Sera of Leprosy Patients with Type 2 Reactions Recognize Selective Sequences in *Mycobacterium leprae* Recombinant LSR Protein

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Type 2 reactions (erythema nodosum leprosum [ENL]) are episodic, reactional states causing significant morbidity in lepromatous leprosy patients. With a view to defining the immunological differences between the stable and reactional forms of lepromatous leprosy, we determined antibody responses to LSR, a recombinant protein of *Mycobacterium leprae* previously described by us (S. Laal, Y. D. Sharma, H. K. Prasad, A. Murtaza, S. Singh, S. Tangri, R. S. Mishra, and I. Nath, Proc. Natl. Acad. Sci. USA 88:1054–1058, 1991), as well as to 10- to 15-mer overlapping peptides synthesized on the basis of the LSR amino acid sequence. We report here the selective recognition of B cell epitopes by sera from patients with ENL as compared with a control group with nonreactional lepromatous leprosy. Peptides 2 and 3, with the sequences GVTYEIDLTNKNAA and IDLTNKNAAKLRGD, respectively, were recognized by >95% of sera from patients with active ENL. Peptide 3 in addition showed reactivity with sera taken from 91.6% of lepromatous leprosy patients who were apparently stable but who were recorded to have had ENL several weeks before or after the sample collection. The core sequence IDLTNKNAA common to both these peptides may be a major target of humoral responses in ENL. In addition, the RGD motif at the C terminus appeared to influence the antigenicity of peptide 3 in enzyme-linked immunosorbent assay. It would appear that humoral responses during ENL are directed to selective antigenic determinants of the leprosy bacillus. The use of such serological markers to identify lepromatous leprosy patients with a high risk for developing ENL would be of clinical and predictive value.

Leprosy has been intensively investigated in recent years with a view to understanding the immunological basis for the diverse clinical manifestations seen in subjects infected with the same pathogen, viz., *Mycobacterium leprae*. The stable forms of both the localized tuberculoid leprosy and the disseminated lepromatous leprosy are characterized by hypopigmentation of the skin and local nerve thickening and are relatively free of systemic symptoms (8, 15). However, serious morbidity develops in some patients because of the onset of episodic leprosy reactions. Of clinical significance is the erythema nodosum leprosum reaction (ENL) (type 2 reaction), seen in 10 to 15% of lepromatous leprosy patients, which leads to systemic manifestations of fever, generalized erythematous dermal nodules, and arthritis (19). ENL often occurs after institution of chemotherapy and acts as a deterrent to specific drug therapy in those with repeated episodes. During active ENL, patients have been reported to have immunological features indicative of polyclonal B cell activation, immune complex deposition (19), and development of antigen-specific T cell functions in previously anergic individuals (10, 12). Information on the antigenic determinants that are the targets for these immune reactions is lacking, and no clinical or laboratory markers are currently available to identify individuals who are prone to ENL.

With the advent of DNA technology, an increasing number of *M. leprae* genes have been cloned and sequenced (4,

21, 22). Recombinant antigens are now available for defining the epitopes involved in immunological perturbation. Our group has previously described a 135-kDa fusion protein designated LSR (11) which had been identified from the lambda gt11 expression library of *M. leprae* (22) by using lepromatous sera. This fusion protein, consisting of 89 amino acids (aa) encoded by *M. leprae* DNA, was highly immunoreactive, being recognized by both T cells and sera of leprosy patients. Sela et al. subsequently identified the full gene from a cosmid library and showed that the total protein had 123 aa with the LSR at the carboxy terminus (17). We sought to identify differences in antibody recognition between stable and reactional leprosy patients by defining the B cell epitopes of LSR. Overlapping peptides synthesized on the basis of the amino acid sequence of LSR were used in enzyme-linked immunosorbent assay (ELISA) with coded serum samples from leprosy and tuberculosis patients as well as healthy subjects from areas of India where leprosy is endemic and from areas where it is not endemic.

MATERIALS AND METHODS

Subjects and sera. Coded sera from 294 leprosy patients classified on the basis of the clinical and pathological criteria of Ridley and Jopling (15) and from 18 healthy family contacts (FC) were collected from areas of South India where leprosy is endemic (VHS Leprosy Project, Shakthi Nagar) as well as from the low-endemicity area of New Delhi. In addition, sera from 40 healthy volunteers from the region where leprosy is not endemic (NC) and from 46 pulmonary tuberculosis patients were included as controls.

