CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, Jan. 2001, p. 150–160 Vol. 8, No. 1
1071-412X/01/$04.00+0 DOI: 10.1128/CDLI.8.1.150–160.2001

Identifying Diagnostic Peptides for Lyme Disease through Epitope Discovery

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Received 15 March 2000/Returned for modification 7 July 2000/Accepted 11 September 2000

Serum antibodies from patients with Lyme disease (LD) were used to affinity select peptide epitopes from 12 large random peptide libraries in phage display format. The selected peptides were surveyed for reactivity with a panel of positive sera (from LD patients) and negative sera (from subjects without LD), thus identifying 17 peptides with a diagnostically useful binding pattern: reactivity with at least three positive sera and no reactivity with any of the negative sera. The peptides define eight sequence motifs, none of which can be matched convincingly with segments of proteins from *Borrelia burgdorferi*, the LD pathogen; evidently, then, they are "mimotopes," mimicking natural pathogen epitopes without matching contiguous amino acids of pathogen proteins. Peptides like these could be the basis of a new diagnostic enzyme-linked immunosorbent assay for LD, with sufficient specificity and sensitivity to replace expensive immunoblotting tests that are currently required for definitive serological diagnosis. Moreover, the method used to discover these peptides did not require any knowledge of the pathogen and involved generic procedures that are applicable to almost any infectious disease, including emerging diseases for which no pathogen has yet been identified.

Serological diagnosis of infectious diseases relies on detection of pathogen-specific antibodies in the sera (or other fluids) of infected subjects. It uses generic processes—primarily detection of immunoglobulin bound to immobilized antigen—that are readily applied to a very wide variety of diseases. Furthermore, because of the tremendous amplification inherent in the antibody response, it can be very sensitive, although it has the intrinsic limitation of being unable to detect infection at extremely early times before the emergence of an antibody response. The widely publicized use of serological tests to screen people and donated blood for signs of human immunodeficiency virus type 1 infection exemplifies the usefulness of this method of diagnosis.

The strengths and weaknesses of serological diagnosis as currently practiced are well illustrated in the case of Lyme disease (LD). Ticks infected with the spirochete *Borrelia burgdorferi* cause about 15,000 cases of LD in the United States each year, making it the most common insect-borne malady in the country (53). About 70% of infected people experience an expanding "bull's-eye" rash (erythema migrans) at the site of the tick bite (68). Within days to weeks, the spirochete may disseminate. Common manifestations of early disseminated infection include migratory joint pain, acute neurological involvement including meningitis, or cardiac abnormalities, particularly atrioventricular (AV) nodal block. Months to years later, untreated patients often develop intermittent or chronic arthritis, primarily affecting one or both knees. Early diagnosis and treatment can prevent subsequent more severe consequences of the infection.

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Unfortunately, current serodiagnostic enzyme-linked immunosorbent assays (ELISAs) are not highly sensitive and selective, hindering detection (1, 3, 5, 8, 12, 14, 29, 32, 38, 41, 50, 67, 70, 74). Definitive serological diagnosis depends on a complex, expensive immunoblot analysis (14, 16, 31, 35, 38, 40, 41, 51, 56, 58, 72, 74).

Most current ELISAs use crude extracts of *B. burgdorferi* as the antigen (30, 32, 42, 43, 61, 67). There are several drawbacks to such bacterial extracts. First, different strains of bacteria have different characteristics, which can change with successive culture passages (30); thus, it is difficult to control quality strictly. Second, as a complex mixture, a bacterial extract invites background reactions that obscure the diagnostic signal. The background reactions can be adventitious, or can represent cross-reaction with antibodies elicited by normal human flora such as *Escherichia coli*. To some extent background signals can be counteracted by preabsorption of the test sera with an *E. coli* extract (20), but this is only a partial remedy, and is an arduous countermeasure to be avoided if possible. Third, in any complex antigen, the most informative epitopes are diluted with numerous less informative or noninformative epitopes (in addition to the misinformative epitopes responsible for background reactions), potentially limiting the informative signal-to-noise ratio. With the advent of a vaccine based on recombinant outer surface protein A (62, 69, 75, 76), a fourth problem arises: distinguishing vaccine-induced from infection-induced antibodies.

