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Critical Residues of the *Mycobacterium leprae* LSR Recombinant Protein Discriminate Clinical Activity in Erythema Nodosum Leprosum Reactions

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We reported earlier (S. Singh, N. P. Shanker Narayan, P. J. Jenner, G. Ramu, M. J. Colston, H. K. Prasad, and I. Nath, Infect. Immun. 62:86–90, 1994) that polyclonal antibodies directed against selective sequences in the *Mycobacterium leprae* recombinant protein designated LSR were present in lepromatous leprosy patients undergoing erythema nodosum leprosum (ENL) reactions (type 2 reactions). In this study using peptides with single-residue deletions from positions 6 to 24, we define three distinct regions, GVTY, NAA, and RGD, which were important for antibody recognition and for the discrimination of clinically silent and active ENL reactions. Antibodies against NAA were found only in patients undergoing active reactions. This is in contrast to the results for the RGD motif, which was recognized in all ENL patients, irrespective of the clinical status. Though GVTY was recognized in both groups of patients, its recognition was masked by the flanking glutamic acid. These findings point towards a specific molecular recognition pattern that emerges when a lepromatous leprosy patient undergoes immune perturbations leading to ENL reactions. Moreover, the fine specificity of immunological recognition changes during the natural evolution of the host-parasite interaction.

Lepromatous leprosy (LL), the disseminated multibacillary form of *Mycobacterium leprae* infection, is characterized by specific T-cell unresponsiveness and enhanced B-cell functions. Some patients with LL also suffer from acute, episodic, reactional states called erythema nodosum leprosum (ENL) reactions (type 2 reactions), which are characterized by erythematous nodules and systemic manifestations of arthritis and pyrexia (5, 15). The mechanisms underlying these reactions have been ascribed variously to polyclonal B-cell activation and enhanced T-cell reactivity (6, 8). In an attempt to unravel the target of such immune perturbation, we mapped the B-cell epitopes of the arginine-rich immunodominant recombinant fusion protein of *M. leprae* which we had earlier cloned, sequenced, and described as LSR (7). Subsequently, the full gene was found by Sela et al. to code for a 15-kDa protein (12). We found that patients who were actively undergoing an ENL reaction had antibodies which recognized two overlapping peptides, peptide 2, GVTYEIDLTNKNAA (positions 6 to 19), and peptide 3, IDLTNKNAAKLRGD (positions 11 to 24), whereas patients who were clinically silent at the time of sampling had antibodies which recognized only the latter sequence. We drew attention to the importance of the RGD motif as a predictor of ENL. In the present study, we provide evidence for two other B-cell epitopes in peptide 2 which are the major targets of the antibody response during clinical activity.

The sera tested were from the individuals previously reported (13). The groups consisted of 44 active reactional patients (ENL patients) bled prior to the institution of steroid therapy, 48 lepromatous patients who had a prior history of ENL reactions (H/O patients) but showed no clinical activity at the time of testing, 125 stable lepromatous patients from areas of endemicity, and 40 healthy volunteers from regions where leprosy is not endemic (NC group). The patients were diagnosed on the basis of Ridley and Jopling classification (10). Sera reached the laboratory within 24 h of bleeding and were stored at −20°C.

Sixteen synthetic peptides with single-amino-acid deletions at either or both termini of the sequence GVTYEIDLT NKNAAKLRGD, spanning both peptide 2 and peptide 3 of LSR (peptide 2/3, corresponding to amino acids 6 to 24 [Fig. 1]), were synthesized by a solid-phase methodology (4). An indirect enzyme-linked immunosorbent assay (ELISA) was performed as described earlier (13) with peptides at a concentration of 200 ng per well and a 1:125 dilution of sera. Peroxidase-conjugated rabbit anti-human total immunoglobulin was used as the second antibody, the color was developed with *o*-phenylenediamine, and the optical density (OD) was read at 492 nm. The serum sample was considered positive when the OD in an ELISA exceeded 3 standard deviations over the mean value obtained with NC sera. A fluid-phase competitive ELISA was performed as described above after prior incubation of sera with various concentrations of the competing RGD peptide at 37°C for 2 h.