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TABLE 1. Synthetic peptides of LSR used in ELISA[a]

Peptide no.	Sequence	Amino acid no.
1	EFGLDGVTYEIDLTNKNA	1–18
2	GVTYEIDLTNKNAA	6–19
3	IDLTNKNAAKLRGD	11–24
4	KNAAKLRGDLRQWVSAG	16–32
5	LRGDLRQWVSAGRRVG	21–36
6	RQWVSAGRRVGGRRRG	26–41
7	AGRRVGGRRRGRSNSG	31–46
8	GGRRRGRSNSGRGRG	36–50
9	GRSNSGRGRGAIDRREQSA	41–58
10	GRGRGAIDRREQSAA	46–59
11	AIDREQSAAIREWA	51–64
12	QSAAIREWARRNGHNV	56–71
13	REWARRNGHNVSTRGRI	61–77
14	RNGHNVSTRGRIPAD	66–80
15	VSTRGRIPADAVIDA	71–84
16	RIPADVIDAFHAAT	76–89
3-1	IDLTNKNAAKL	11–21
5-1	KLRGDLRQWVSAGRRVGG	20–37

[a] LSR sequence: E-1FGLDGVTYEIDLTNKNAAKLRGDLRQWVSAGR RVGGRRRGRSNSGRGRGAIDREQSAAIREWARRNGHNVSTRGRI PADVIDAFHAAT-89. Regions of pepties with significant β-turn potential ($P > 1.5 \times 10^{-4}$) are underlined.

Sera from areas where leprosy is endemic were sent by air on ice packs to arrive in the laboratory within 24 h of the sample collection. All sera were stored at −20°C prior to testing and were decoded by the clinicians after the ELISA results became available.

ELISA. ELISA was performed in 96-well plates (Maxi-sorb-Immunoplates; Nunc Intermed, Kamstrup, Denmark) coated overnight at 4°C with 50 μl of antigen (4 μg/ml) per well in 50 mM bicarbonate buffer, pH 9.6. Antigen-coated wells were washed three times with Tris-buffered saline (50 mM Tris and 150 mM NaCl, pH 7.4), and the uncovered reactive sites were blocked with 1% fat-free, spray-dried milk for 2 h at room temperature. Duplicate samples of sera at 1:125, 1;250, and 1:500 dilutions were added (50 μl per well), and the plates were incubated for 1 h at 37°C. The plates were washed five times with Tris-buffered saline and further incubated for 1 h at 37°C with 50 μl of a 1:1,000 dilution of peroxidase-conjugated rabbit anti-human total immunoglobulin (Dakopatts A/S, Glostrup, Denmark). The plates were washed as before, and a color reaction was developed with 50 μl of 50 mg% o-phenylenediamine in 50 mM citrate-phosphate buffer, pH 5.0. After an incubation of 20 min at 37°C, the assay was terminated with 8 N H_2SO_4. The optical density (OD) was determined at 492 nm. ELISA data reported here refer to results obtained with sera diluted to 1:125.

Antigens. Lysates of *Escherichia coli* expressing the LSR antigen, control lysates containing the vector without the insert, and sonicated armadillo-derived *M. leprae* were prepared as described earlier. Eighteen peptides were synthesized on the basis of the sequence of the LSR antigen (Table 1). Sixteen were overlapping peptides of 15 to 19 amino acids, while peptides 3-1 and 5-1 represent defined modifications of peptides 3 and 5, respectively. The peptides were synthesized by the solid-phase method using t-Boc chemistry (6) in polypropylene bags (74-μm pore size) with 150 mg of p-methylbenzhydrylamine-HCl resin (Bachem Inc., Torrance, Calif.). The resin was deprotonated by the addition of 5% diisopropylethylamine (Merck, Darmstadt, Germany) in dichloromethane (DCM) before introduction of

the first amino acid. The coupling cycle was initiated by submerging the bags in a solution containing equimolecular amounts of t-Boc amino acids (Bachem Inc.) and diisopropylcarbodiimide (Merck) in 10 M excess over the available amine in the bag. The reaction was allowed to proceed for 60 min, and the product was washed with DCM. The t-Boc group of the newly coupled amino acids was removed with 55% trifluoroacetic acid (TFA) (Pierce, Rockford, Ill.) in DCM. The reaction products were washed, and amino groups were deprotonated with diisopropylethylamine. The peptides were cleaved by treatment with 2 ml of 10% anisole in anhydrous HF (Air Products and Chemicals Inc., Allentown, Pa.) for 60 min at 0°C. The peptides were subsequently extracted with 5% acetic acid (Merck), analyzed, and purified by high-pressure liquid chromatography.

