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Characterization of Murine B-Cell Epitopes on the *Mycobacterium leprae* Proline-Rich Antigen by Use of Synthetic Peptides

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Using synthetic peptides representing overlapping sequences of the 100-amino-acid-long N-terminal region of the proline-rich antigen of *Mycobacterium leprae* (PRA), we have mapped the epitopes in the primary structure of PRA recognized by four monoclonal antibodies. The *M. leprae*-specific monoclonal antibody F47-9 recognized the amino acid sequence LGSAYP (residues 34 to 39). Both monoclonal antibodies F67-1 and F67-5 recognized the sequence YPPP within the repeated sequence of PRA at four sites (residues 38 to 41, 50 to 53, 60 to 63, and 70 to 73). Monoclonal antibody F126-5 recognized the sequence SYPPP, also within the repeat, at three sites (residues 49 to 53, 59 to 63, and 69 to 73). All three epitopes appeared to be linear as far as can be determined by this approach.

Characterization of the protein antigens of *Mycobacterium leprae* would make it possible to identify the functional epitopes involved in protective and suppressive immune responses and is a prerequisite for the development of vaccines and immunodiagnostic assays. Previously, we reported the identification of a 36,000-molecular-weight protein antigen (referred to as proline-rich antigen [PRA]) recognized by both patients sera (9) and species-specific (4, 8) and cross-reactive (12) murine monoclonal antibodies.

The DNA sequence of the gene was shown to code for a polypeptide of 249 amino acids with a predicted molecular mass of 26, 299 Da (12). The deduced amino acid sequence revealed a proline-rich amino-terminal region (amino acids 19 to 95) containing a number of repeated sequences similar or identical to the sequence PGGSYPPPPP, in which the binding sites of all PRA-reactive monoclonal antibodies could be localized. Here, we report the exact mapping of murine B-cell epitopes, using synthetic peptides representing overlapping sequences of the 100-amino-acid-long N-terminal region of PRA.

Monoclonal antibodies to PRA were produced as described previously (8, 12). Monoclonal antibody F47-9 recognizes an *M. leprae*-specific determinant, whereas F67-1, F67-5, and F126-5 recognize broadly cross-reactive determinants on PRA (4, 8, 12).

Overlapping decapeptides were synthesized and tested with the Epitope Scanning Kit (Cambridge Research Biochemicals, Cambridge, United Kingdom), which is a modification of the technique of multiple peptide synthesis on polyethylene rods, originally described by Geysen et al. (5). Peptides were synthesized according to the sequence of the 100 N-terminal amino acids of PRA (12). The peptides still coupled to their solid-phase supports were tested against the monoclonal antibodies in an ELISA (enzyme-linked immunosorbent assay). Absorbances were plotted at the position in the PRA sequence of the N-terminal amino acid of the peptides.

Conventional peptides were synthesized on a SAM2 synthesizer (Biosearch) by E. Freund, Hubrecht Laboratory, Utrecht, The Netherlands, using a solid-phase methodology (11). Four peptides were synthesized: P8905—PSELGSAY PP, corresponding to the sequence of PRA between positions 31 and 40; P9021—LGSAYPC, corresponding to positions 34 to 39 of the sequence of PRA, with an extra C-terminal cysteine residue attached; P8906—GGSYPPPPP PC, corresponding to positions 47 to 56 (repeated at positions 57 to 66), with an extra cysteine; P9019—GGSYPPP PPPGGSYPPPPPP, corresponding to positions 47 to 66.

Thirty-nine sera from leprosy patients throughout the spectrum were kindly provided by R. V. Cellona, Leonard Wood Memorial Center for Leprosy Research, Cebu, The Philippines.