Antibodies against peptide 2/3, which spanned both peptides 2 and 3 (positions 6 to 24), were observed in >86% of reactional patients, irrespective of disease activity (Fig. 1), and sera of the ENL and H/O groups showed significant elevation in OD levels in comparison with sera of stable LL patients ($P < 0.001$) (Table 1). The seropositivity to this peptide was marginally lower than that observed with peptide 2 and peptide 3 used alone (Fig. 1; Table 1).

With peptide 2/3 as the central sequence, peptides incorporating deletions on either the NH_2 or COOH terminus or both

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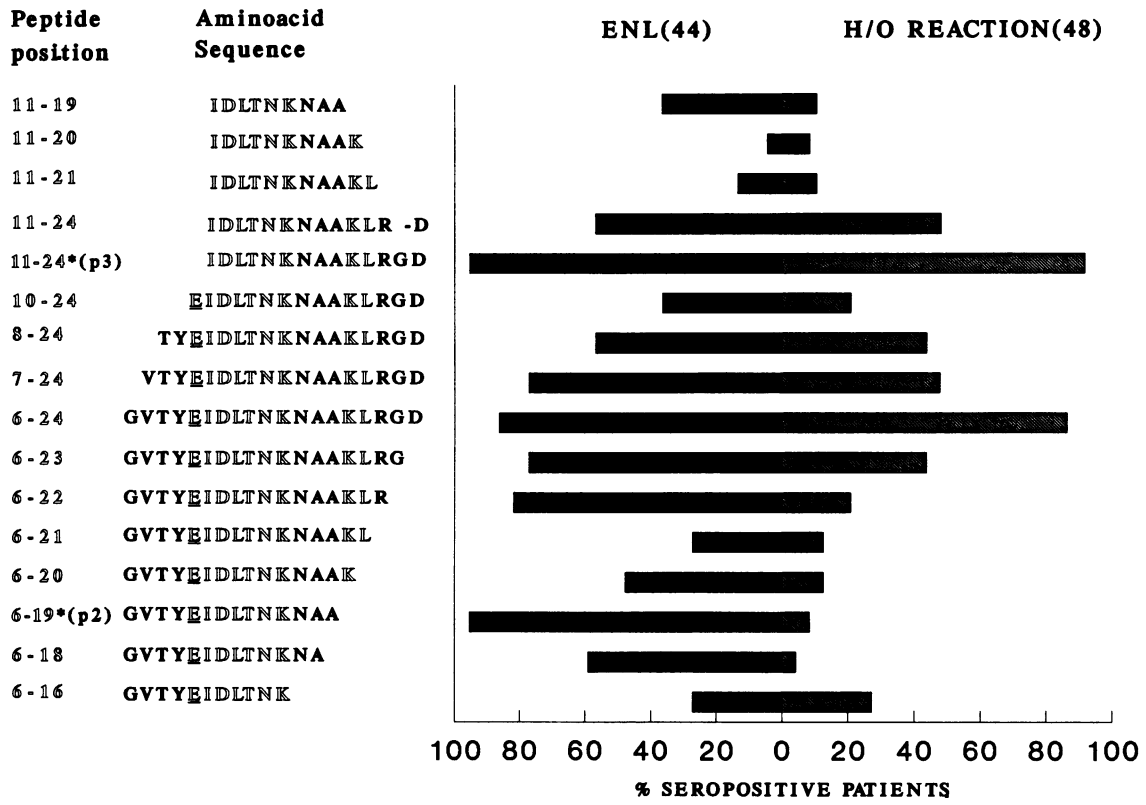

FIG. 1. Percentage of reactional leprosy patients showing seroreactivity to peptides spanning residues 6 to 24 of LSR. Sera from ENL patients (■) and LL H/O patients (▨) were screened at a dilution of 1:125. The asterisks indicate the peptides designated peptide 2 and peptide 3 by Singh et al. (13). Critical and masking residues are boldfaced and underlined, respectively. Numbers in parentheses represent the number of patients screened.

termini were used to identify the critical residues in immune recognition during ENL reactions.

