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Solid phase peptide synthesis of epitopes that react with monoclonal antibodies to the 65,000 dalton protein of *Mycobacterium leprae*

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Introduction

The gene coding for the 65,000 dalton protein of *M. leprae* has recently been sequenced (1). Subclones of this gene which expressed a peptide that reacted with monoclonal antibodies (MOAbs) IIC8 or IIIC8 (2,3) were compared to subclones that expressed nonreactive peptides. From these comparisons it was predicted that the epitopes recognized by MOAb IIC8 and IIIC8 would be contained within separate domains of 12 and 13 amino acids respectively. The capabilities of solid phase peptide synthesis (4,5), available within the Immunology Research Laboratory at the Pacific Medical Center at the University of Washington, made it possible to synthesize these peptides and variants thereof to test the accuracy and specificity of the predictions made by DNA sequencing. In addition, as part of a collaborative study of all known monoclonal antibodies to the 65,000 dalton protein, MOAb F67-2 was found to be closely related but different from MOAb IIC8 (6). The IIC8 peptide and its variants were therefore also tested against the F67-2 MOAb. this article reports the peptides synthesized and the results of tests of their antigenicity.

Materials and methods

Using the predicted sequence for the epitopes recognized by MOAbs IIC8 and IIIC8 (1), peptides were synthesized using a BioSearch SAM II automated peptide synthesizer. The SAM II uses carbodiimide coupling of t-boc-N-protected L-amino acids. The peptides are bound either to a capped 4-methylbenzhydrylamine resin (peptide amides) or to the standard Merrifield resin (peptide acids). The alpha amino groups are protected by tertbutyloxycarbonyl (t-Boc) moieties, and most reactive side chain functional groups of the amino acids used in the synthesis are protected by benzyl-derived side-chain blocking groups developed by Merrifield and coworkers and cleavable by HF at the same time as the fully synthesized peptide is cleaved from the resin support. A standard synthetic cycle consists of a deblocking step (45 % trifluoroacetic acid, 2.5 % anisole in methylene chloride) to remove the t-Boc group followed by a base wash (10 % distilled diisopropyl ethylamine in methylene chloride) to remove and neutralize the TFA used for deprotection, followed by coupling for 2 hours of the next t-Boc protected amino acid to the peptide with the activator diisopropylcarbodiimide (DIPCDI), followed by capping by N-acetylation (0.3M l-acetylimidazole in dimethylforma-

mide) to block any unreacted alpha amino groups to prevent growth of failure sequences and give a product that is easier to purify. Numerous washing steps are inserted between the major reaction steps to insure that excess reagent from each of these steps is removed before the next reagent is introduced. Synthesis involves single- or double-couple routines, depending upon the sequence (e.g. I,V,N,T and Q are routinely double-coupled using 0.4M t-boc amino acids, as are polar stretches of amino acids, the first amino acid added to the resin, etc.). After deprotection, peptides are cleaved from the resin in an HF cleavage apparatus (Peninsula Labs) using the Tam-Merrifield low-high HF cleavage protocol (7) for peptides containing M,C,Y,W or D, or the standard high HF procedure (90 % anhydrous HF, 10 % anisole v/v, 0°C, 60 min) for all other peptides. After evacuation of the HF and drying overnight, the peptides are extracted from the resin with 10-20 % acetic acid and lyophilized. Of 16 separate peptides of >20 amino acids in length that were synthesized each taking an average of 6-7 days, the yields ranged from 107-620 mg of crude peptide obtained from 0.3 mmol of derivatized resin (median 316 mg). Multiple peptides were derived from a single synthesis by stopping the program at the desired point, deblocking the N-terminal t-boc-amino acid of the peptide chain, removing some resin (which was cleaved separately from the rest of the resin), and proceeding with the synthesis using the remaining resin.

A portion of the crude peptides were analyzed by reversed-phase HPLC over a C-4 (Synchropak) column. The peptide containing peaks were hydrolyzed at 110°C for 24 hours in 6N HCl + 10mM phenol + 0.5 % mercaptoethanol. The hydrolyzed peaks were then reacted with phenylisothiocyanate to give PTC-amino acids according to Heinrikson and Meredith (8). The PTC-amino acids were identified using a room-temperature acetonitrile elution protocol developed in the Immunology Research Lab which employs a 4.5x250mm IBM C-18 column.

The methionines in the synthesized peptides were reduced (except as noted in Table II) as a result of the use of the Tam-Merrifield low-high HF cleavage protocol. Methionines were oxidized (as noted) to the sulfoxide form using 0.3M hydrogen peroxide in 20mM phosphate buffer, pH 2.1, at 30°C for 70 minutes. The reaction was terminated by adjusting the pH to 6.2 with NaOH and adding 10 micrograms of catalase (9).