RESULTS

Antibody responses to LSR recombinant antigen. In conformity with our earlier data (11), LSR lysates reacted with sera from >50% of all leprosy patients and FC but not with any of the NC (Table 2). A given serum was designated positive when the OD in ELISA exceeded 3 standard deviations (SD) of the mean value obtained with sera from NC. Sixty-five percent of sera from tuberculosis patients also reacted to LSR, indicating the presence of cross-reactive epitopes in the total protein.

Of significance was the enhanced (95%) seroreactivity observed in ENL patients. All except 2 of 64 individuals showed positive reactivity, with an OD (mean ± SD) of 0.537 ± 0.265. Of the 173 polar lepromatous leprosy patients who appeared stable at the time of the sampling, 48 had a history or clinical record of ENL. Of these, 44 (91.6%) reacted with the LSR antigen (mean OD ± SD, 0.309 ± 0.207). Eleven patients in this group developed clinical ENL 3 to 6 weeks after being tested. The others had single or multiple reactional episodes from 4 weeks to several months before the sampling.

Antibody response to synthetic peptides. In order to study the fine specificity of the antibody recognition in the above-described patients, 10- to 15-mer synthetic peptides spanning the sequence of LSR (Table 1) were used as antigens in ELISA. As expected, a higher percentage of ENL patients than of stable lepromatous leprosy subjects reacted with most peptides (Table 2). Of particular interest were peptides 2, 3, and 13, which were recognized by 93 to 95% of ENL sera. Peptide 2 (GVTYEIDLTNKNAA) was recognized only by sera from patients in the active phase of ENL. In contrast, peptide 3 (IDLTNKNAAKLRGD), which shared a core sequence of IDLTNKNAA, showed a broader reactivity with sera from both patients with active ENL and those with a record of previous reactions. In addition, a lower proportion (22 to 28.5%) of stable lepromatous, tuberculoid leprosy patients and FC also showed seropositivity with peptide 3. Interestingly, tuberculosis patients and NC did not show antibodies to this peptide. It was not possible to establish whether this subset of lepromatous leprosy patients were undergoing subclinical reactions at the time of the sample collection.

Peptide 13, which had a sequence that did not overlap with those of peptides 1 and 3, also reacted in ELISA with 93% of sera from patients with active ENL. However, it was inferior as a predictor of ENL, as antibodies to this peptide were detectable in only 12.5% of asymptomatic patients with a history of reactions. The mean OD values obtained in ELISA supported these observations (data not shown).

TABLE 2. Percentages of seropositive subjects showing antibodies to overlapping peptides of LSR[a]

Antigen	% of subjects with antibodies						
	ENL (44)	Lepromatous (173)		Tuberculoid (77)	TB (46)	FC (18)	NC (40)
		H/O (48)	Stable (125)				
Peptide no.							
1	47.7	8.3	4.8	3.8	0	5.5	0
2	**95.4**	**8.3**	**8.0**	**6.4**	**0**	**0**	**0**
3	**95.4**	**91.6**	**28.0**	**28.5**	**0**	**22.2**	**0**
4	59.0	47.9	32.8	42.8	0	16.6	0
5	81.8	12.5	48.8	55.8	63.0	44.4	10.0
6	86.3	20.8	40.8	57.1	89.0	27.7	10.0
7	77.2	87.5	74.4	79.2	0	61.1	2.5
8	36.3	64.5	20.8	14.2	0	5.5	0
9	56.8	43.7	25.6	18.1	17.3	16.6	2.0
10	47.7	25.0	12.8	7.7	0	5.5	2.5
11	79.5	22.9	28.8	41.5	69.5	22.2	0
12	88.6	18.7	29.6	22.0	69.5	11.1	0
13	**93.1**	**12.5**	**22.4**	**12.9**	**17.3**	**22.2**	**2.5**
14	40.9	16.6	10.4	22.0	0	5.5	0
15	27.2	18.7	31.2	16.8	80.4	22.2	0
16	45.4	14.5	38.5	40.2	0	22.2	0
LSR	95.4	91.6	61.6	51.0	63.0	56.0	0
MLS	97.7	93.7	96.8	71.0	69.5	83.0	13.0

[a] The mean OD ± SD obtained by ELISA with 1:125 dilutions of sera from NC was 0.047 ± 0.016. Sera showing ODs of >0.1 (3 SD above the mean) were considered seropositive. H/O, history of reactions; TB, pulmonary tuberculosis; lepromatous and tuberculoid, include both polar and borderline leprosy types (15); MLS, sonicated *M. leprae*. Numbers in parentheses indicate the number of individuals tested.