Deletion of residues (G, V, T, and Y) at the NH_2 terminus led to a loss of seroreactivity in both active (ENL) and inactive (H/O) groups of patients. On deletion of the flanking residue E (at which point the peptide 3 sequence appears), the reactivity not only was recovered but also reached maximal levels both in terms of OD levels (Table 1) and percentage of reactors (Fig. 1), suggesting that glutamic acid had a negative effect on binding. Thus, though GVTY was present in peptide 2, its effect was masked by E and it was not recognized by antibodies of inactive patients until E was deleted.

Sequential deletion of the amino acids in the motif NAA also led to sequential and selective loss of seroreactivity in patients with active ENL (Fig. 1). Since peptide 2 contains this sequence and is recognized only by antibodies of patients undergoing reactions, NAA would appear to be the critical site targeted by the antibody response during clinical activity. Interestingly, algorithms predict significant β-turn potential ($P > 1.5 \times 10^4$) in this region (NKNA) (1, 3, 9).

Deletions at the COOH end of peptide 2/3 further confirmed the importance of the RGD motif for antibody recognition (13) in type 2 leprosy reactions (Fig. 1; Table 1), with both inactive and active reactional patients showing a loss of seroreactivity with peptides lacking RGD. The importance of RGD was further strengthened by competitive inhibition assays, in which preadsorption with RGD significantly removed antibody activity against peptides 3 and 2/3 but not peptide 2 (Fig. 2). Interestingly, there were differences in the pattern of antibody reactivity between the two groups of reactional patients. Deletion of each residue (R, G, and D) from the COOH end led to a sequential loss of reactivity in the inactive reactional patients. In contrast, active ENL sera showed an

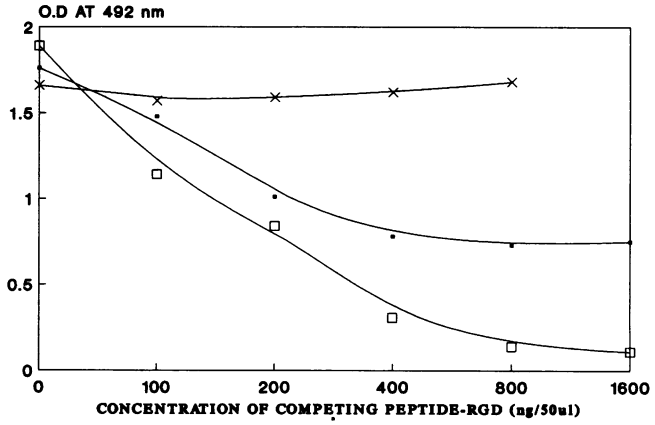

FIG. 2. Competitive ELISA with pooled sera from five type 2 (ENL) reactional leprosy patients. Sera were preadsorbed with RGD and reacted with p2 (GVTYEIDLTNKNAA) (×), p3 (IDLT NKNAAKLRGD) (□), and p2/3 (GVTYEIDLTNKNAAKLRGD) (•) in an ELISA. Inhibition was observed only with peptides containing the RGD sequence (p3 and p2/3).

TABLE 1. Seroreactivities of multibacillary leprosy patients showing selective recognition of GVTYE, NAA, and RGD motifs in the position 6 to 24 region of LSR2 recombinant protein during reactional states only

