

Combinatorial search for diagnostic agents: Lyme antibody H9724 as an example

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Two peptide libraries, Ac-MXXXXXBBRM and Ac-VXXXXXBBRM, were constructed on TentaGel solid support to search for ligands that bind tightly with the H9724 Lyme antibody. By using an on-bead ELISA, approximately 120 ligands were selected as candidates for further study. Matrix-assisted laser desorption ionization mass spectrometry analysis of the candidate ligands indicated a high rate of occurrence of certain amino acids at the randomized positions. On the basis of the initial screening results, a small library was designed and iteratively synthesized. Subsequent library screenings led to the identification of four peptides, Ac-PQEEGX-NH₂ (X = R, K, A, D), that showed specific affinity to the antibody. This combination of solid-phase screening and iterative synthesis is an effective strategy for rapid identification of ligands that bind tightly with disease-specific antibodies and should be applicable, at least in principle, to other ligand-receptor systems. This combinatorial library approach can also be a useful tool for the discovery of novel diagnostic agents.

Sera from patients with an infectious disease are known to often contain a large number of different antibodies, generated by the host immune system, that are directed against the infectious agent. The degree of immune activation may be monitored by measuring the serum concentrations of these antibodies. However, in many cases in which the etiologic agents either are not readily available in large quantity or cross-react with other disease-unrelated antibodies, it is difficult to diagnose reliably and therefore treat the diseases. Our goal was to exploit the combinatorial library as a valuable tool for the discovery of small molecules as diagnostic agents that bind tightly with disease-specific antibodies. Small molecules such as peptides are useful in serodiagnosis and vaccine

development for infectious diseases. Peptides are excellent diagnostic agents because of their long shelf life and ease of manipulation in electrophoresis (described below). Also, to increase specificity of serodiagnosis, it is preferable to use defined peptide epitopes or mimotopes within bacterial proteins in which other parts of the sequences may be recognized by antibodies to other proteins of other microorganisms. Such nonspecific binding interactions often produce false-positive results due to the presence of other antibodies cross-reacting with the studied bacterial antigens. Using the Lyme antibody as an example, we report here our initial effort in developing combinatorial methods for the rapid identification of novel ligands.

Lyme disease is an infectious disease caused by a tick-transmitted spirochete *Borrelia burgdorferi*. Its multi-system syndrome includes erythema migrans (EM) and headaches in early stages and subsequent severe dermatologic, arthritic, rheumatologic, cardiac, and neurologic manifestations in later stages [1-3].¹ Lyme disease has been called a "great imitator" because of its broad spectrum of clinical symptoms in common with other diseases (e.g., influenza and rheumatoid arthritis) [1, 4]. Its diagnosis is heavily dependent on clinical description, but in the absence of EM, it can be difficult. Serologic tests are also used for Lyme disease diagnosis, but most exhibit low specificity and (or) sensitivity [4-9]. The importance of the early-stage diagnosis of Lyme disease is apparent as treatment with antibiotics at this stage shows a higher rate of success than that at later stages [4, 10].

Among the abundant antigenic proteins of *B. burgdorferi*, the internally localized flagellin (a flagellar protein) is

¹ Nonstandard abbreviations: EM, erythema migrans; ACE, affinity capillary electrophoresis; HOBt, 1-hydroxybenzotriazol; HBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; DIEA, *N*-ethyl-diisopropylamine; TFA, trifluoroacetic acid; DMF, *N,N*-dimethylformamide; BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine and nitroblue tetrazolium chloride; *p*NPP, *p*-nitrophenyl phosphate; dd, double distilled; PBST, PBS with Tween 20; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry; TBS, Tris-buffered saline; IC₅₀, concentration of soluble peptides at 50% inhibition; and AP, alkaline phosphatase.

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of particular interest for the purpose of diagnosis [11–15]. This protein is an important antigenic target of the immune response, as antibodies to the flagellin are detected in most Lyme patients, even at early stages [16]. The monoclonal antibody H9724 has been previously prepared against *B. burgdorferi* flagellin and the minimum epitope was identified as hexamer peptides: VQQEGA, MLQGV D, VQEGVQ, QQEGAQ [17–19]. Using a combinatorial approach, we were interested in searching for new epitopes against H9724 that could be used as diagnostic agents in the development of Lyme serodiagnosis by affinity capillary electrophoresis (ACE) [20–23]. Using ACE for the disease diagnosis has the following advantages: the technique is economical, rapid, experimentally straightforward and versatile, and, most significantly, it is performed in homogeneous aqueous solution, so that nonspecific interactions are minimized [20–23]; peptides can be readily separated because capillary electrophoresis is a high-resolution separation technique and charge manipulation of peptide is easily achievable; to a particular disease (Lyme borreliosis, for example), with ACE, several epitope or mimotope peptides can be used to achieve reliable diagnosis (one disease, multiple probes) in solutions.