Inhibition assays were performed in an ELISA, using peroxidase-labeled antibodies, essentially as described previously (3, 8, 12). Synthetic peptides (0.1 M) or *M. leprae* sonic extract (1.5 μg/ml) was coated onto polystyrene microtiter plates by adding to each well 100 μl of antigen in carbonate buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, pH 9.6) and incubating for 18 h at 37°C. Plates were washed with phosphate-buffered saline containing 0.1% (vol/vol) Tween 20. Peroxidase-labeled monoclonal antibody and unlabeled monoclonal antibody (various amounts of purified immunoglobulin), synthetic peptides (various concentrations), or patient sera diluted 1:5 in phosphate-buffered saline containing 0.1% (vol/vol) Tween 20 and 2% (wt/vol) bovine serum albumin were added simultaneously to the coated plates. Plates were then incubated for 3 h at 37°C. After washing, wells were incubated with 100 μl of tetramethylbenzidine-substrate solution (12 mg of 3,3′, 5,5′-tetramethylbenzidine in 5 ml of ethanol added to 15 ml of 0.1 M citrate–phosphate buffer, pH 5.0; H_2O_2 added to a final concentration of 0.015%). The reactions were stopped after 15 min by adding 50 μl of 2 M H_2SO_4, and the A_{450} was measured.

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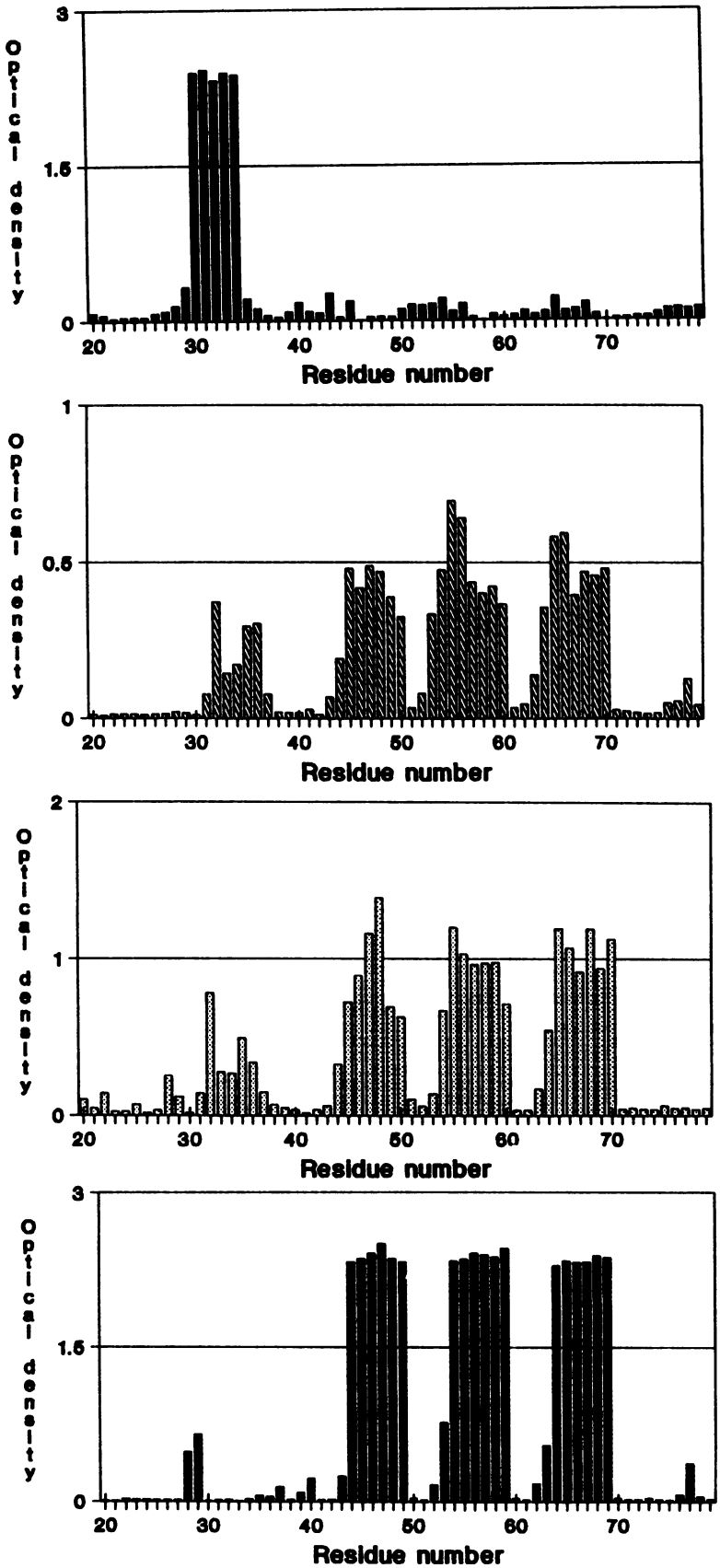

FIG. 1. Peptide scans of the N-terminal region of PRA with (top to bottom) monoclonal antibodies F47-9 (■), F67-1 (▨), F67-5 (▩), and F126-5 (▨), shown here for residues 20 to 79. Peptides in this scan are 10 amino acids long. Absorbances obtained with ELISA are plotted at the position in the PRA sequence of the N-terminal residue of the peptides.