Peptide positions	Amino acid sequence[a]	Mean OD ± SD for group[b]:		
		ENL (44)	LL H/O type 2 (48)	Stable LL (125)
11–19	-----IDLTNK**NAA**-----	0.10 ± 0.01	0.06 ± 0.07	0.05 ± 0.01
11–20	-----IDLTNK**NAA**K----	<0.01 ± 0.01	0.01 ± 0.03	<0.01 ± 0.05
11–21	-----IDLTNK**NAA**KL---	0.02 ± 0.06	0.01 ± 0.03	<0.01 ± 0.01
11–24	-----IDLTNK**NAA**KL**R-D**	0.34 ± 0.17	0.14 ± 0.16	0.04 ± 0.06
11–24 (p3)[c]	-----IDLTNK**NAA**KL**RGD**	0.50 ± 0.34	0.18 ± 0.14	0.10 ± 0.23
10–24	----EIDLTNK**NAA**KL**RGD**	0.21 ± 0.12	0.06 ± 0.10	0.03 ± 0.01
8–24	--**TYE**IDLTNK**NAA**KL**RGD**	0.29 ± 0.18	0.13 ± 0.12	0.06 ± 0.14
7–24	-**VTYE**IDLTNK**NAA**KL**RGD**	0.35 ± 0.21	0.14 ± 0.11	0.06 ± 0.09
6–24 (p2/3)	**GVTYE**IDLTNK**NAA**KL**RGD**	0.41 ± 0.27	0.35 ± 0.18	<0.01 ± 0.07
6–23	**GVTYE**IDLTNK**NAA**KL**RG**-	0.31 ± 0.19	0.20 ± 0.11	0.03 ± 0.01
6–22	**GVTYE**IDLTNK**NAA**KL**R**--	0.39 ± 0.25	0.10 ± 0.15	0.02 ± 0.05
6–21	**GVTYE**IDLTNK**NAA**KL---	0.19 ± 0.10	0.06 ± 0.04	0.01 ± 0.02
6–20	**GVTYE**IDLTNK**NAA**K----	0.21 ± 0.09	0.03 ± 0.02	0.01 ± 0.02
6–19 (p2)[d]	**GVTYE**IDLTNK**NAA**-----	0.34 ± 0.20	0.03 ± 0.02	0.04 ± 0.02
6–18	**GVTYE**IDLTNK**NA**------	0.27 ± 0.15	0.01 ± 0.02	0.01 ± 0.01
6–16	**GVTYE**IDLTNK--------	0.09 ± 0.03	0.05 ± 0.04	<0.01 ± 0.01

[a] Critical and masking residues are boldfaced and underlined, respectively.
[b] Results are the mean OD ± standard deviation (SD) obtained in an ELISA, with a 1:125 dilution of NC sera having a value of 0.047 ± 0.016. Sera showing an OD of >0.1 (3 SDs above the mean) were considered positive. Numbers in parentheses represent the number of patients tested. Statistical analysis for the test of significance between responses to peptides within a group was determined by nonparametric analysis (Kruskal-Wallis test [14]).
[c] Peptide 3 as reported by Singh et al. (13).
[d] Peptide 2 as reported by Singh et al. (13).

abrupt drop in reactivity only after the deletion of all three residues (Fig. 1 and Table 1), indicating that the humoral immune response during clinical activity was discriminatory for conformational differences in this part of the antigen.

When used alone, RGD showed poor reactivity with active (11%) and inactive (8%) ENL sera, which may be related to its small size leading to steric hindrance when absorbed on plastic plates (2).

In our earlier study, by a method of exclusion we had concluded that the core sequence of IDLTNKNAA was common to the peptides recognized by antibodies of both active and inactive reactional patients (13). When this peptide was formally tested by ELISA, the reactivity was found to be low (Fig. 1), thereby confirming that flanking sequences at the NH_2 and COOH ends were important for antibody recognition.

Molecular recognition of living organisms may vary during the course of the natural immune response. In this study, we show that even over a small stretch of 19 amino acids, multiple sites are involved in antibody reactivity, and that patients with different immunopathological manifestations of disease have different fine specificities. Some sites (NAA) seem important for evaluating activity, while others, such as RGD, seem important for predicting a propensity to develop ENL. This motif is considered the basic recognition unit for adhesion to fibronectin receptors (11). When these results are considered in the light of the many potential sites of immunological recognition in the total organism, of which the 89-amino-acid LSR forms only a part, one would predict considerably more immunoreactivity. It is therefore interesting that over 90% of reactional patients have antibodies predominantly recognizing these sites. Thus, selectivity seen in the natural human immune response to intracellular pathogens may provide leads to the molecular mechanisms which underlie the host-pathogen interaction leading to silent or active disease and could be used to predict morbidity. Relapses in leprosy can be confused with mild or silent reactional states, and a diagnostic marker for the latter would have clinical utility in determining the mode of treatment.

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