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Major Proteins of Mycobacterial Strain ICRC and Mycobacterium leprae, Identified by Antibodies in Sera from Leprosy Patients and Their Contacts

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Sera from leprosy patients across the clinical spectrum, healthy contacts, tuberculosis patients, and healthy donors were tested for their reactivity with antigens of mycobacterial strain ICRC (a cultivable mycobacterium) and Mycobacterium leprae by immunoprecipitation technique. Using M. leprae antigens, it was not possible to distinguish between reactivities of sera from lepromatous, borderline lepromatous, borderline tuberculoid, and tuberculoid leprosy patients. All these sera identified M. leprae antigens with molecular masses of 47, 36, 21, and 14 kDa. When the same sera were tested for their reactivities with antigens of mycobacterial strain ICRC, several differences were observed. The 21-kDa antigen of mycobacterial strain ICRC was exclusively precipitated by sera from all lepromatous leprosy patients and from those undergoing erythema nodosum leprosum reaction. Sera from all the other donors tested failed to identify the 21-kDa antigen of mycobacterial strain ICRC. The 14-kDa protein of mycobacterial strain ICRC was identified by sera from a few lepromatous leprosy patients (5 of 26) and all their contacts. Our studies indicate that antigens present on cultivable mycobacteria rather than species-specific antigens may prove to be useful in the serodiagnosis of leprosy.

Cell-mediated immune responses are known to play an important role in conferring protective immunity against mycobacterial infections (12, 17). The role played by the humoral arm of the immune system in combating mycobacterial infections remains undefined. The uncontrolled multiplication of Mycobacterium leprae in multibacillary leprosy patients elicits strong antibody production, while in paucibacillary patients, the antibody response seems to be smaller (1, 4). Although in this disease the antibodies may play a minor role in the overall effector mechanism, they are associated with progressive pathology. A knowledge of the antigenic components of M. leprae capable of eliciting humoral immune responses is a prerequisite for understanding the pathogenesis of leprosy. Several reports on the presence of common mycobacterial antigens and species-specific antigenic determinants associated with M. leprae have appeared (3, 4, 15, 16, 18, 22, 23). M. leprae-specific phenolic glycolipid I (6) and lipoarabinomannan B (14) have been identified as major target antigens for humoral responses in multibacillary leprosy patients. Despite several efforts, it is still difficult to define M. leprae protein antigens which may have serodiagnostic potential.

ICRC, a cultivable mycobacterium isolated from human lepromata, is undergoing clinical trials as an antileprosy vaccine in areas in India in which leprosy is endemic (8). The vaccine brings about persistent lepromin conversion in 55% of lepromatous leprosy (LL) patients and in 95% of their healthy household contacts (8). Crossed-immunoelectrophoresis experiments using polyclonal anti-ICRC serum have indicated antigenic relatedness between ICRC and M. leprae antigens (5). Comparable T-cell responses to antigens of ICRC and M. leprae have also been reported (10).

Emmrich and Kaufmann (9) have demonstrated that M. leprae-specific T-cell clones established from tuberculoid leprosy (TT) patients showed significant cross-reactivity with antigens of ICRC.

The present investigations were aimed at studying the reactivity of sera from leprosy patients across the clinical spectrum to antigens of ICRC and M. leprae by using a highly sensitive immunoprecipitation technique. Our studies demonstrate that the 21-kDa protein of ICRC shows exclusive reactivity with sera from LL patients. The identification and characterization of such immunodominant antigens may provide an important tool for serodiagnosis of leprosy.

MATERIALS AND METHODS

Antigens. ICRC bacilli, isolate C-44, were maintained in Dubos' modified medium as described by Chirmule et al. (5). The cultures at passages 50 to 65 were harvested and washed five times with Tris-acetate buffer (20 mM, pH 7.2). Bacilli (12 × 1010) were suspended in 10 ml of the buffer and sonicated at 80 W on ice for 60 min at 50% duty cycle in a Branson sonifier (Branson Ultrasonics Corp., Danbury, Conn.). The bacterial extract was then centrifuged at 218,200 × g for 1 h at 4°C (5). The supernatant, which contained soluble proteins and was referred to as the sonicate of ICRC, was collected. After determination of the protein concentration (20), aliquots of the sonicate were lyophilized and stored at −20°C.

