Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 1054–1058, February 1991
Medical Sciences

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

(human pathogens/patient sera/λgt11 expression/human immune response)

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

ABSTRACT **Pooled polyvalent sera from lepromatous leprosy patients were used to screen a λgt11 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 tuberculoid (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 λgt11 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 λgt11 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.

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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 [leprosin, 10 μg/ml; courtesy of R. J. W. Rees through the Immunology of Leprosy (IMMLEP) Program of the World Health Organization (WHO)]. Ten sera showing strong reactivity with 10- and 20-ng dots of leprosin 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 lysogenized with λgt11, 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 λgt11 *M. leprae* DNA Expression Library. The λgt11 *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 SA2D 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 tuberculoid; TT, tuberculoid.

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§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 λgt11 (without insert) were grown on a lawn of *E. coli* Y1090. Bacteriophage DNA was transferred onto 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 *Eco*RI (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, Udhagamandalan, 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 5×10^6 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 mg/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 λgt11, 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 tuberculoid (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 (10^5 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% CO_2. 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* (5×10^6 bacteria). [*methyl*-^{3}H]Thymidine (2 Ci/mmol, Amersham; 1 Ci = 37 GBq) was added (1 μCi per well) and the cells were harvested 16 hr later. Incorporation of [^{3}H]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 λgt11 (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 *Eco*RI. The *Eco*RI fragment from LSR1 and the smaller *Eco*RI–*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 λgt11 *M. leprae* DNA expression library were screened with pooled pread-

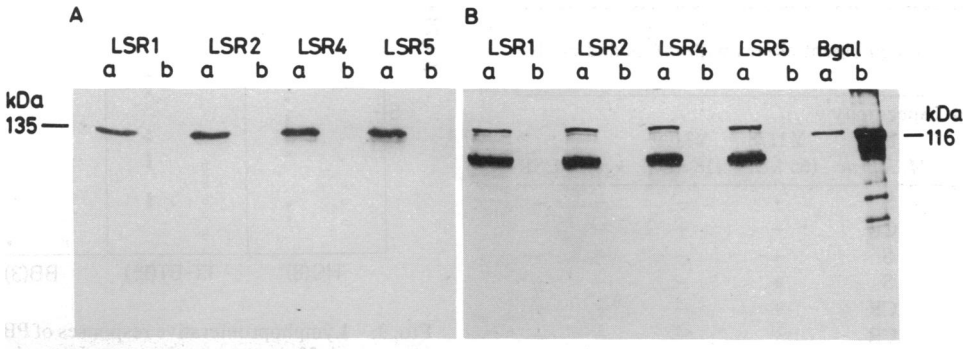

FIG. 1. Immunoblot analysis of recombinant proteins from clones LSR1, -2, -4, and -5, showing reactivity to both polyvalent sera from LL leprosy patients (*A*) and mAb to β-galactosidase (*B*). Lysates from induced (lanes a) and uninduced (lanes b) lysogens of recombinants were tested. The fusion protein bands for all four clones were at 135 kDa and were detected only in induced lysates. In *B*, the Bgal lanes contained lysate from induced λgt11 vector in *E. coli* Y1089 (lane a) or commercial β-galactosidase marker (lane b). The second band in *B* indicates reactivity with degraded fusion proteins.

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 produced a 135-kDa protein that reacted with LL sera (Fig. 1*A*) 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. 1*B*) and was present only in induced cultures (lanes a), indicating that it was a fusion protein.

*Eco*RI 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. 2*B*). The nick-translated *Eco*RI fragment from LSR2 hybridized strongly with phage DNA from all four clones (Fig. 2*A*). It did not hybridize with DNA from the control λgt11 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 BT-TT patients showed optimal [^{3}H]thymidine incorporation in response to both antigens (>2000 cpm). Though individual dose responses were variable, the major response to the native *M. leprae* appeared to be contributed by the LSR2 protein. PBMC cultures from a few individuals did not respond to the native *M. leprae* but showed a low proliferative response with LSR2 antigen. In contrast, BB and BL-LL showed low to nil responses with both antigens. When the Δcpm values obtained in all subjects with both antigens were compared by Spearman's rank correlation, a highly significant ($P < 0.001$) correlation coefficient of 0.64 was obtained. A few responder individuals who were concurrently tested with LSR1 lysates showed responses similar to those with LSR2 antigen (data not shown).