In this paper, two libraries, Ac-MXXXXXBBRM and Ac-VXXXXXBBRM (B = β -alanine, X = all L-amino acids but C, M, and I; $17^5 = 1.4 \times 10^6$ decapeptides for each library), were synthesized on the TentaGel resin by using both the method of split synthesis [24] and the strategy of partial chain termination [25]. By combining the power of a high-throughput on-bead ELISA with iterative deconvolution, four ligands with high binding affinities to H9724 were rapidly identified.

Materials and Methods

REAGENTS

TentaGel S NH₂ resin used in the synthesis of peptide libraries was purchased from Advanced ChemTech. PAL resin was from PerSeptive Biosystems. Fmoc amino acids, 1-hydroxybenzotriazol (HOBt), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), and ninhydrin test kits were obtained from AnaSpec. *N*-ethyl-diisopropylamine (DIEA), 1,2-dithioethane, trifluoroacetic acid (TFA), thioanisole, and guanidine hydrochloride were available from Fluka Chemicals. *N,N*-dimethylformamide (DMF) and acetic anhydride were from Fisher Scientific. Ethyl ether was from J. T. Baker. Acetylglycine, mercaptoacetic acid, phenol, gelatin, goat anti-mouse whole IgG-alkaline phosphatase conjugate, tablets containing 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine and nitroblue tetrazolium chloride (BCIP/NBT), *p*-nitrophenyl phosphate (*p*NPP), and cyanogen bromide were purchased from Sigma Chemical Co. Multiscreen[®] 96-well filtration plates were from Millipore. Mouse monoclonal antibody H9724 supernatant was generously provided by Stanley Stein and Leonard H. Sigal at the University of Medicine and Dentistry of New Jersey.

PREPARATION OF PEPTIDE LIBRARIES

The libraries were synthesized on TentaGel S NH₂ resin (4 g; 80–100 μ m, 0.26 mmol/g substitution, 2.86×10^6 beads/g) by using standard Fmoc chemistry and applying the divide–couple–recombine method (split synthesis) to generate the one-bead–one-compound libraries [24, 25]. To construct the peptide libraries Ac-MXXXXXBBRM and Ac-VXXXXXBBRM, where X = A, D, E, F, G, H, K, L, N, P, Q, R, S, T, V, W, Y, and B = β -alanine, a BBRM spacer was first flanked to the C terminus of the libraries. Fmoc amino acids (4 mol equivalents) with 10% (mol/mol) acetylglycine were activated by HOBt/HBTU/DIEA in DMF (4 mol equivalents) and added to each reaction vessel. The coupling reaction was carried out for 2 h with double coupling used at the randomized positions to ensure a complete coupling. After the Fmoc deprotection with 200 mL/L piperidine in DMF for 20 min, the resin was combined and mixed in DMF before division into 17 equal parts for the next cycle. Split synthesis was carried out for five cycles. For the last randomized position, the resin was divided into halves to couple M and V. The N termini were acylated by using acetic anhydride (10 mol equivalents) with HOBt (10 mol equivalents), HBTU (5 mol equivalents), and DIEA (10 mol equivalents) in DMF for 2 h. With the cleavage cocktail of 820 mL/L TFA, 40 mL/L water, 40 mL/L thioanisole, 60 mL/L mercaptoacetic acid, and 40 g/L phenol, the side chains of the peptides were deprotected for 4 h (100 mg resin/mL) followed by five washings with ethyl ether.

