Recombinant fusion protein identified by lepromatous sera mimics native Mycobacterium leprae in T-cell responses across the leprosy spectrum

(Human pathogen/patient sera/Agt11 expression/human immune response)


*Department of Biotechnology, All India Institute of Medical Sciences and ‡Department of Dermatology, Safdarjung Hospital, New Delhi 110 029, India

Communicated by V. Ramalingaswami, October 15, 1990 (received for review March 29, 1990)

ABSTRACT Pooled polyclonal sera from lepromatous leprosy patients were used to screen an Agt11 recombinant DNA expression library of Mycobacterium leprae in order to identify the relevant antigens recognized by the human immune response. Of the 300,000 phages screened, 4 clones were identified that coded for fusion proteins of the same molecular mass. The fusion protein from clone LSR2 was tested for immunoreactivity in assays using peripheral blood cells and sera from 11 laboratory personnel and 105 patients across the leprosy spectrum. LSR2 protein appears to be predominantly a T-cell antigen. It evokes similar lymphoproliferative responses as the native bacillus both at the individual level and in the leprosy spectrum as a whole. Though only 50% of patient sera with anti-M. leprae antibodies reacted with the fusion protein, the pattern of reactivity in the antibody responses was also similar for the various clinical types. The coding regions of clones LSR1 and LSR2 are identical. They show no homology with sequences stored in data banks and encode a protein of 89 amino acids with a calculated molecular mass of ~10 kDa.

Leprosy is a chronic infectious disease caused by noncultivable Mycobacterium leprae. The clinicopathological spectrum observed in this disease reflects the variability in the host immune responses to the pathogen (1). Protective immunity is mainly effected by cellular responses as evidenced by the presence of optimal T-cell functions in the localized paucibacillary form of tuberculosis (TT) leprosy. In contrast, the generalized multibacillary lepromatous (LL) leprosy shows antigen-specific T-cell anergy concomitant with the presence of high levels of specific and crossreactive mycobacterial antibodies (2, 3). Young et al. (4) constructed a genomic library of M. leprae in the Agt11 expression vector. By using monoclonal antibodies (mAbs) as screening reagents, genes coding for 12-, 18-, 28-, 36-, and 65-kDa proteins of M. leprae were identified. Whereas some of these have been shown to share homology with the heat shock proteins of various species (5–8), the 18-kDa protein has been found to be stimulatory for human T helper clones (9) and for peripheral blood cells from healthy contacts (10).

With a view to identifying genes expressing proteins recognized by the human immune response to natural M. leprae infection, we have used polyclonal antibodies obtained from pooled sera of lepromatous patients to screen the Agt11 DNA expression library. We have identified four clones coding for a fusion protein of the same molecular mass. It appears to be a dominant T-cell antigen and mimics the native bacillus in lymphoproliferative responses of all clinical types of leprosy patients. It is also recognized by the sera of 50–70% of the patients having anti-M. leprae antibodies.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Subjects. The study included 105 leprosy patients attending the Hansen disease clinic of Safdarjung Hospital (New Delhi) and 11 healthy laboratory personnel with >3 years of constant contact with patients. The type of leprosy was diagnosed on the basis of clinical and histopathological criteria of Ridley and Jopling (11), and the bacterial index was assessed by slit skin smears. Patients were bled prior to or within 6 months of treatment with a multidrug regimen consisting of 600 mg of rifampicin monthly, 100 mg of clofazimine on alternate days, and 100 mg of dapsone daily.

Sera. Sera from untreated LL patients were screened for the presence of anti-M. leprae antibodies by a dot ELISA using sonicated M. leprae as antigen [lepromin, 10 μg/ml; courtesy of R. J. W. Rees through the Immunology and Leprosy (IMMLEP) Program of the World Health Organization (WHO)]. Ten sera showing strong reactivity with 10- and 20-ng dots of lepromin were pooled. Horseradish peroxidase-conjugated anti-human IgG (1:200 dilution, Dakopatts, Denmark) and 4-chloro-1-naphthol (Sigma) were used for detection. The LL serum pool was depleted of anti-Escherichia coli antibodies by adsorption with lysates of E. coli Y1083 lysiogonized with Agt11, immobilized on nitrocellulose paper (NCP). This serum pool was stored at −20 °C and used at 1:200 dilution for screening the M. leprae DNA library.