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*.

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

mAb or serum	*M. leprae* antigen, kDa	Specificity for *M. leprae*	Y3178 (65 kDa)	Y3179 (18 kDa)	λgt11	LSR
MC 2404	65	S	+	−	−	−
MC 0401	65	CR	+	+	−	−
MC 1723	68	S	+	−	−	−
MC 4243	65	S	+	−	−	−
MC 4220	68	CR	+	−	−	−
MC 2009	35–70	CR	+	−	−	−
SA2 D7C	65	CR	+	−	−	−
MC 8026	18	S	−	+	−	−
LL serum	−	−	−	−	−	+

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

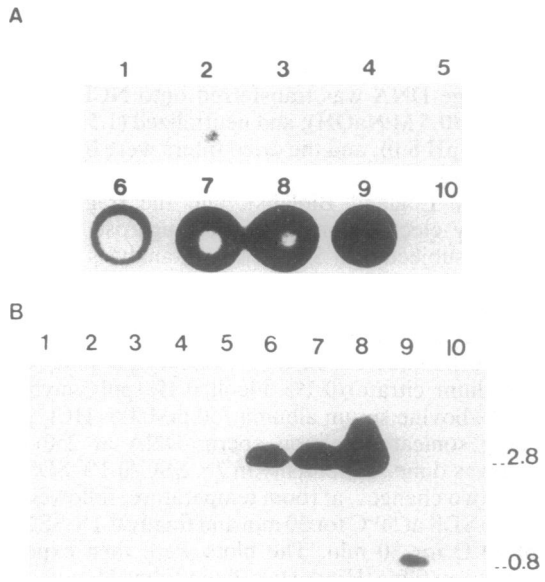

FIG. 2. (*A*) Hybridization of [α-^{32}P]dATP-labeled *Eco*RI insert from clone LSR2 to phage DNA from clones identified with murine mAbs to *M. leprae* antigens of defined molecular mass [1, Y3164 (28 kDa); 2, Y3178 (65 kDa); 3, Y3179 (18 kDa); 4, Y3180 (36 kDa); 5, Y3184 (12 kDa)], from LSR clones (6, LSR1; 7, LSR2; 8, LSR4; 9, LSR5), and from phage λgt11 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 *Eco*RI 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.

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

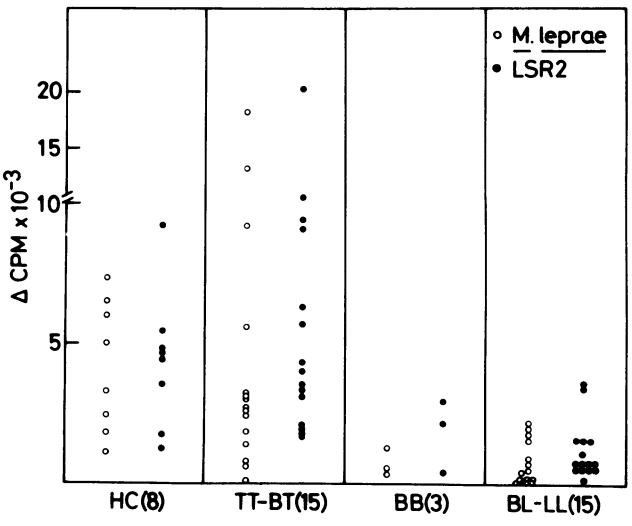

FIG. 3. Lymphoproliferative responses of PBMCs from 8 healthy contacts and 33 leprosy patients to integral *M. leprae* (○) and immobilized LSR2 antigen particles (●). The healthy contacts (HC) and TT-BT patients showed positive lymphoproliferative responses to both antigens. The multibacillary BB and BL-LL patients showed poor or nil responses to both antigens. For both antigens Δcpm was calculated as described in *Materials and Methods*.