Solid phase ELISA immunoassays were performed as previously described (10 - 12) employing a sonicate antigen preparation of whole *M. leprae* or purified peptides bound to polystyrene plates and inhibiting binding of the MOAb to these antigen coated plates with the peptides being evaluated.

Results

Table 1 illustrates the 12mer peptide synthesized based upon the predicted sequence for the epitope recognized by IIC8 MOAb, and eleven variants of this peptide based upon the 12mer sequence. The antigenic reactivity of the 12mer and the 11 peptide variants with IIC8 and F67-2 MOAbs was quantitated by immunoassay. The 12mer peptide EYEDLLKAGVAD was highly antigenic and only 2 nanograms of this peptide were required to produce 40 % inhibition of IIC8 MOAb binding. Six peptide variants inhibited the IIC8 MOAb binding (Figure 1), and eight peptide variants inhibited F67-2 binding (Figure 2). Shortening the sequence from the amino terminal side of the peptide produced approximately five fold less antigenicity for the 11mer, 100 fold less antigenicity for the 10 mer and total loss of antigenicity for the 9mer (DLLKAGVAD, Table 1, Figure 1). These same peptides when reacted with F67-2 showed 3-fold less antigenicity with the 11mer, 50-fold less antigenicity with the 10mer and 300-fold less antigenicity with the 9mer (Table 1, Figure 2). Variants of the 11mer which were shortened at the C-terminus (YEDLLKAGVA AND YEDLLKAGV) had approximately 10-fold less antigenicity for IIC8 and 5-fold less antigenicity for F67-2 and

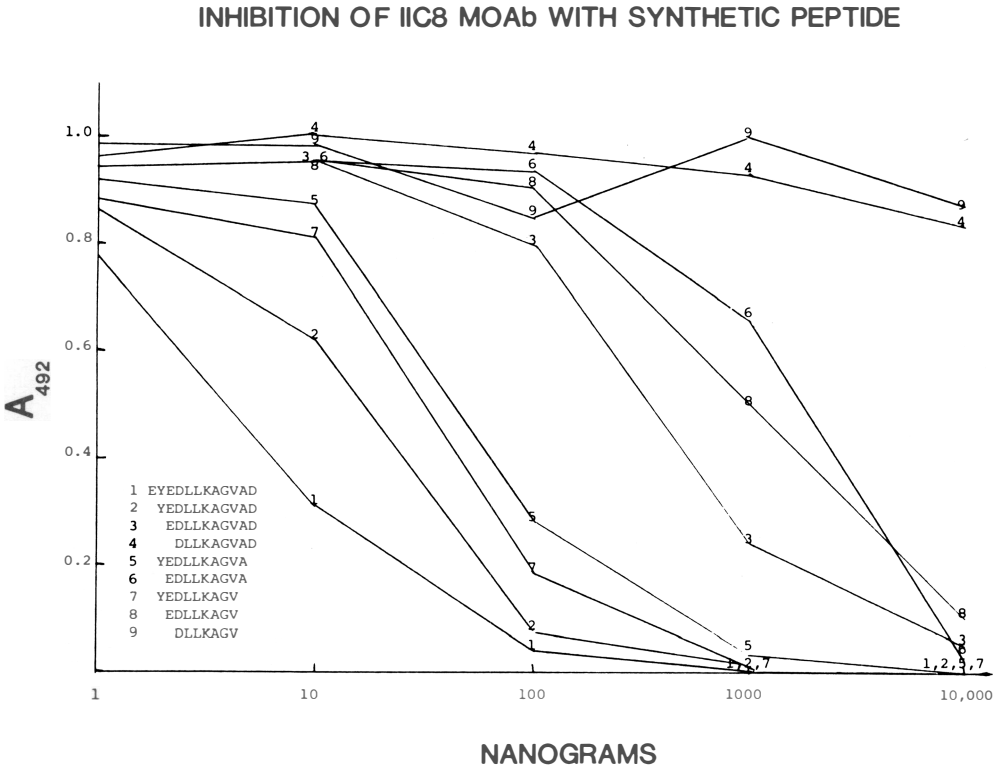

Figure 1.
Inhibition of IIC8 MOAb with nine different synthetic peptides. E = glutamic acid, Y = tyrosine, D = aspartic acid, L = leucine, K = lysine, A = alanine, G = glycine, V = valine.