TABLE 1. Cross-inhibition of reactivity of monoclonal antibodies with peptide P8906

Peroxidase-labeled monoclonal antibody	Amt (μg) of unlabeled antibody giving 50% inhibition[a]			
	F47-9	F67-1	F67-5	F126-5
F47-9	NA[b]	NA	NA	NA
F67-1	>500	25	50	<4
F67-5	>500	15	75	100
F126-5	>500	>500	>500	10

[a] Binding of peroxidase-labeled antibodies F47-9 (1/125), F67-1 (1/500), F67-5 (1/3,750), and F126-5 (1/20,000) to 0.1 M peptide P8906 (GGSYPPPPPPC), giving an A_{450} of 1.0.
[b] NA, Not applicable; F47-9 did not give binding to peptide P8906.

The reactivity of human sera to the synthetic peptides was determined in an ELISA, essentially as described previously (3). Peptides were coated onto polystyrene microtiter plates at a concentration of 0.1 mM (100 μl per well) in carbonate buffer and incubated for 18 h at 37°C. Antibody binding was visualized by using peroxidase-labeled goat anti-human polyvalent immunoglobulin, followed by substrate as described above.

In the peptide scan of overlapping decapeptides, monoclonal antibody F47-9 reacted with peptides 30 to 34, covering the 30- to 43-amino-acid sequence of PRA (Fig. 1). The epitope recognized by F47-9 could be deduced from the common sequence within these peptides: LGSAYP (residues 34 to 39 of PRA). Monoclonal antibodies F67-1 and F67-5 are probably identical, since they gave an identical reaction pattern in the peptide scan (Fig. 1), i.e., with peptides 32 to 36, 44 to 50, 54 to 60, and 64 to 70. Both monoclonal antibodies recognized the epitope YPPP, which is located within the repeated sequence of PRA at positions 38 to 41, 50 to 53, 60 to 63, and 70 to 73. Monoclonal antibody F126-5 reacted with peptides 44 to 49, 54 to 59, and 64 to 69 (Fig. 1). The deduced epitope SYPPP, is located at positions 49 to 53, 59 to 63, and 69 to 73 within the repeated sequences of PRA. Thus, F67-1/F67-5 and F126-5 recognize overlapping epitopes.

To characterize the overlapping epitopes further, cross-inhibition of the binding of the monoclonal antibodies to synthetic peptide P8906 (GGSYPPPPPPC), containing the overlapping epitopes, was assessed. Monoclonal antibody F126-5 was able to inhibit the binding of peroxidase-labeled monoclonal antibodies F67-1 and F67-5 (Table 1). Although monoclonal antibodies F67-1 and F67-5 were able to inhibit the binding of each other to P8906, they could not inhibit the binding of F126-5. Functioning as a control, monoclonal antibody F47-9 did not inhibit the binding of any of the other monoclonal antibodies to the peptide (Table 1). Similar results were obtained with microtiter plates coated with *Escherichia coli* lysate containing cro-β-galactosidase-PRA fusion protein (12), when the epitopes are presumed to be present in their natural conformational state. One possible explanation could be that F126-5 has a much higher affinity for its epitope than the other monoclonal antibodies. Binding of F126-5 to the three copies of its epitope could prevent the subsequent binding of F67-1 or F67-5 to their additional epitope through steric hindrance.