ON-BEAD SCREENING OF PEPTIDE LIBRARIES

Solid-phase ELISA was used for library screening. In brief, the peptide libraries were washed three times each with double-distilled (dd) H₂O and 1 \times PBST buffer (Na₂HPO₄, 8 mmol/L; KH₂PO₄, 1.5 mmol/L; NaCl, 137 mmol/L; KCl, 2.7 mmol/L; and 1 mL/L Tween 20, pH 7.2). To minimize nonspecific binding, the secondary antibody, goat anti-mouse IgG-alkaline phosphatase conjugate, 1:1000 diluted in blocking buffer (2 \times PBS buffer containing 1 g/L gelatin, 0.5 mL/L Tween 20, pH 7.5), was first added to the peptide libraries and incubated for 1 h, followed by three washings with 1 \times PBST, two washings with 2 \times PBS, and one washing with 1 \times TBS (Tris, 2.5 mmol/L; NaCl, 13.7 mmol/L; and KCl, 0.27 mmol/L; pH 8.0). The beads were then incubated for 30 min with a standard alkaline phosphatase substrate, BCIP/NBT, and several purple-colored beads were manually removed from the libraries under microscope (60 \times magnification). Subsequently, the library beads were regenerated by washing with DMF and isopropanol to reduce the background color. The libraries were incubated with mouse monoclonal antibody H9724 supernatant (1:50 diluted in the blocking buffer), treated with the secondary antibody, and substrate screened as described above. The purple-colored beads were manually selected and incubated with guanidine hydrochloride (6 mol/L, pH 1.0) for 30 min to dissociate the bound antibodies.

DETERMINATION OF LIGAND SEQUENCES BY MATRIX-ASSISTED LASER DESORPTION IONIZATION MASS SPECTROMETRY (MALDI-MS)

Purple-colored beads were extensively washed with dd H₂O. The peptides were released from each bead into individual microtubes by CNBr (20 μ L, 100 μ g/ μ L) in 700 mL/L formic acid for 16–24 h in the dark. Excess CNBr was removed by lyophilization and the peptide residues in each tube were dissolved in 1 mL/L TFA (20 μ L) followed by MALDI-MS analysis. Analysis was performed on a Kratos Kompact MALDI-III mass spectrometer by mixing sample solution (1 μ L) with saturated α -cyano-4-hydroxycinnamic acid (1.0 μ L) in 1:1 (by vol) water:acetonitrile.

ITERATIVE SELECTION OF BINDING PEPTIDE LIGANDS

On the basis of peptide sequences determined from MALDI-MS analysis, a small peptide library was designed and synthesized on the TentaGel resin to search for tight-binding ligands through deconvolution by using the ELISA as described above. A soluble substrate of alkaline phosphatase, *p*NPP, was used to obtain quantitative data. The absorbance was recorded at 405 nm after terminating the reaction with NaOH (3 mol/L).

DETERMINATION OF IC₅₀ VALUES BY A CAPTURE ELISA

Individual peptides were synthesized on PAL resin (0.33 mmol/g substitution) by using standard Fmoc chemistry. The peptides were cleaved from the resin with the same cleavage cocktail used for peptide libraries, precipitated, and washed five times with cold ethyl ether. After purification by reversed-phase HPLC, the quality of each peptide was ascertained by MALDI-MS.

The solid-phase antigen Ac-VQQEGABBRM on TentaGel resin suspended in blocking buffer was evenly distributed into a Multiscreen[®] 96-well filtration plate (approximately 0.25 mg/well) in triplicate and incubated for 2 h. After blocking buffer drainage, preequilibrated soluble peptide antigen/antibody mixtures (1:1 serial dilutions from 1.28 mmol/L to 625 nmol/L) were added to individual wells and agitated for 30 min. After the resin was washed three times with 1 \times PBST buffer, the secondary antibody (100 μ L, 1:1000 dilution in the blocking buffer) was added and agitated for 1.5 h. Finally, the resin was drained and washed three times with 1 \times PBST buffer, twice with 2 \times PBS buffer, and once with 1 \times TBS buffer. Soluble substrate *p*NPP (200 μ L) was added to each microtiter well, followed by color development. After adding NaOH (50 μ L, 3.0 mol/L), the solutions in each well were diluted to 1 mL with dd H₂O and the absorbance was measured on a Shimadzu UV-1201 UV-VIS spectrometer at 405 nm. The percentage of inhibition was calculated as

$$\% \text{ inhibition} = 100 - [100 \times (A_i - A_b)/(A_0 - A_b)]$$

where A_b is the blank absorbance (without incubation with the primary antibody) and A_i and A_0 are the absorbances with and without the soluble peptides in the antibody solutions, respectively. The IC₅₀ values were determined as the concentrations of soluble peptides at 50% inhibition.