Of the other peptides, peptide 7 bound to 74 to 87% of sera from leprosy patients or their FC. It showed no cross-reactivity with sera from tuberculosis patients and reacted with only 1 of 40 sera from healthy NC. Peptides 5, 6, 11, and 12 reacted with sera from pulmonary tuberculosis patients, indicating the presence of cross-reactive epitopes.

Antibody recognition of RGD motif in LSR. The relevance of antibody binding by leprosy sera to the RGD motif of LSR was explored by using synthetic peptides (Table 3). Peptide 3, which contained this motif, showed maximal binding particularly in reactional patients. Peptide 3-1, in which RGD was deleted, showed a significant reduction in binding ($P < 0.001$). Of further interest was the decrease in antibody binding when the position of this motif was altered. Thus, peptide 4, which had RGD near the middle of the sequence, showed a marked reduction, whereas peptide 5, with RGD near the amino terminus, showed a lesser reduction in binding to antibodies from the same leprosy patients. The modified peptide 5-1, which had an additional lysine and glycine at either end, showed a further loss in reactivity with the same sera. The percentage of seropositive individuals

reacting to these peptides reflected the general pattern observed with OD values.

These data taken together indicate that the major B cell epitopes recognized by sera from ENL patients lie between amino acids 11 and 24 of the LSR sequence and include IDLTNKNAA and the RGD motif.

Relationship of local hydrophilicity regions in LSR to antibody recognition. On the basis of algorithms (3, 5), five regions of local hydrophilicity were identifiable in the LSR protein (Fig. 1). Of these, the first region from the N terminus, i.e., aa 15 to 18, contains the core sequence common to peptides 2 and 3. The second region contains RGD. These areas correspond to the sequence mapped by ENL sera in ELISA. The fifth region (aa 63 to 73) is represented by peptide 13, which had also shown high seroreactivity in ENL patients. The third region, spanning aa 35 to 46 (peptides 6 and 7), appears to represent epitopes commonly recognized by sera from patients with all leprosy types but not by sera from tuberculosis patients. The fourth region, represented by peptide 11, was not discriminatory

TABLE 3. Seroreactivity of stable and reactional lepromatous leprosy patients to peptides with and without the RGD motif[a]

Peptide no.	Mean OD ± SD (% seropositive subjects)		
	ENL (n = 44)	Lepromatous (n = 173)	
		H/O (n = 48)	Stable (n = 125)
3	0.501 ± 0.338 (95.4)	0.181 ± 0.139 (91.6)	0.103 ± 0.236 (28)
3-1	0.023 ± 0.059 (13.6)	0.010 ± 0.032 (10.4)	0.001 ± 0.012 (1.6)
4	0.148 ± 0.166 (59)	0.091 ± 0.141 (47.9)	0.079 ± 0.159 (32.8)
5	0.325 ± 0.325 (81.8)	0.024 ± 0.074 (12.5)	0.143 ± 0.204 (48.8)
5-1	0.084 ± 0.034 (27.2)	0.013 ± 0.043 (14.5)	0.0562 ± 0.087 (21.6)

[a] Sera were diluted to 1:125. Peroxidase-conjugated rabbit anti-human total immunoglobulin was diluted to 1:1,000. H/O, history of reaction. $P < 0.001$ for peptide 3 versus peptide 3-1 for all three patient groups. (Freidman's test); $P < 0.001$ for peptide 3 versus peptide 18 for ENL and H/O patients.
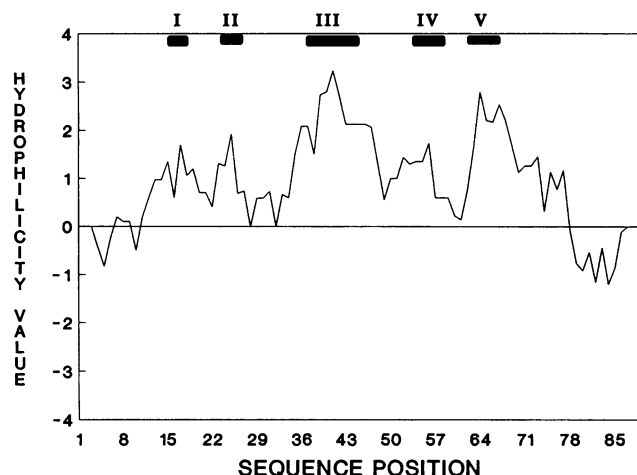
FIG. 1. Hydrophilicity graph of the 89-aa recombinant LSR antigen of *M. leprae*, showing five regions of local hydrophilicity (solid bars).

and showed variable antigenicity. Thus, the experimental data correlated with the hydrophilicity regions of LSR.