Screening of Agt11 M. leprae DNA Expression Library. The Agt11 M. leprae DNA library (courtesy of R. A. Young through the IMMLEP Program of WHO) was screened with the above pool of preadsorbed LL sera (12). The positive clones, designated LSR, were purified and checked for crossreactivity with murine mAbs MC 2404, 0401, 1723, 2009, 4243, 4220 (obtained from WHO; ref. 13), and SAD2 7C (courtesy of D. B. Young, Medical Research Council Unit, Hammersmith Hospital, London; ref. 14), defining epitopes on the 65-kDa antigen, and MC 8026 (WHO), defining the 18-kDa antigen of M. leprae.

Characterization of the Recombinant LSR Proteins. Lysogens of LSR clones were established in E. coli Y1089 and induced to produce recombinant proteins, as described (12). Lysates thus obtained were subjected to SDS/8% PAGE (15) followed by Western blot analysis (16) using the pooled LL sera or anti-β-galactosidase mAb (Promega). DNA Hybridization. Samples (2 μl) of the phage stocks of LSR clones, the five clones (Y3164, Y3178, Y3179, Y3180, and Y3184) previously identified by using anti-M. leprae

Abbreviations: mAb, monoclonal antibody; WHO, World Health Organization; NCP, nitrocellulose paper; PBMC, peripheral blood mononuclear cell; LL, lepromatous; BL, borderline lepromatous; BB, borderline; BT, borderline tuberculosis; TT, tuberculoid.

To whom reprint requests should be addressed.

The sequence reported in this paper has been deposited in the GenBank data base (accession no. X53487).
murine mAbs (courtesy of R. A. Young) that define the 28-, 65-, 18-, 36-, and 12-kDa antigens, respectively, and Agt11 (without insert) were grown on a lawn of E. coli Y1090. Bacteriophage DNA was transfected into NCP, denatured (1.5 M NaCl/0.5 M NaOH), and neutralized (1.5 M NaCl/0.5 M Tris-HCl, pH 8.0), and the dried filters were baked at 80°C for 2 hr. Phage DNA from the above clones was cut with EcoRI (New England Biolabs), and the fragments were separated by electrophoresis in 0.8% agarose gel (type 2, Sigma) and subjected to Southern blot analysis (17).

Purified and nick-translated insert DNA from clone LSR2 was used to probe the plaque replica and the Southern blot. Hybridizations were carried out at 42°C for 16 hr in 50% (vol/vol) formamide/5× SSC (1× SSC is 150 mM NaCl/15 mM trisodium citrate)/0.1% Ficoll/0.1% polyvinylpyrrolidone/0.1% bovine serum albumin/50 mM Tris-HCl, pH 7.5, containing sonicated salmon sperm DNA at 250 μg/ml. Washing was done sequentially in 2× SSC/0.1% SDS for 20 min, with two changes, at room temperature, followed by 2× SSC/0.1% SDS at 50°C for 30 min and finally 0.1× SSC/0.1% SDS at 64°C for 30 min. The blots were then exposed to polyester x-ray films (Hindustan Photo Films Manufacturing, Udhagamandalam, India) for 16 hr at –70°C.

**M. leprae Antigens.** Armadillo-derived M. leprae (courtesy of R. J. W. Rees through the IMMLEP Program of WHO) was used as integral and sonicated form for the lymphoproliferation assay and dot ELISA, respectively. Except where stated 5 × 10⁵ bacilli per ml and 10 and 20 ng of leprosin were used. The expressed recombinant proteins were isolated from lysates of E. coli Y1089 lysogenized with LSR2 and tested at concentrations indicated below. Thirty microliters of lysate (1 ng/ml) of LSR2 lysogen was resolved by SDS/PAGE and transferred to NCP. The fusion protein band identified in a position complementary to the Western blot was cut out and converted into antigen-bearing particles by dissolution in dimethyl sulfoxide, precipitation with carbonate/bicarbonate buffer (pH 9.6), and resuspension in 1 ml of medium RPMI 1640 (GIBCO) (18). A β-galactosidase band, obtained from lysates of E. coli Y1089 lysogenized with Agt11, and a piece of NCP of similar size were treated in an identical manner for use as controls.