Since ICRC belongs to the Mycobacterium avium complex (8), soluble antigens of M. avium serovar 3, kindly provided by J. Ivanji (M.R.C., London, England), were also used in these studies. Sonicate of M. leprae (CD 122) was a gift from R. J. W. Rees through the World Health Organization-Immunology of Leprosy Programme.

Patients and controls. Blood samples were obtained from

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leprosy patients across the clinical spectrum who were attending the outpatient clinic at Richardson Leprosy Hospital, Miraj, India. The patients were grouped as newly diagnosed untreated LL patients \( (n = 6) \), LL patients on multiple drug therapy (MDT) showing good response (MDT-R, \( n = 11 \)), LL patients clinically classified as nonresponders to MDT (MDT-NR, \( n = 9 \)), LL patients in erythema nodosum leprosum (ENL) reaction \( (n = 7) \), borderline tuberculoid patients \( (BT, n = 7) \), borderline lepromatous patients \( (BL, n = 8) \) and TT patients \( (n = 7) \). Blood samples were also collected from healthy household contacts of leprosy patients \( (n = 12) \), tuberculosis \( (TB) \) patients \( (n = 6) \), and laboratory personnel who served as healthy controls \( (n = 6) \). The diagnoses of the patients were established by using clinical and histopathological criteria based on the Ridley-Jopling classification (21).

Blood samples were allowed to clot at 4°C overnight. Serum was collected by centrifugation at 400 \( \times g \) for 10 min at 4°C. Aliquots of serum samples were stored at \(-20°C\). Sera were tested both as individual and pooled samples.

**Radiolabeling of soluble antigens.** The sonicates of ICRC, *M. leprae* and *M. avium* were labeled with Na\(^{125}\)I (Amersham Int., Amersham, Bucks, England) by using the chloramine T method as described by Greenwood et al. (11). The radiolabeled protein fraction was separated from free iodine by using Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) gel chromatography.

**Immunoprecipitation.** Radiolabeled mycobacterial antigens \( (4 \times 10^9 \text{ cpn}) \) in Tris HCl buffer \( (50 \text{ mM}, \text{pH} 8.3) \) containing 0.5% Triton X-100 were incubated with 15 \( \mu \text{L} \) of test serum for 2 h on ice (3). The immune complexes were precipitated by incubation for 30 min on ice with 25 \( \mu \text{L} \) of a 10% \((\text{wt/vol})\) suspension of *Staphylococcus aureus* Cowan (Sigma Chemical Co., St. Louis, Mo.). The precipitates were washed three times with Tris HCl buffer \( (50 \text{ mM}, \text{pH} 8.3) \), with a change of microtube for the last wash. Bound antigen was eluted by boiling the pellet in 30 \( \mu \text{L} \) of electrophoresis sample buffer consisting of 2% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol, 10% glycerol, and 0.0001% bromophenol blue in 62.5 mM Tris HCl, pH 6.8.

**Electrophoresis and autoradiography.** The immunoprecipitated antigens were separated by using 5 to 20% gradient SDS-polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (19). For visualization of protein bands, dried gels were exposed to Fuji X-ray films (Fuji Photo Film Co., Ltd., Tokyo, Japan) for 24 to 72 h at \(-70°C\).

The protein patterns of the sonicates were analyzed by separating unlabeled sonicates on the gradient SDS-PAGE and staining the gels by silver stain as described by Blum et al. (2).

**Molecular mass estimation.** The molecular masses of proteins were determined as described by Hames (13). For all the proteins, the relative mobility \( (R_f) \) was measured with reference to a tracking dye. Using linear regression analysis, a standard curve of the molecular mass versus the \( R_f \) values of the standard marker proteins was plotted. Molecular masses of the unknown proteins were calculated from the standard curve.

**RESULTS**

**Characterization of ICRC and *M. leprae* antigens.** Silver staining of the ICRC sonicate separated by 5 to 20% gradient SDS-PAGE revealed several protein bands in the molecular mass range of 153 to 14 kDa (Fig. 1, lane 1). Radioiodination of the ICRC sonicate exhibited protein bands of 81, 60, 55, 29, 25, 21, and 14 kDa (Fig. 1, lane 2). The 48- and 16-kDa proteins appeared as very faint bands on the autoradiogram. *M. leprae* sonicate, after fractionation on the gradient SDS-PAGE, exhibited protein bands in the range of 174 to 14 kDa (Fig. 1, lane 3). On iodination, the proteins with molecular masses of 63, 57, 56, 23, 21, and 14 kDa were visible. The 14-kDa protein of *M. leprae* was prominently seen in both the silver-stained gel and the autoradiogram of the labeled *M. leprae* sonicate (Fig. 1, lane 4).

