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COMBINATORIAL EPITOPE SEARCH: PITFALLS OF LIBRARY DESIGN

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Abstract: *Borrelia burgodorferi* flagellin epitopes of various formats were presented on a TentaGel support. ELISA binding assays of these epitopes showed that a flexible linker, β-Ala-β-Ala, was better than a rigid linker, Pro-Pro, for peptide-antibody recognition. The previously reported 12-mer epitope was optimally minimized to a hexamer. Copyright © 1996 Elsevier Science Ltd

In an effort to develop combinatorial methods for the discovery of ligands that bind tightly with receptor molecules, we studied an epitope from *Borrelia burgdorferi* flagellin, a prevalent Lyme disease antigen, as the model system. This epitope consists of 12 amino acids with a sequence of EGVQQEGAQQPA (residues 213-224)[1,2] and binds to H9724, a mouse monoclonal antibody generated against the whole flagellin protein. We constructed a peptide library of Ac-XXXXXPAPRM on TentaGel S NH_2 resin using the partial chain-termination strategy,[3] in which five positions toward the *N*-terminus were randomized with 17 amino acids (A, D, E, F, G, H, K, L, N, P, Q, R, S, T, V, W, and Y). A common linker PAPRM was flanked at the *C*-terminus where the methionine residue was engineered to mediate the cleavage of peptides from the resin for sequence determination by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) and the arginine was included in peptides to ensure greater efficiency of ionization when peptides cleaved from a single bead were detected by MALDI-MS.[3] PAP served as a spacer to present the randomized peptides. To our surprise, after screening the whole library using ELISA (H9724 as the primary antibody, goat antimouse IgG conjugated with alkaline phosphatase as the secondary antibody, and BCIP/NBT as the substrate), only a limited number of colored (purple) beads were discovered from sublibraries containing Leu, Val, Pro, and Tyr at their *N*-termini. None of the identified peptides resembled a part of the natural epitope.[4]

We speculated that either the flanking residues or the length of the peptides, or both, were not optimally designed, resulting in a lower affinity and the inability to capture H9724. The importance of flanking residues has been well documented in the literature. For example, Dervan et al. recently reported that the addition of a *C*-terminal β-alanine in hairpin polyamides enhances both DNA-binding affinity and sequence specificity, while addition of a *C*-terminal glycine residue reduces both the binding affinity and sequence specificity.[5] Also, it was suggested that short peptides may not bind tightly with antibodies.[3] To further explore the underlying reasons, the full flagellin epitope was synthesized on TentaGel using various spacers such as Pro-Pro, Gly-Gly, and β-Ala-β-Ala. We chose Pro-Pro dipeptide as a spacer because of its rigidity, Gly-Gly because it is the simplest residue, and β-Ala-β-Ala as another spacer because it is flexible in conformation. A long linker could adversely affect the binding outcome,[6] so only duplicate amino acids were used as the spacer in this study.

In our experiments, peptides were assembled on TentaGel S NH_2 resin (0.26 mmol/g loading) using the standard Fmoc chemistry. Each coupling step was monitored by the ninhydrin test. The *N*-termini of the final peptides were acetylated using acetic anhydride. Deprotection of side chain functional groups was conducted for 4 h using a solution containing TFA (82%), water (4%), thioanisole (4%), mercaptoacetic acid (6%), and phenol (4%). A small portion of the beads was cleaved with CNBr (100 mg/mL) in 70% formic acid for 24 h, the resulting solution was lyophilized to remove excessive cyanogen bromide and then redissolved in TFA (0.1%)/water. The peptide solution (1 μL) was mixed with the saturated 2,5-dihydroxybenzoic acid in water (1 μL) for MALDI analysis. For ELISA experiments, peptide beads were washed with the PBST buffer (1x; 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, and 0.1% Tween-20, pH 7.2) three times, then blocked with gelatin (0.1%) in PBS buffer (2x) for 1 h. The supernatant containing mouse monoclonal antibody H9724 was diluted 1:1000 with the blocking buffer and incubated with the beads for another hour. After washing with PBST (1x) three times, the peptides on the beads were incubated with a diluted (1:10,000) goat antimouse IgG-alkaline phosphatase conjugate for an additional hour. The final washing included PBST (1x) three times, PBS (2x) twice, and TBS (1x; 2.5 mM Tris, 13.7 mM NaCl, and 0.27 mM KCl, pH 8.0) once. A standard alkaline phosphatase substrate, 5-bromo-4-chloro-3′-indolyl phosphate *p*-toluidine salt and nitro-blue tatrazolium chloride (BCIP/NBT), was added and the staining reaction was allowed to proceed for 30 min.