**Lymphoproliferation Assays.** Ficoll/Hypaque-separated (19) peripheral blood mononuclear cells (PBMCs) obtained from 8 healthy contacts and from 15 tuberculoid–borderline tuberculosis (TT-BT), 3 borderline (BB), and 15 borderline lepromatous–lepromatous (BL-LL) patients were resuspended in RPMI 1640 containing 10% human AB serum, 2 mM L-glutamine, 25 mM Hepes buffer, and 100 units of penicillin and 100 μg of streptomycin, sulfate per ml. Cells were cultured (10⁵ cells per well) in 96-well round-bottomed microtiter plates (Nunc, Intermed, Kanstrup, Denmark) for 5 days at 37°C in humidified air with 5% CO₂. Quadruplicate cultures were exposed to medium only or to 25 μl of LSR2 antigen particles (undiluted or diluted 1:2.5, 1:5, or 1:10). NCP particles alone (diluted 1:5), β-galactosidase-blotted NCP particles (diluted 1:2.5 or 1:5), or integral M. leprae (5 × 10⁸ bacteria). [methyl-³H]Thymidine (2 Ci/mmol, Amer sham; 1 Ci = 37 GBq) was added (1 μCi per well) and the cells were harvested 16 hr later. Incorporation of [³H]thymidine was expressed as cpm (counts per minute). For cultures stimulated with the integral M. leprae antigen, Δcpm was calculated as mean cpm of M. leprae-stimulated cultures – mean cpm of unstimulated PBMCs. For recombinant LSR2 antigen-stimulated cultures, Δcpm was calculated as mean cpm of LSR2-stimulated cultures – mean cpm of β-galactosidase-stimulated cultures.

**Antibody Assays.** Sera from 11 healthy contacts and from 38 TT-BT, 9 BB, and 34 BL-LL patients were depleted of anti-E. coli antibodies and tested in a dot ELISA to detect antibodies to LSR2 protein. Samples (2 μl) containing 0.4–1.6 μg of total protein from lysates of uninduced LSR2, induced LSR2, or induced Agt11 (producing β-galactosidase, negative control) lysogens or 10 and 20 ng of leprosin (positive control) were dotted on NCP. Sera from healthy contacts and TT-BT patients were used at dilutions of 1:25 to 1:200; sera from BL-LL patients were tested at 1:150 and 1:300. Sera were considered positive when an unequivocal color reaction developed in the concentration range of the LSR2 protein used.

**DNA Sequence Analysis.** DNAs from clones LSR1 and -2 were prepared by standard methods and cloned separately into pBluescript (Stratagene) (17). The insert DNA was isolated on low-melting-point agarose after digestion with EcoRI. The EcoRI fragment from LSR1 and the smaller EcoRI–Pst I fragment from LSR2 were subcloned into M13mp18 or M13mp19 (Bethesda Research Laboratories) as suggested by the supplier. DNA sequence was determined in both directions by the dideoxy chain-termination method (20) using universal primers. Band compressions due to secondary structures were resolved using Taq polymerase (Sequenase, United States Biochemical) and the primer extension method. Sequence data were analyzed using IBI/Pustell sequence-analysis programs and the amino acid sequence was inferred.

**Statistical Analysis.** The correlation coefficient for optimal responses to M. leprae and LSR2 protein was estimated by Spearman’s rank correlation (21).