Results and Discussion

CONSTRUCTION AND SCREENING OF THE PEPTIDE LIBRARIES

The size of a chemically synthesized library is limited with the method of split synthesis [26]. To ensure the presence of all possible peptides at a 95% confidence level in the libraries, the total number of beads needed is at least three times the number of possible peptides. In our case, a total randomized hexapeptide library with 17 amino acids (17⁶ peptides) would require approximately 25 g of resin, which is costly and difficult to manipulate experimentally. To control the library size and aid in the search of ligands, the libraries of Ac-M/VXXXXXBRRM were designed with the two amino acids M and V (based on the native epitope sequences) fixed at the N termini [18, 19]. At the C termini, methionine was flanked for the ease of peptide release with CNBr from the TentaGel resin for MALDI-MS sequencing. Arginine was included to ensure a greater efficiency of ionization upon MS analysis. Two β -alanines were introduced as the linker because of its flexible conformation [19]. Isoleucine, cysteine, and methionine were omitted from the randomized positions to simplify the sequence determination, prevent the formation of disulfide bonds, and eliminate undesired cleavage, respectively.

With ELISA, the color density of positive beads is important for the success of the library screening. Because of chain termination, the color of positive beads was less intense than those with only the full-length peptides. The discrepancies may come from (a) the reduced quantity of full-length binding peptide on a single bead, (b) weaker affinity of shorter peptides to H9724 [19], or (c) less divalent binding of the antibody from overpopulated peptides on beads. To maximize the chances of identifying lead molecules from the libraries and to eliminate the ambiguity of positive beads, a 1:50 dilution of H9724 was used for the library screening on beads. As a result, approximately 120 colored beads were selected (Fig. 1) and subsequent MALDI-MS analysis decoded 15 sequences in the V sublibrary and 37 sequences in the M sublibrary (Fig. 2). Because MALDI-MS decoding relied on the molecular mass of each amino acid, accurate assignment of amino acid identities with similar masses (e.g., Q, K, and E) was difficult. As most of the ligands identified from both the M and V sublibraries contained X = Q/E/K, verification of these ligands individually would be labor intensive. Since only a limited number of amino acids occurred with high frequency at the randomized positions, the strategy of deconvolution was used to identify the best ligands.

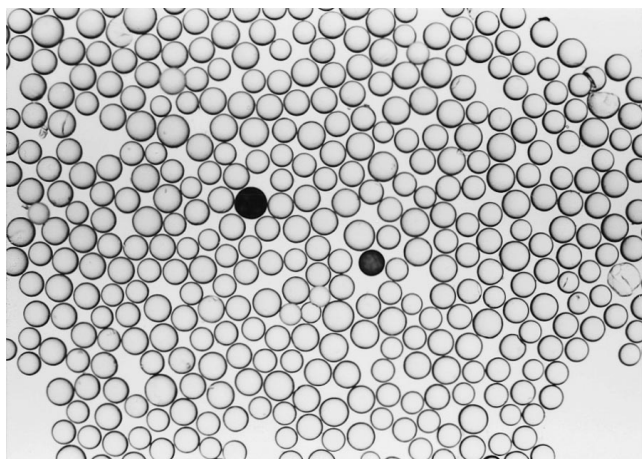


Fig. 1. An ELISA of a peptide library of Ac-MXXXXBBRM against the Lyme antibody H9724.

Only a portion of the beads in the library is shown in the photograph (60 \times). The dark beads in the center were identified as the hits.

ITERATIVE DECONVOLUTION OF TIGHT-BINDING LIGANDS

A small library of 5120 peptides Ac- $X_1X_2X_3X_4X_5X_6$ BBRM ($X_1 = M, V$; $X_2 = X_3 = Q, E, K, L$; $X_4 = Q, E, K, G$; $X_5 = Q, E, K, G, V$; $X_6 = Q, E, K, L, D, R, F, A$) was synthesized for deconvolution. Eight sublibraries (Ac-M/VQX $_3$ X $_4$ X $_5$ X $_6$ BBRM, Ac-M/VEX $_3$ X $_4$ X $_5$ X $_6$ BBRM, Ac-M/VKX $_3$ X $_4$ X $_5$ X $_6$ BBRM, and Ac-M/VLX $_3$ X $_4$ X $_5$ X $_6$ BBRM) randomized at $X_3, X_4, X_5,$ and X_6 positions were prepared to assay the binding abilities to H9724 by ELISA. The sublibraries containing Q at X_2 were found to be most active in both M and V sublibraries (Fig. 3, step 1). Therefore, X_2 was fixed as Q, and a new set of sublibraries were synthesized and screened until each position, in turn, was deconvoluted. Because individual peptides have respective binding affinities, a strong signal often can occur from the additive effects of tight-binding ligands and (or) a combination of moderate- and tight-binding ligands. By lowering the concentration of the target molecule (e.g.,