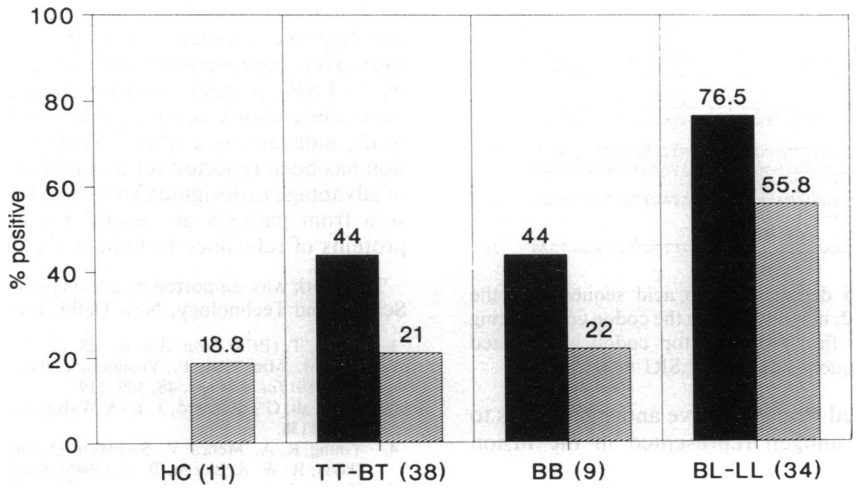

FIG. 4. Individual sera from 11 healthy contacts (HC) and 81 leprosy patients assessed for presence of anti-LSR2 protein antibodies (hatched bars) and anti-*M. leprae* antibodies (solid bars) in a dot ELISA. The relative proportion of patients showing antibodies to both antigens increased from the TT-BT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

frame extending from the *Eco*RI 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 (22) of the protein sequence showed hydrophobic and possible amphipathic regions. No significant homology was found up to September 1990 with sequences stored in the GenBank, 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 polyvalent sera from LL patients to screen the λgt11 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-

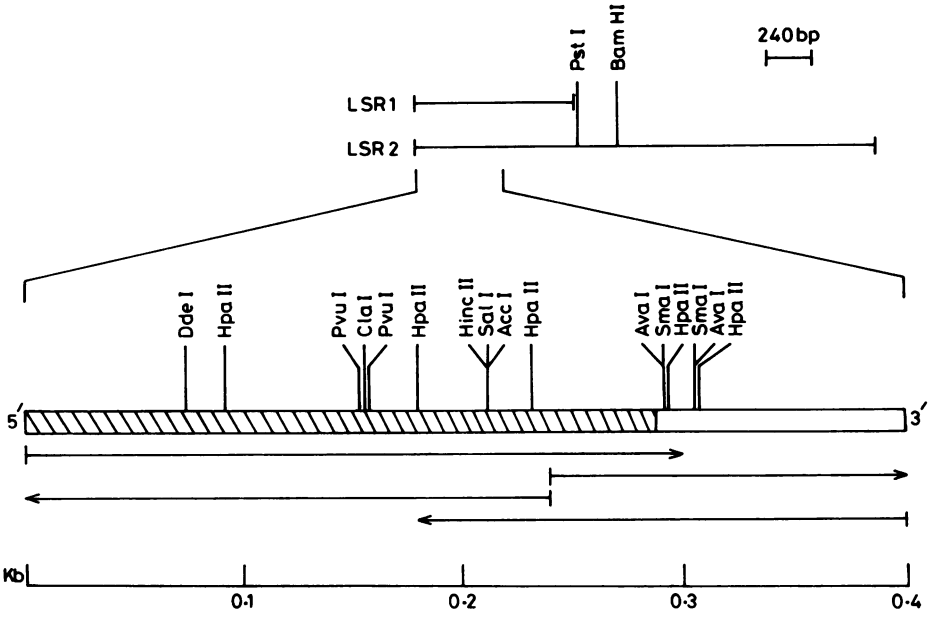

FIG. 5. Restriction map of clones LSR1 and -2. Hatched bar represents the coding region. Length and direction of sequenced restriction fragments are illustrated by the arrows.