**RESULTS**

**Recombinant Protein from M. leprae DNA Expression Library.** Approximately 300,000 phages of a Agt11 M. leprae DNA expression library were screened with pooled pread-
sorbed LL sera selected on the basis of high antibody levels to sonicated *M. leprae*. Four clones (LSR1, -2, -4, and -5) were identified. In contrast, pooled sera from TT patients failed to give unequivocal signals. All four clones probed a 135-kDa protein that reacted with LL sera (Fig. 1A) but not with pooled sera from TT patients or with mAbs directed against 65- and 18-kDa antigens of *M. leprae*. That these sera and mAbs were immunoreactive was indicated by positive reaction with the appropriate controls in a dot ELISA (Table 1). The 135-kDa protein reacted with a mAb directed against β-galactosidase (Fig. 1B) and was present only in induced cultures (lanes a), indicating that it was a fusion protein.

EcoRI digestion of LSR1 DNA yielded an insert of 800 base pairs (bp) whereas LSR2, -4, and -5 had inserts of about 2.8 kilobase pairs (kb) (Fig. 2A). The nick-translated EcoRI fragment from LSR2 hybridized strongly with phage DNA from all four clones (Fig. 2A). It did not hybridize with DNA from the control Agt11 or with the five earlier reported clones identified with murine mAbs (4). The protein from LSR2 was investigated further for its biological relevance in cellular and humoral responses of leprosy patients.

**Lymphoproliferative Responses to the Recombinant LSR2 Protein.** Since protective immunity in leprosy is associated with cell-mediated immune responses, we examined the ability of LSR2 protein to induce proliferation of PBMCs from all clinical types of leprosy patients during the natural course of *M. leprae* infection. Healthy laboratory personnel with >3 years of constant exposure to patients were also studied. For each individual, the response to the native integral *M. leprae* at a predetermined concentration was compared with the proliferation induced by the recombinant antigen-bearing NCP particles.

Fig. 3 indicates the overall data obtained on 33 leprosy patients and 8 healthy contacts. A striking similarity in the pattern of responses was observed between the native and the recombinant antigens. In general, PBMCs from healthy subjects and TT-PT patients showed optimal [3H]thymidine incorporation in response to both antigens (>2000 cpm).

Thus it can be seen that the 135-kDa protein isolated by affinity chromatography on LSR2 is not only crossreactive with LL sera from leprosy patients. For each individual, the response to the native integral *M. leprae* at a predetermined concentration was compared with the proliferation induced by the recombinant antigen-bearing NCP particles.

**Table 1. Reactivity of recombinant clones with mAbs and pooled LL sera**

<table>
<thead>
<tr>
<th>mAb or serum</th>
<th>M. leprae Specificity antigen, kDa</th>
<th>Y3178</th>
<th>Y3179</th>
<th>Y3179 (65 kDa)</th>
<th>Y3179 (18 kDa)</th>
<th>Agt11</th>
<th>LSR</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC 2404</td>
<td>65</td>
<td>S</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>MC 0401</td>
<td>65</td>
<td>CR</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MC 1723</td>
<td>68</td>
<td>S</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MC 4243</td>
<td>65</td>
<td>S</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MC 4220</td>
<td>68</td>
<td>CR</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MC 2009</td>
<td>35-70</td>
<td>CR</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SA2 D7C</td>
<td>65</td>
<td>CR</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MC 8026</td>
<td>18</td>
<td>S</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LL serum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

S, specific; CR, crossreactive. For Y3178 and Y3179, see ref. 4; for LSR clones 1, 2, 4, and 5, refer to this paper.

**Antibody Responses to the Recombinant LSR2 Protein.** The percentage of individuals showing antibodies to LSR2 increased from the TT to the LL pole. Only 2 of 11 healthy contacts had detectable levels of LSR2 antibodies (Fig. 4). Thus it appears that in leprosy patients the pattern of antibody response to the recombinant protein closely follows that observed with sonicated native *M. leprae*.