antibody), the moderate-binding and tight-binding ligands may be differentiated; therefore, H9724 was sequentially diluted from 1:100 to 1:5000 during deconvolution. As a result, it was found that the peptides Ac-M/VQEEGX ($X = R, K, D, A$) were identified as the motif with the strongest binding to the Lyme H9724 antibody (Fig. 3, step 5). Because the N terminus region was only randomized with M and V, binding ligands with other amino acids at this position would have been omitted. Thus, the N termini of X_1 QEEGXBBRM with the remaining 17 amino acids (except C) were further deconvoluted. The results indicated the preference for aromatic, hydrophobic amino acids W, Y, P, M, H, and I (Fig. 4). From the P sublibrary, Ac-PQEEGXBBRM ($X = R, K, A, D$) peptides were identified to be the best binding ligands for H9724 with higher affinities than those of two native epitopes with the on-bead ELISA (Fig. 5).

CONFIRMATION AND EVALUATION OF NEW BINDING SEQUENCES

It has been well documented that, with the on-bead binding assays, the solid support and linker may interfere with the ligand-receptor interactions [27–29]. For example, hepatitis B surface antigen mimotopes presented on bacteriophage coat protein were much better immunogens than those presented as multiple antigenic peptides or coupled to a protein carrier [30]. To demonstrate the specific binding to H9724, the newly discovered ligands, along with two native epitopes (Ac-VQQEGA-NH $_2$ and Ac-MLQGVD-NH $_2$), were individually synthesized and their binding to H9724 in solution evaluated. Ac-VQQEGABBRM, covalently attached to a TentaGel resin, was used as the solid-phase antigen. With a capture ELISA, the new ligands were able to competitively inhibit the binding of H9724 to the solid-phase antigen, and this allowed the estimation of the relative affinities of the antibody to these binding peptides (Table 1). From the IC $_{50}$ values, four new ligands showed stronger binding affinities than that of the natural epitope of *B. burgdorferi* flagellin (Ac-VQQEGA-NH $_2$), however, comparable with that of the native epitope of the cross-reactive human Hsp60 (Ac-MLQGVD-NH $_2$) (Table 1). The unrelated peptide Ac-FLTDGT-NH $_2$ showed no inhibition effect at concentrations up to 6 mmol/L in solution. Therefore, we concluded that the interactions between Ac-PQEEGX-NH $_2$ ($X = R, K, A, D$) and H9724 were specific.

The four newly identified ligands show no obvious sequence homology to the native epitopes, although they are more similar to the flagellin epitope than that of human Hsp60 in sequence. The interactions of the new ligands and antibody are likely through the concept of a mimotope: As long as the complementarity between the antigen-binding site of antibody and the molecular surface of the binding peptide is maintained (in regard to both shape and charge), binding interactions can occur [31]. Therefore, antibody-binding peptides are not necessarily an accurate reproduction of the epitopes [32, 33].

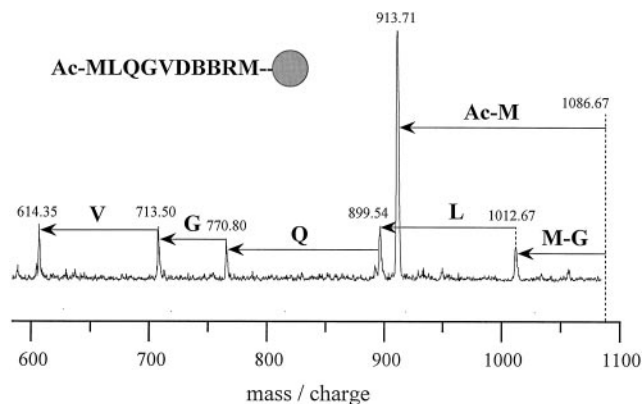


Fig. 2. MALDI mass spectrum of peptides cleaved from a single bead. This bead was recognized by H9724 and contained a sequence of Ac-MLQGVD-BBRM. Note that only 5% of the peptides from a single bead was used for sequence decoding.