The epitope Ac-EGVQQEGAQQPA presented on TentaGel through BBRM (B = β-Ala), GGRM and PPRM were tested against H9724 under identical ELISA conditions. The peptide having BBRM showed a dark purple color, while the color was only light purple for the peptide with a rigid spacer of PPRM. The peptide presented using GGRM as a spacer gave an intermediate purple color. This difference in color between BBRM and PPRM was consistently observed when other truncated epitopes were presented, such as Ac-VQQEGAQQPA. Therefore, we concluded to use BBRM as the linker for further evaluating the effect of the peptide length.

To determine the minimum length of peptide required for binding to H9724 antibody, the peptide was systematically extended from ABBRM to the full sequence EGVQQEGAQQPABBRM. The most noticeable change in color on beads came from the addition of one Gln to QEGAQQPABBRM, which changed color substantially from white to purple (Table 1). Further extension of the peptide from QQEGAQQPABBRM up to EGVQQEGAQQPABBRM gave an indistinguishable purple color. To further reduce the peptide length, the peptide QQEGAQQPABBRM was shortened one by one from the *C*-terminus. It was found that after Pro was removed, bead color was substantially lightened: QQEGAQBBRM gave light pink color and QQEGABBRM did not bind to H9724 (Table 1). Using the same approach, the peptide VQQEGAQQPABBRM was shortened accordingly starting from its *C*-terminus. The hexamer VQQEGABBRM was found to still show strong binding, but pentamer VQQEGBBRM presented no affinity to the antibody (Table 1). Results reported here clearly demonstrate that the minimum peptide length required for H9724 antibody recognition depends on what the *N*-terminal residue is: VQQEGABBRM vs. QQEGAQQPBBRM. To exclude the possibility that long peptide causes nonspecific interactions, QEVEGAQQPABBRM, a slightly altered version of

VQQEGAQQPABBRM, was synthesized. No positive response was observed in ELISA assay, demonstrating that the recognition of antibody was highly sequence specific.

Table 1. ELISA Assays of a Lyme-Specific Monoclonal Antibody H9724 Binding to Peptides of Various Formats Presented on TentaGel Beads.

Peptide	Color	Peptide	Color
Ac-EGVQQEGAQQPABBRM	purple	Ac-QQEGAQQPBBRM	purple
Ac-GVQQEGAQQPABBRM	purple	Ac-QQEGAQQBBRM	light purple
Ac-VQQEGAQQPABBRM	purple	Ac-QQEGAQBBRM	light pink
Ac-QQEGAQQPABBRM	purple	Ac-QQEGABBRM	white
Ac-QEGAQQPABBRM	white	Ac-QQEGBBRM	white
Ac-EGAQQPABBRM	white	Ac-QQEBBRM	white
Ac-GAQQPABBRM	white	Ac-VQQEGAQQPBBRM	purple
Ac-AQQPABBRM	white	Ac-VQQEGAQQBBRM	purple
Ac-QQPABBRM	white	Ac-VQQEGAQBBRM	purple
Ac-QPABBRM	white	Ac-VQQEGABBRM	purple
Ac-PABBRM	white	Ac-VQQEGBBRM	white
Ac-ABBRM	white	Ac-VQQEBBRM	white

Peptide libraries are typically randomized in less than six residues (i.e., 20^5 = 3.2 million peptides, which are experimentally feasible for handling). Useful ligands have been identified from these libraries for certain receptors, such as the epitope peptide YGGFQ for a monoclonal antibody against β-endorphin,[7] the peptide RRWWCR as antimicrobial reagent against *S. aureus*,[8] and the peptide HPQ(F) for streptavidin as our and other groups have researched.[3] However, ligands to protein receptors such as enzymes and antibodies are often composed of longer peptides. In this work, it was found that the minimum length of peptides essential for the H9724 antibody recognition depends on the first residue at its *N*-terminus. Since the randomized positions only covered five amino acids in our Ac-XXXXXPAPRM library and the shortest epitope sequence required for binding to H9724 is Ac-VQQEGAPAPRM, no ligands resembling the natural epitope were found in Val sublibrary.[4] Recently, affinity capillary electrophoresis (ACE) has demonstrated its usefulness in studying ligand-receptor interactions of both weak- and strong-binding biochemical systems.[9] It is our interest to understand how the addition or deletion of single amino acid affects the antibody binding. Quantitative binding studies of the previously reported 12-mer epitope and newly optimized 6- and 8-mer peptides to the H9724 antibody in solution are currently in progress.

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