DISCUSSION

Sera from lepromatous leprosy patients during the active phase of type 2 reactions (ENL) show enhanced reactivity to the recombinant LSR antigen compared with sera from patients in the stable state. That this antigen may be differentially recognized by individuals prone to type 2 reactions is indicated by the increased seroreactivity of patients who were clinically stable at the time of the sample collection but had a record of previous or subsequent ENL. As no definitive tests are currently available, it was not possible to rule out subclinical episodes of ENL in these patients. That a proportion of lepromatous leprosy patients were reacting to cross-reactive epitopes in LSR was indicated by the fact that 63% of sera from tuberculosis patients also showed reactivity to the total antigen.

With a view to defining the fine specificity of the B cell epitopes recognized by sera from ENL patients, overlapping peptides spanning the LSR sequence were used as antigens. As expected, there was a general enhancement in the humoral responses of ENL patients to many of the peptides. Of interest was the recognition of overlapping peptides 2 (GVTYEIDLTNKNAA) and 3 (IDLTNKNAAKLRGD) by >95% of patients with active ENL. Whereas peptide 3 was recognized by sera from both ENL patients and clinically silent patients with a history of reactions, peptide 2, which shared a core sequence of IDLTNKNAA, selectively reacted with the sera from patients in the active phase of ENL. It would appear, therefore, that humoral responses during active ENL are directed at selective epitopes as compared with responses during the stable lepromatous state. The sequence spanned by these peptides correlated with local hydrophilicity regions in the algorithms generated for LSR. In addition, these peptides had β-turn potentials of $P > 1.5 \times 10^{-4}$ (Table 1).

Furthermore, peptide 3 had an RGD motif at the C terminus. This sequence is considered to be the basic unit of the cell recognition system for adhesion to extracellular

matrices such as fibronectin and laminin. Monocytic cells have fibronectin receptors that recognize the RGD sequence (16). The presence of this motif in LSR may indicate cell attachment sites in *M. leprae* which facilitate its entry into macrophages, wherein it resides and multiplies. ENL patients have antibodies which appear to recognize the RGD motif. Deletion of RGD significantly ($P < 0.001$) reduced the antigenicity of peptide 3. Positional alterations of this motif within the peptide also influenced seroreactivity. The ability of antibodies to bind to the peptides was lowered when RGD was present near the amino terminus or in the middle of the overlapping peptides. Additional residues such as lysine and glycine also hindered antibody binding. This conforms with reports in which neighboring amino acids have been shown to influence the conformation of peptides and subsequent binding by antibodies (7, 13, 20). Taken together, the data indicate that both the core sequence of 9 aa and the RGD motif may be the major targets for immune recognition by B cells in reactional leprosy patients.

It has been suggested that the majority of antibody-binding epitopes on native proteins have discontinuous sequences (1, 2). Recent studies on B cell epitope analysis of mycobacterial proteins have also indicated that sera from infected patients primarily bound to nonlinear epitopes, whereas murine monoclonal antibodies and hyperimmune sera bound to linear epitopes (9, 14, 18). It is interesting that polyclonal sera from ENL patients showed strong reactivity to continuous or linear sequences from 6 to 24 aa of the LSR. The emergence of antibodies to these epitopes during reactional states may reflect a "hyperimmune" state. That this hyperimmune state may also affect T cells is indicated by our previous studies, in which we observed the emergence of short-lived antigen-specific T cell responses in ENL patients (10). Such perturbations of the immune response during infection may be crucial for the development of ENL. Whether this is due to immunoregulatory disturbances or due to random uncovering of hitherto hidden or cryptic epitopes requires further investigation. Alternatively, it is possible that ENL patients have an inherent predisposition to developing B cell responses to "harmful" epitopes. Supporting this are follow-up studies which showed that 11 of the lepromatous leprosy patients with antibodies to peptide 3 and with no clinical evidence of reaction at the time of testing developed ENL 3 to 6 weeks later.

The humoral immune response to a living organism is the result of a complex array of cellular and molecular interactions. Mapping studies such as these may help in elucidating the antigenic determinants of importance in discriminating immune system events from pathologic events during natural infections with whole organisms. In conclusion, peptides 2 and 3 may be candidate markers for identifying a subgroup of lepromatous leprosy patients likely to develop debilitating ENL. Identification of such patients prior to institution of antileprosy treatment would help in better management of lepromatous leprosy.

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