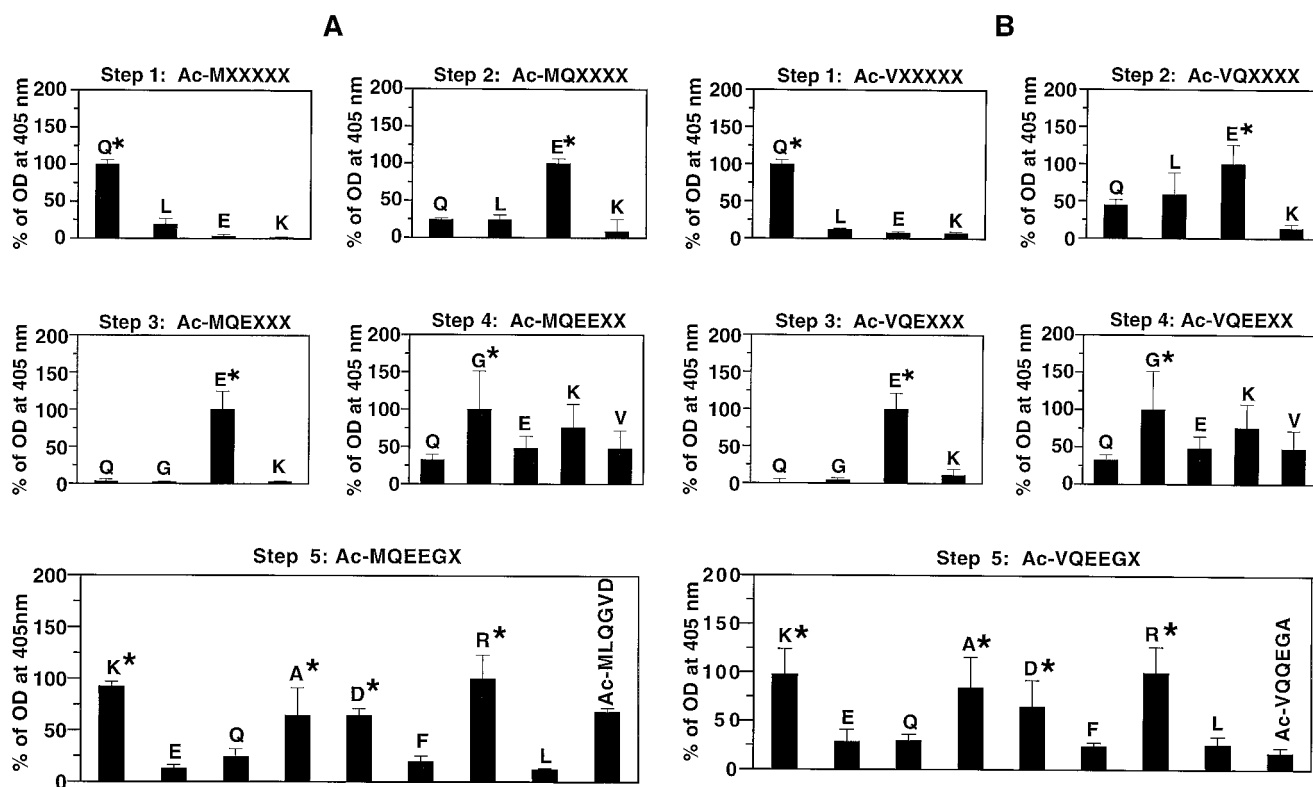


Fig. 3. Deconvolution of M and V sublibraries (Ac-MX₂X₃X₄X₅X₆BBRM and Ac-VX₂X₃X₄X₅X₆BBRM, X₂ = X₃ = Q, E, K, L; X₄ = Q, E, K, G; X₅ = Q, E, K, G, V; X₆ = Q, E, K, L, D, R, F, A) with on-bead screening.

(A) M sublibrary; (B) V sublibrary. Amino acids labeled with an asterisk (*) were selected for subsequent deconvolutions. *Step 1*: first round of iterative selection; *step 2*: second round of iterative selection with a defined amino acid (Q) at X₂ obtained from step 1; *step 3*: third round of iterative selection with two defined amino acids (QE) at X₂X₃ obtained from steps 1–2; *step 4*: fourth round of iterative selection with three defined amino acids (QEE) at X₂X₃X₄ obtained from steps 1–3; *step 5*: fifth round of iterative selection with four defined amino acids (QEEG) at X₂X₃X₄X₅ obtained from steps 1–4. Conditions used for on-bead ELISA: *step 1*: H9724 (1:100), goat anti-mouse whole IgG–alkaline phosphatase conjugate (IgG-AP) (1:1000); *steps 2 and 3*: H9724 (1:500), IgG-AP (1:2000); *step 4*: H9724 (1:600), IgG-AP (1:2400); *step 5*: H9724 (1:1000), IgG-AP (1:5000).