variants of the 10 mer vith a shortened C-terminus (EDLLDAGVA and EDLLKAGV) showed 425 fold (EDLLKAGVA) and 255 fold (EDLLKAGV) less antigenicity for IIC8 and 100 fold (EDLLKAGVA) and 50 fold (EDLLKAGV) less antigenicity for F67-2. The 9mer variant with the sequence DLLKAGV required 233 times more protein than the predicted 12mer to inhibit the F67-2 immunoassay by 40 % (Figure 2) but this same peptide variant produced no inhibition of the IIC8 immunoassay even in amounts more than 5000 times in excess of the weight of predicted 12mer capable of producing 40 % inhibition of the IIC8 immunoassay (Table 1, Figure 1). Thus the minumum sequence at the amino terminus of the peptide appears to require glutamic acid (E) for the IIC8 MOAb but not for the F67-2 MOAb, and the tyrosine (Y) at the amino terminus is also very contributory to antigenicity for each MOAb. For the IIC8 MOAb loss of the tyrosine resulted in 23fold, 21fold, and 23fold decreases in antigenicity for the peptides YED..VAD converting to ED..VAD, YED..VA converting to ED..VA, and YED..V converting to ED..V respectively. For the F67-2 MOAb these same peptide variants resulted in 16fold, 20fold, and 10fold reductions in antigenicity respectively with loss of the tyrosine. These results are consistent with our earlier observation that radiolabelling with ^{125}I of the tyrosine on the 12mer results in complete loss of antigenicity. Loss of the glutamic acid in the peptides ED..VAD and ED..V caused 6-fold and 5-fold reductions in antigenicity respectively for the F67-2 MOAb and mo-

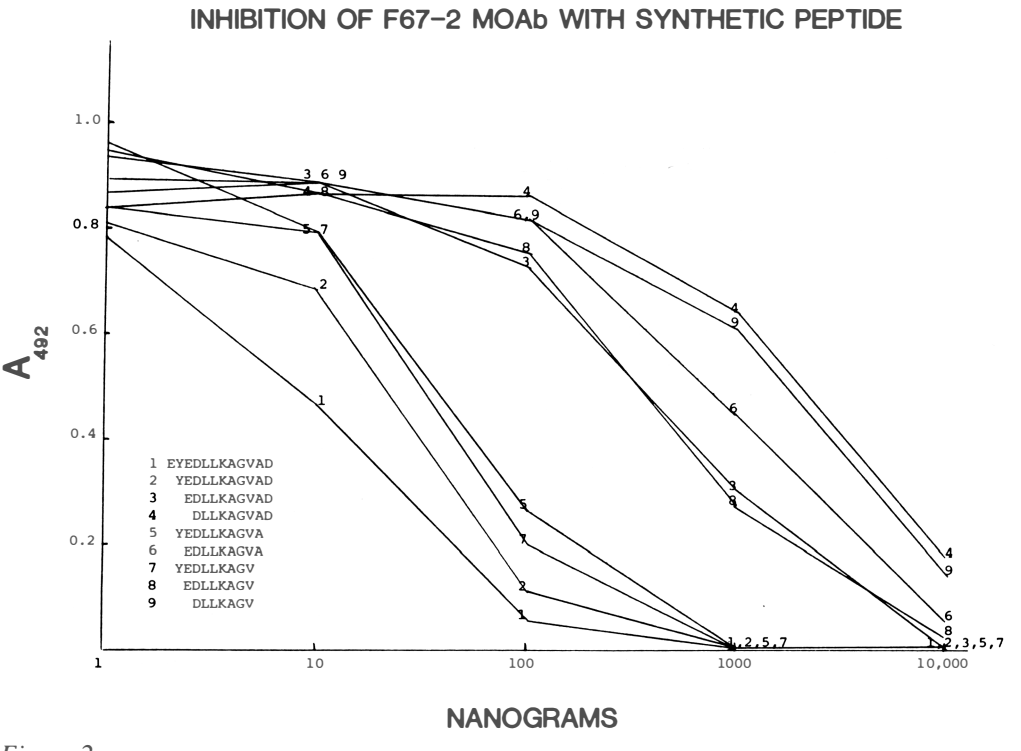

Figure 2.
Inhibition of F67-2 MOAb with nine different synthetic peptides. E = glutamic acid, Y = tyrosine, D = aspartic acid, L = leucine, K = lysine, A = alanine, G = glycine, V = valine.

re than 50-fold and 20-fold reductions respectively in the antigenicity for the IIC8 MOAb. Further peptides are required to define the minimum amino terminus for antigenicity for the F67-2 MOAb and to define the minimum C-terminal amino acid for antigenicity of both the IIC8 and F67-2 MOAbs. With the peptides shown in Table 1, antigenicity can be demonstrated for the IIC8 MOAb with an 8mer with the sequence EDLLKAGV and for the F67-2 MOAb with a 7mer with the sequence DLLKAGV.

Table 2 illustrates the twelve peptides synthesized based upon the predicted sequence of the epitope recognized by MOAb IIIC8, and the antigenicity of each peptide as assessed by immunoassay.

