incubation with 2% bovine serum albumin in PBS-Tween 20 for 2 h and a subsequent washing step.

Serial dilutions of human sera up to 1:10,000 in 10% goat serum and 0.1% Tween 20 were added to achieve antigen-antibody binding by incubating for 1 h at 37°C; this procedure was followed by thorough washing. The plates were further incubated for 1 h at 37°C with preevaluated dilutions of peroxidase-labeled affinity-purified secondary goat anti-human IgG or IgM (Cappel Laboratories, West Chester, Pa.) in PBS containing 10% goat sera and 0.1% Tween 20. After washing, the enzyme activities were revealed with the substrate 3′,5′-tetramethylbenzidine plus dimethyl sulfoxide and H2O2 by continuing the reaction for 10 to 15 min at 25°C. The color development was stopped with 50 μl of 2 N H2SO4, and optical density at 450 nm was measured in a Bio-Rad ELISA-reader (Bio-Rad Laboratories, Richmond, Calif.) coupled to a Macintosh computer. The endpoint titers of IgG antibody in the sera of 35 L-lep and 18 T-lep patients,

TABLE 1. Percentage of sera from patients with various diseases and normal subjects reacting to specified antigens of M. tuberculosis sonic extracts in an immunoblotting assay

<table>
<thead>
<tr>
<th>Disease (no. of patients)</th>
<th>Reaction (% of samples for antigens of)</th>
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<tbody>
<tr>
<td></td>
<td>64 kDa</td>
</tr>
<tr>
<td>L-lep (40)</td>
<td>+ (30)</td>
</tr>
<tr>
<td></td>
<td>± (10)</td>
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<tr>
<td>T-lep (28)</td>
<td>+ + + (85)</td>
</tr>
<tr>
<td></td>
<td>+ + (15)</td>
</tr>
<tr>
<td>Leprosy contacts and normal controls (42a)</td>
<td>± (60)</td>
</tr>
<tr>
<td>TB (20)</td>
<td>± (70)</td>
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<tr>
<td></td>
<td>± (30)</td>
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a + + + to ± indicates the degree of reactivities with a 1:100 serum dilution as judged visually; e.g., + + + and ± are very intense and very weak bands, respectively.

b Reacted to the 29- or 33-kDa antigen singly but never to the doublet.

c Only 1 of 15 leprosy contacts reacted to the 29/33-kDa doublet.
15 leprosy contacts, 30 normal Dutch controls, and 20 TB patients were determined by extrapolating the titration curve to an optical density value equal to or greater than two times that for the reagent blanks. A significantly high mean IgG antibody titer of 5,500 \( \pm \) to 29/33-kDa and 64-kDa \( M. \) \textit{tuberculosis} antigens was found among L-lep and T-lep patients, respectively, whereas a mean titer of about only 800 \( \pm \) to 1,200 \( \pm \) was observed in other groups. In certain L-lep patients, a raised IgM antibody titer to either or both of the 29/33-kDa and 64-kDa antigens was also encountered.

On the basis of such observations, we conducted another set of serology ELISAs by using 29/33-kDa doublet and 64-kDa \( M. \) \textit{tuberculosis} antigens with one 1:400 dilution of sera at random. The results are shown in Fig. 2A and B. We observed the mean optical density values at 450 nm for IgG 29/33-kDa doublet and 64-kDa antigens were 0.92 \( \pm \) 0.19 and 0.38 \( \pm \) 0.11 and 0.25 \( \pm \) 0.18 and 1.20 \( \pm \) 0.41 in the sera of L-lep and T-lep patients, respectively. These values among nonleprosy patients and controls appeared to be within the range of 0.16 to 0.28 and 0.23 to 0.28 for 29/33-kDa doublet and 64-kDa antigens, respectively.

From the results given above (both ImBA and ELISA), it is quite evident that L-lep and T-lep patients can be distinguished from each other and possibly from patients with other mycobacterial diseases.

Since these \( M. \) \textit{tuberculosis} antigens are present in \( M. \) \textit{leprae}, \( M. \) \textit{avium}, and \( M. \) \textit{bovis} BCG (Fig. 1A and B), they are considered immunologically cross-reactive antigenic components of mycobacteria (mycobacterial ImCRAC). The distinct nature of antibody activities of L-lep and T-lep sera to 29/33-kDa doublet and 64-kDa mycobacterial ImCRAC prompted us to name these antigens L-lep- and T-lep-specific mycobacterial ImCRAC, respectively.

The present report clearly shows that the antibody responses to 29/33-kDa and 64-kDa mycobacterial ImCRAC are specific for leprosy. However, one close leprosy contact showed significantly high seroreactivities to 29/33-kDa antigens. Therefore, we are presently engaged in a serological survey in which both ELISA and ImBA are being used for the diagnosis of different stages of leprosy, particularly those of indeterminate and nonpolar groups, as well as individuals at greater risk for developing the disease.

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LITERATURE CITED