**Immunoprecipitation of ICRC antigens with patients' sera.** Sera obtained from LL patients (untreated, MDT responders, and MDT nonresponders) immunoprecipitated ICRC antigens with molecular masses of 94, 81, 58, 48, 32, 25, 23, 21 (21 to 22), and 14 kDa (Fig. 2A). The 58- and 14-kDa proteins of ICRC were immunoprecipitated by respectively 3 of 26 and 5 of 26 serum samples from LL patients. The 21-kDa protein of ICRC was consistently precipitated by all the LL sera tested. Sera from LL patients in ENL showed an intense reactivity with the 21-kDa protein of ICRC, but none of them reacted with the 58- and 14-kDa antigens of ICRC (Fig. 3C).

**Immunoprecipitation of ICRC antigens with sera from TT patients** also revealed several bands of proteins with molecular masses of 94, 81, 48, 32, and 25 kDa (Fig. 2B). It was observed that none of the sera from TT patients reacted with the 21-kDa protein of ICRC.

Sera from BL patients immunoprecipitated almost all the major antigens of ICRC precipitated by sera of LL patients but with varying intensities (Fig. 3A). At the time of testing, the diagnosis of BL received by us had been made on the
basis of the clinical picture. Three of these BL sera exhibited reactivity with the 21-kDa protein, whereas sera from other BL patients failed to precipitate the 21-kDa antigen of ICRC (Fig. 3A).

Although sera from BT (Fig. 3B) and TB (Table 1) patients and healthy donors (Fig. 3D) immunoprecipitated several antigens of ICRC, none of them precipitated the 21-kDa protein.

**Immunoprecipitation of M. leprae antigens with patients' sera.** Sera from untreated, MDT-responsive, and MDT-nonresponsive LL patients identified *M. leprae* antigens with molecular masses of 47, 36, 21, and 14 kDa (Fig. 4A).

The 21- and 14-kDa proteins of *M. leprae* were strongly precipitated by all the LL sera. Immunoprecipitation profiles of *M. leprae* antigens obtained by using sera of TT patients were comparable to those observed with LL sera. The 21- and 14-kDa proteins of *M. leprae* were strongly precipitated by sera of TT patients (Fig. 4B). Sera obtained from BT, BL, LL in ENL, and TB patients and healthy donors showed a similar pattern of reactivity (Table 1).

**Immunoprecipitation of ICRC and M. leprae antigens with sera from contacts of leprosy patients.** Reactivity of sera obtained from healthy household contacts of LL, BT, and TT patients with ICRC antigens was examined. It was interesting to note that all the sera showed strong reactivity with the 14-kDa protein of ICRC (Fig. 5A). We have earlier observed that the 14-kDa protein of ICRC was also identified by sera from LL patients (5 of 26; Fig. 2A). The 21-kDa protein of ICRC was faintly precipitated by sera from the contacts tested (Fig. 5A). Other ICRC antigens recognized had molecular masses of 94, 81, 48, 32, 29, 25, and 18 kDa.

The same serum samples were tested for reactivity with 125I-labeled *M. leprae* antigens. The sera precipitated proteins of 47, 36, 29, 21, and 14 kDa (Fig. 5B). The 47- and 36-kDa proteins of *M. leprae* were weakly precipitated by sera from contacts of leprosy patients. No significant differences were observed in the reactivity patterns of sera obtained from the contacts of LL, BT, or TT patients.