For example, the lectin concanavalin A binds the YPY-containing peptides identified from the screening of a combinatorial library; this binding is specific and can compete with the natural ligand methyl- α -D-mannopyranoside. When applying a combinatorial strategy, the results are most clearly defined if each residue in the epitope sequence makes a distinct contribution to anti-

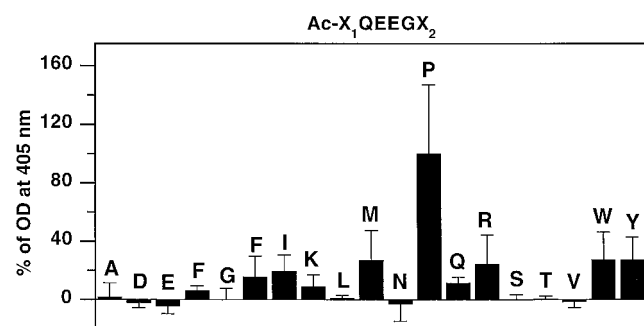


Fig. 4. Preference of amino acid at the N terminus of a newly identified motif Ac-X₁QEEGX₂BBRM (X₁ = 19 amino acids, except C; X₂ = A, D, K, R).

Condition used for on-bead ELISA: H9724 (1:3000), goat anti-mouse whole IgG-AP (1:10 000).

body binding. In some cases, the precise epitopes were able to be identified from peptide libraries [34, 35]. The screening criteria may also influence the selection of candidate ligands. Under harsh screening conditions, the tight-binding ligands are more likely identified from a library. If the native epitopes only have moderate-binding affinity, they would most likely be excluded as candidate ligands with an on-bead screening and would be missed during deconvolution. As a result, the identified ligands could have completely different sequences from the par-

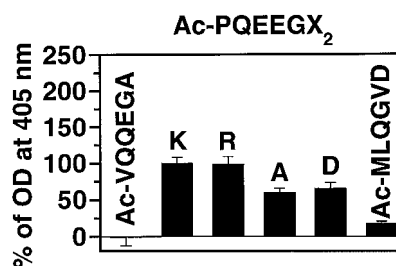


Fig. 5. Comparison of new ligands with two native epitopes by using an on-bead ELISA.

Condition used: H9724 (1:5000), goat anti-mouse whole IgG-AP (1:10 000).

Table 1. IC₅₀ values for the natural epitopes and peptide ligands identified from the combinatorial libraries.

	Sequence	IC ₅₀ , μmol/L
Peptide ligand	Ac-PQEEGA-NH ₂	45 ± 8
	Ac-PQEEGD-NH ₂	39 ± 7
	Ac-PQEEGK-NH ₂	33 ± 10
	Ac-PQEEGR-NH ₂	64 ± 22
Native flagellin epitope	Ac-VQQEGA-NH ₂	155 ± 27
Native Hsp60 epitope	Ac-MLQGVD-NH ₂	62 ± 10

ent antigens, but with higher or comparable affinities [36]. In addition, although the epitopes are restricted to relatively small sequences, some conformational constraint may be needed for high-affinity peptides [37]. Therefore, many ligands with similar binding affinities could be identified from short peptide (e.g., hexapeptide) libraries. In some cases, monoclonal antibodies have displayed polyspecific binding abilities to short peptides [36, 38].

In conclusion, four new ligands with improved affinity to H9724 were identified by using a combinatorial approach. These new sequences may have potential applications in the serodiagnosis of Lyme disease. The tight-binding epitopes, in principle, could increase the sensitivity of serodiagnosis. On the basis of our new epitope sequences, second-generation peptide libraries may be designed by fixing new sequences at either the N or C terminus to increase the peptide length. Therefore, longer peptide ligands may be discovered with an even higher affinity to H9724. Divalent interactions between antibodies and peptide ligands may also be applied in the design of new antibody ligands for Lyme serodiagnosis. Our combination of on-bead ELISA and iterative synthesis has proven to be an efficient method in the identification of antibody epitopes.

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