A peptide of 11 amino acids in length with the structure DPTGGMGGMDF demonstrated maximal antigenicity. Substitution to an amide form of the C-terminal phenylalanine was of almost equivalent antigenicity to the acid form which is present in the native molecule since this is the carboxy terminus of the molecule, and the 10mer without the C-terminal phenylalanine (DPTGGMGGMD) was approximately 50-fold less antigenic than peptides containing this amino acid (DPTGGMGGMDF, Table 2). All antigenicity was lost with removal of the amino-terminal aspartate (D). A striking finding was that oxidation of the methionines to the sulfoxide resulted in almost complete loss of antigenicity. Loss of functional activity for many proteins has been observed secondary to oxidation of the methionine residues, including *E. coli* ribosomal protein L12 (13,14), lysozyme (15), pepsin (16), ribonuclease

Table 1.

INHIBITION OF IIIC8 MOAb BINDING TO PEPTIDE COATED PLATES BY SYNTHETIC PEPTIDES CONTAINING THE PREDICTED EPITOPE AND POSSIBLE VARIANTS

Synthetic Peptide Used		ng Required for 40% Inhibition
DKPEKTAAPASDPTGGMGGMDF-amide	reduced	24
DKFEKTAAFASDPTGGMGGMDF-acid	reduced	40
KTAAPASDPTGGMGGMDF-amide	reduced	13
APASDPTGGMGGMDF-amide	reduced	11
ASDPTGGMGGMDF-acid	reduced	14
SDPTGGMGGMDF-amide	reduced	16
DPTGGMGGMDF-acid	reduced	8
PTGGMGGMDF-amide	reduced	>10,000
TGGMGGMDF-amide	reduced	>10,000
DPTGGMGGMD -amide	reduced	500
ASDPTGGMGGMDF-acid	oxidized	900
ASDPTGGMGGMDF-amide	oxidized	3,500

MINIMUM SEQUENCE	methionines reduced
maximal antigenicity	DPTGGMGGMDF
minimal antigenicity	DPTGGMGGMD

E = glutamic acid, Y = tyrosine, D = aspartic acid, L = leucine, K = lysine, A = alanine, G = glycine, V = valine.

(17), alpha-chymotrypsin (18,19), alpha-l-proteinase inhibitor (20-22), calmodulin (23), ACTH (19) and the chemotactic factors fMet-Leu-Phe (24) and complement C5A (24).

Discussion

The synthesized peptides reported above help to more precisely define the structural requirements of the epitopes recognized by the MOAbs IIC8 and IIIC8. The use of peptide synthesis allows many manipulations to identify the structural requirements of an epitope. Single amino acid substitutions within an epitope may identify the crucial residues within the epitope. In some instances this role may be related to secondary structure. For example, since antibody reactive epitopes have been found in beta sheets or with beta turns, if the substituted amino acid is one which breaks beta structure it may have a more adverse effect upon antibody binding than a different amino acid substitution which may still conform to a beta sheet secondary structure. The data in Table 2 suggest that the C-terminal phenylalanine does not

Table 2.

INHIBITION OF IIC8 AND F67-2 MOAb BINDING TO PEPTIDE COATED PLATES BY SYNTHETIC PEPTIDES CONTAINING THE PREDICTED EPITOPE AND POSSIBLE VARIANTS

Synthetic Peptide Used	ng Required for 40% Inhibition IIC8	F67-2
EYEDLLKAGVAD-amide	2	6
YEDLLKAGVAD-amide	10	18
EDLLKAGVAD-amide	230	280
DLLKAGVAD-amide	>10,000	1800
YEDLLKAGVA -amide	28	30
EDLLKAGVA -amide	850	600
YEDLLKAGV -amide	22	28
EDLLKAGV -amide	510	280
DLLKAGV -amide	>10,000	1400
KAGVAD-amide	>10,000	>10,000
EYEDLL -amide	>10,000	>10,000
EYEDLL + KAGVAD	>10,000	>10,000
MINIMUM SEQUENCE		
maximal antigenicity		EYEDLLKAGVAD
minimal antigenicity		DLLKAGV

D = aspartic acid, K = lysine, P = proline, F = phenylalanine, E = glutamic acid, T = threonine, A = alanine, S = serine, G = glycine, M = methionine.

need a charged carboxylate for binding to the IIIC8 monoclonal. The data also suggest that the oxidation state of methionines in the IIIC8 epitope is critical for binding to the monoclonal antibody. We do not yet know which of the two methionines (or both) is more important. Since there are numerous examples of loss of protein function upon oxidation of critical methionines, and since methionines are readily oxidized in solution upon exposure of this residue to oxygen in the presence of divalent trace metal ions, the state of oxidation of this residue and thus the assignment of the epitope «active» sequence cannot be assumed without verification. For example, exposure of this part of the 65,000 dalton protein upon ingestion of *M. leprae* by macrophages may lead to both reduced and oxidized states of these methionines *in vivo*.

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