**DNA Sequence Analysis.** To further define the LSR clones insert DNA from the LSR1 and LSR2 clones was sequenced according to the strategy shown in Fig. 5. An open reading

![Fig. 2. (A) Hybridization of [α-32P]dATP-labeled EcoRI insert from clone LSR2 to phage DNA from clones identified with murine mAbs to *M. leprae* antigens of defined molecular mass (1, Y3164 (28 kDa); 2, Y3178 (65 kDa); 3, Y3179 (18 kDa); 4, Y3180 (36 kDa); 5, Y3184 (12 kDa)), from LSR clones (6, LSR1; 7, LSR2; 8, LSR4; 9, LSR5), and from phage Agt11 without insert (spot 10). Positive signals were unique to LSR clones. (B) Southern blot of LSR clones hybridized with the same probe as in A, showing EcoRI fragments of 0.8 kb for LSR1 (lane 9) and 2.8 kb for LSR2, -4, and -5 (lanes 8, 7, and 6, respectively). Lanes 1–5 and 10 are numbered as in A and do not show hybridization.**

![Fig. 3. Lymphoproliferative responses of PBMCs from 8 healthy contacts and 33 leprosy patients to integral *M. leprae* (○) and immobilized LSR2 antigen particles (●). The healthy contacts (HC) and TT-BT patients showed positive lymphoproliferative responses to both antigens. The multibacillary BB and BL-LL patients showed poor or nil responses to both antigens. For both antigens Δcpm was calculated as described in Materials and Methods.**
frame extending from the EcoRI site was found that coded for the same 89 amino acids in both clones (Fig. 6), indicating thereby that this open reading frame corresponds to the fusion protein. The calculated size of the protein from the predicted amino acid sequence was 9810.280 Da (≈10 kDa), with an isoelectric point of 12.17. The predicted restriction map is shown in Fig. 5. The hydropathy plot of J. (colony courtesy of J. Colston, National Biomedical Research Foundation, National Institute for Medical Research, Mill Hill, London) data bases (courtesy of J. Colston).

**DISCUSSION**

With a view to identifying the dominant protein antigens recognized by the human immune response to natural *M. leprae* infection, we used preadsorbed polyclonal sera from LL patients to screen the Agt11 DNA expression library of *M. leprae*. Four clones were identified that coded for a lacZ promoter-dependent fusion protein of the same molecular mass. Clone LSR1 had an 800-bp insert, whereas the other clones had inserts of about 2.8 kb. With the LSR2 insert as a probe, hybridization was shown to be uniquely restricted to LSR clones, with no detectable homology to phage DNA derived from the five previously reported clones identified by murine antibodies (4, 13). That the recombinant protein does not correspond to any of the known stress proteins is indicated by the hybridization data.

To evaluate the immunoreactivity of the fusion protein, lysates from clone LSR2 were tested in lymphoproliferation assays using PBMCs from patients across the leprosy spectrum as well as control healthy responders who had been exposed to *M. leprae*. Significantly, there was marked similarity in the lymphoproliferative responses to the fusion protein and the native integral bacilli both at the individual level and for the clinical type of leprosy. Maximal lymphoproliferative responses were observed for the healthy and BT-TT leprosy subjects, with a marked decline in responsiveness for the BB and BL-LL patients. Most of the prolif-

---

**Fig. 4** Individual sera from 11 healthy contacts (HC) and 81 leprosy patients assessed for presence of anti-LSR2 protein antibodies (hatched bars) and anti- *M. leprae* antibodies (solid bars) in a dot ELISA. The relative proportion of patients showing antibodies to both antigens increased from the TT-BT to the BL-LL pole. Fifty percent or more of the patients showing anti-*M. leprae* antibodies were positive for LSR2 antibodies.

**Fig. 5** Restriction map of clones LSR1 and -2. Hatched bar represents the coding region. Length and direction of sequenced restriction fragments are illustrated by the arrows.
Such mimicry would be amenable to exploitation for understanding the protective immune mechanisms in leprosy. Moreover, the responder individuals showing T-cell reactivity to LSR2 protein were unrelated, indicating that major histocompatibility complex (MHC) restriction did not apply to the molecule as a whole. Such permissive MHC association has been reported for malarial peptides (34) and may be of advantage in designing vaccines. It appears that polyclonal sera from patients are useful for identifying recombinant proteins of relevance to human T-cell responses.

This work was supported by grants provided by the Department of Science and Technology, New Delhi, India.