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GAATTCGGGCTTGACGGGGTGACCTACGAGATCGACCTTACGAACAAGAATGCCGCGAAA   60
GluPheGlyLeuAspGlyValThrTyrGluIleAspLeuThrAsnLysAsnAlaAlaLys

CTGCGTGGCGATCTGAGGCAATGGGTGTCCGCCGGACGGCGCGTCGGCGGTCGGCGGCGA  120
LeuArgGlyAspLeuArgGlnTrpValSerAlaGlyArgArgValGlyGlyArgArgArg

GGGCGTTCCAATTCTGGACGCGGCCGTGGGGCGATCGATCGCGAACAGAGCGCGGCGATC  180
GlyArgSerAsnSerGlyArgGlyArgGlyAlaIleAspArgGluGlnSerAlaAlaIle

CGGGAATGGGCTCGTCGGAACGGACATAATGTGTCGACTCGTGGTCGTATTCCGGCCGAC  240
ArgGluTrpAlaArgArgAsnGlyHisAsnValSerThrArgGlyArgIleProAlaAsp

GTCATTGACGCATTCCACGCGGCGACTTAAAATAAAAAGTTCTGTACTGACGCCCGGGCT  300
ValIleAspAlaPheHisAlaAlaThr+++

CTAGGACCCGGGCGTCAGTACTTTAAAAAAATATGTTGCTTTCGTTGCTGGCGAACTGAT  360
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FIG. 6. Nucleotide and deduced amino acid sequence of the coding region of clone LSR2, numbered from the codon commencing from the *Eco*RI site. The first in-frame stop codon is indicated (+++). The nucleotide sequence of clone LSR1 was identical.

erative response observed with the native antigen appears to be contributed by the antigen represented in the fusion protein.

Previous studies on recombinant proteins from the same library had used T-cell lines and clones to study immunoreactivity (9, 23, 24). These may not truly reflect the antigens relevant to the biology of the disease or the total T-cell repertoire, since not all T cells may have survived the *in vitro* selection pressures inherent in the current methodologies. Similar to the present report, Dockrell *et al.* (10) also used unfractionated PBMCs of leprosy patients and healthy contacts for studying T-cell responses to another recombinant protein of *M. leprae*, which has been shown to be an 18-kDa stress protein. They found that 50% of tuberculoid patients and 95% of healthy contacts showed lymphoproliferation to this antigen. Our LSR2 protein stimulated lymphoproliferation of a higher proportion (90%) of BT-TT patients and healthy contacts.

In antibody assays the LSR2 protein was recognized by only half of the leprosy patient sera having detectable levels of antibodies to sonicated *M. leprae*. However, the pattern of reactivity in the clinical groups was similar for both antigens in that the number of patients with antibodies was lowest at the TT pole and increased towards the LL pole. Though the recombinant protein had been selected on the basis of reactivity with pooled LL sera having mycobacterial antibodies, at the individual patient level the reactivity was limited. This suggests that the humoral response to LSR2 may reflect individual variation in antibody profiles. Alternatively, the sonicated antigen is likely to have a larger number of epitopes of both specific and crossreactive types. The lower-than-expected antibody reactivity in patients appears to be common to many recombinant proteins of *M. leprae* (25, 26).

In both the LSR1 and the LSR2 clone, an open reading frame extending from the *Eco*RI site was identified that coded for 89 amino acids with a calculated molecular mass of ≈10 kDa. With the 114 kDa for the truncated β-galactosidase encoded in λgt11, the fusion protein would be 124 kDa. Within the limitations in estimating molecular mass from mobilities in SDS/polyacrylamide gels, this compares well with 135-kDa fusion protein detected in the Western blots.

The nature of the gene coding for this protein is not known, as a search in the sequence banks did not reveal significant homology with any known gene or protein, including the 18-, 28-, 36-, 65-, and 70-kDa protein identified recently (8, 27–30). It does not appear to have sequences reminiscent of signal peptides (31), nor does it share homology with the 28-kDa recombinant protein identified by Cherayil and Young (32) using lepromatous sera. Sathish *et al.* (33) recently detected 45 recombinant clones with leprosy patients sera whose further characterization is awaited.

The recombinant protein identified by us mimics the native *M. leprae* in T-cell responses across the leprosy spectrum. 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 polyvalent 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.

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