**Immunoprecipitation of M. avium antigens.** 125I-labeled *M. avium* sonicate exhibited several prominent bands on the gradient SDS-PAGE in the range of 94 to 14 kDa (Fig. 6). Pooled sera from leprosy patients across the clinical spectrum, i.e., LL, BL, BT, TT, and TB patients and healthy donors were tested for their reactivity with 125I-labeled *M. avium* antigens. It was evident that LL sera immunoprecipitated *M. avium* antigens of 94, 84, 51, 46, 43, 36, 30, 27, 26, 24, 21 (a doublet), and 14 kDa. *M. avium* antigens of 84, 26, 21, and 14 kDa were exclusively precipitated by LL sera (Fig. 6).
TABLE 1. Predominant antigens of ICRC and M. leprae identified by sera from different donors

<table>
<thead>
<tr>
<th>Source of serum</th>
<th>ICRC antigen of:</th>
<th>M. leprae antigen of:</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>58 kDa</td>
<td>48 kDa</td>
</tr>
<tr>
<td>LL</td>
<td>++b</td>
<td>+++</td>
</tr>
<tr>
<td>BL</td>
<td>+++</td>
<td>±</td>
</tr>
<tr>
<td>TT</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Contacts</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>TB</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Healthy people</td>
<td>+++</td>
<td>±</td>
</tr>
</tbody>
</table>

* Intensity of bands is indicated from greatest (+++) to lowest (±).
* Precipitated by some sera only.

DISCUSSION

In the present investigations, we have assessed the antibody responses of leprosy patients across the clinical spectrum to antigens of ICRC and M. leprae. Sera from LL patients immunoprecipitated ICRC antigens with molecular masses of 94, 81, 58, 48, 32, 25, 23, 21, and 14 kDa. The same sera immunoprecipitated M. leprae antigens of 47, 36, 21, and 14 kDa. It was clearly evident that the 21-kDa protein of ICRC was exclusively precipitated by sera from LL patients. Sera from three of eight BL patients tested showed reactivity to the 21-kDa protein of ICRC. On histopathological classification, these patients were diagnosed as LL cases. The sera of LL patients in ENL reactions immunoprecipitated ICRC and M. leprae antigens with high intensity. The reactivity of these sera to the 21-kDa protein of ICRC was much stronger than that of sera from LL patients.

Klatser et al. (18), using Western blotting (immunoblotting) techniques, showed that sera from LL patients could identify M. leprae antigens of 86, 41, 36, 28, 22, and 12 kDa. Using the same technique, Vega-Lopez et al. (22) reported that M. leprae antigens of 33, 25, 18, 15, and 12 kDa were identified by sera from LL patients. Immunoprecipitation of M. leprae antigens by sera from untreated leprosy patients revealed proteins with molecular masses of 48, 36, 32 to 33, 27, 15, and 12 kDa (3). Our results appear to be closer to those reported by Britton et al. (3). The variability in the antigen recognition patterns could be due to differences in detection techniques used. Vega-Lopez et al. (22) have suggested that genetic differences among human populations may also affect the immune response to M. leprae, thereby yielding different antibody profiles. Exposure to other environmental mycobacteria and the diversity of epidemiological patterns of leprosy are apparently important aspects in the serological diagnosis of leprosy.

In our studies, the antigen recognition patterns of antibodies obtained from untreated, MDT-treated, or MDT nonresponder LL patients were comparable. Klatser et al. (18) showed that although the intensity of antigen binding was affected in LL patients under prolonged MDT treatment, the overall antibody pattern remained the same. Using M. leprae antigens, we were unable to distinguish between the serological reactivities of LL, BL, BT, TT, and

FIG. 4. Immunoprecipitation of [125I]-labeled M. leprae antigens with sera from LL (A) and TT (B) patients. Right lane in panel B shows immunoprecipitation with pooled sera from LL patients. Molecular mass markers in kilodaltons are shown to the right of each gel. Arrow indicates the location of the 21-kDa protein.
TB patients and healthy donors. Das et al. (7) have emphasized that immunologically cross-reactive antigenic components present on other mycobacteria rather than species-specific antigens may provide important reagents for the serodiagnosis of leprosy. The immunoprecipitation of the 21-kDa protein of ICRC exclusively by sera from LL patients may be useful in serodiagnosis of LL. ICRC is a cultivable mycobacterium belonging to the M. avium complex (8). Immunoprecipitation of M. avium antigens with sera from LL patients has also revealed the presence of a 21-kDa antigen.

The 14-kDa antigen of ICRC seems to be another important protein moiety involved in humoral responses. Sera from a few LL patients (5 of 26) and all healthy household contacts showed a strong reactivity with the 14-kDa protein of ICRC. It still remains to be investigated if detection of the 14-kDa protein of ICRC has any relation to the presence of subclinical infection.

It is thus felt that analysis and characterization of the 21-kDa soluble protein of ICRC, a cultivable mycobacterium, may prove to be important in the serodiagnosis of leprosy.

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