To evaluate the relationship between the epitopes represented by synthetic peptides and, in their native configuration, by *M. leprae*, we determined the inhibition of monoclonal antibody binding to *M. leprae* by synthetic peptides. Peptides P8906 (GGSYPPPPPPC) and P9019 (GGSYPPPP PPGGSYPPPPPP) equally inhibited the binding of monoclonal antibody F126-5 to *M. leprae*, giving an A_{450} of 1.0 (in both cases 4 μM peptide was required to give 50% inhibition). However, the amount of peptide P9021 (LGSAYPC) (0.6 mM) required to inhibit 50% of the binding of monoclonal antibody F47-9 to *M. leprae* was approximately 100-fold greater than that of peptide P8905 (PSELGSAYPP) (4 μM). Neighboring residues may alter the antigenic conformation of a peptide (7, 14), so the additional residues present in peptide P8905 may have contributed to a conformation which more closely resembles the conformation of the epitope in PRA in its natural state. This may be equally true for peptides 30 to 34 in the peptide scan, in which the epitope LGSAYP was embedded in decapeptides. However, we cannot exclude the possibility that the extra cysteine residue in peptide P9021 prevented the epitope from taking its natural conformation. It is known that monoclonal antibody F47-9 reacts with a β-galactosidase fusion protein encoded by recombinant phage Y3180 (12). Although this fusion protein is not related to PRA, both proteins share four of six amino acids (SAYP) of the epitope of F47-9. In this case also, the additional residues present in the Y3180-derived protein, including the C-terminal β-galactosidase residues (12), may have contributed to a conformational structure recognizable by F47-9. These findings together suggest that amino acid residues SAYP of the epitope of monoclonal antibody F47-9 are essential for binding.

Previously, we have described an inhibition ELISA that uses monoclonal antibody F47-9 (8), which was presumed to detect human antibodies to the species-specific epitope of PRA. The relationship between the ability of sera of leprosy patients to inhibit binding of anti-PRA monoclonal antibodies to *M. leprae* and their binding to peptides containing the epitopes is being studied. Binding of sera from 39 untreated leprosy patients to the synthetic peptides containing the epitopes for monoclonal antibodies F47-9 and F126-5 and inhibition of binding of these monoclonal antibodies to *M. leprae* sonic extract by these sera were determined in an indirect ELISA and an inhibition ELISA, respectively. Preliminary results from these experiments revealed a poor correlation between the inhibition of binding of F126-5 to *M. leprae* by these sera and the binding of the sera to peptides P8906 and P9019 (Pearson correlation coefficients: $r = 0.389$, $P < 0.05$; and $r = 0.473$, $P < 0.01$, respectively). No significant relationship between the ability of the patient sera to inhibit binding of monoclonal antibody F47-9 to *M. leprae* and the binding of these sera to peptides P8905 and P9021 was found (Pearson correlation coefficients: $r = 0.148$, $P > 0.05$; and $r = 0.258$, $P > 0.05$, respectively). One possible explanation may be that most sera recognize epitopes that are discontinuous and, thus, effective binding of antibodies in the sera requires additional residues not included in the synthetic peptides. Alternatively, binding of human antibodies to epitopes in the vicinity of the F47-9 or F126-5 epitope could cause steric hindrance of monoclonal antibody binding. A similar discrepancy between binding of human sera to a peptide representing an epitope and inhibition of monoclonal antibody binding has been reported for the 65K antigen (10, 13).

Since all four anti-PRA monoclonal antibodies reacted with linear sequences in the peptide scan, it could be inferred that the respective epitopes are sequential. This would be in agreement with the finding that PRA could be identified by all monoclonal antibodies after Western blotting (immunoblotting) of the denatured protein. However, the minimal length of sequential epitopes has generally been assumed to

be at least six amino acids. Furthermore, it has been suggested that a majority of epitopes on native proteins are discontinuous (1, 2). Since the peptide scan methodology only reveals minimal linear amino acid sequences recognized by monoclonal antibodies, it cannot be excluded that the linear epitopes on PRA reported here are part of assembled epitopes.

The antibody response to an antigen which follows uptake, processing, and presentation of that antigen is determined by the genetic makeup and environmental history of the host. The antibody responses to PRA in inbred mice immunized with sonicated mycobacteria are likely to be quite different from those in humans with clinical leprosy (6). Further study on the epitopes on PRA recognized by human lymphocytes during the phases of infection with *M. leprae* might provide a basis for development of diagnostics.

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