Certainly one sensible response to these criticisms is to use recombinant pathogen proteins as the antigens (6, 13, 21, 22, 24, 27, 28, 36, 37, 43, 44, 52, 57). Such proteins can be propagated and expressed by standard recombinant DNA technology, and their sequences can be monitored frequently to head off variability. Nevertheless, this approach is limited to proteins whose antigenic structures have been investigated. Moreover, the informative epitopes in such proteins are still diluted with

noninformative or misinformative epitopes. Lastly, even recombinant antigens are somewhat expensive to produce.

Use of individual peptide epitopes as diagnostic antigens answers many of the criticisms that have been leveled against crude bacterial extracts or whole recombinant proteins (39, 73, 81, 82). By focusing on single subspecificities, they hold out the possibility of avoiding dilution of the informative epitopes with noninformative or misinformative epitopes. They are also cheaply produced and of high quality, and allow for strictly controllable, chemically simple formats for ELISA and other serological reactions.

Commonly used methods of identifying peptide epitopes are laborious and limited to known antigenic proteins. For example, Yu and coworkers (82) synthesized dodecamers spanning the amino acid sequences of four immunodominant *B. burgdorferi* surface proteins and screened them with several LD patient sera, thereby identifying a panel of eight peptide epitopes. A diagnostic test based on these peptides had a sensitivity of 75% and a specificity of 71% on a panel of 46 independent serum samples not used for identifying the epitopes—a performance roughly comparable to those of commercial assays tested with the same panel of sera.

Epitope discovery is a new approach for identifying peptide diagnostics (10, 11, 23, 59). The source of the peptides in this strategy is a panel of large random peptide libraries (RPLs) in phage display format. Each peptide in such a library is displayed as a "guest" fused to a surface protein of a filamentous phage carrier. Because the viral DNA includes the peptide coding sequence, guest peptides can be propagated and cloned at will simply by infecting fresh bacterial cells with the carrier phage. Using simple microbiological procedures, antibodies from a panel of human sera—both positive sera from patients with the disease and negative sera from other donors—are used to affinity select peptide ligands from these libraries. The selected peptides are then screened for the desired pattern of reactivity: a positive reaction with at least some of the positive sera and no reaction with any of the negative sera. This approach to diagnostics is covered by U.S. and other patents (10, 11, 23, 59).

Epitope discovery has yielded very promising results in several systems (9, 10, 17–19, 23, 34, 45, 46, 48, 55, 63, 64, 71). In this paper, we report the use of an improved implementation of the approach to identify promising candidate diagnostics for LD.

MATERIALS AND METHODS

Solutions and preparations. A 10 mM stock solution of biotin was made by dissolving at 10 mM in water and adjusting the pH to 6 to 9 with NaOH; it was then filter sterilized and stored at −20°C. Blocking solution consisted of 0.1 M $NaHCO_3$, 5 mg of dialyzed bovine serum albumin (BSA)/ml, 0.1 μg of streptavidin/ml, and 200 μg of NaN_3/ml; it was filter sterilized and stored at 4°C and could be reused until microbial contamination was evident. Dialyzed BSA (Sigma [St. Louis, Mo.] A6793; presumed to be free of biotin) was dissolved at 50 mg/ml to make a stock solution, which was then filter sterilized and stored at 4°C. Dulbecco's phosphate-buffered saline (D-PBS) consisted of 2.67 mM KCl, 1.15 mM KH_2PO_4, 138 mM NaCl, 8.06 mM Na_2HPO_4, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$ (pH 7.2). A solution containing all components but $CaCl_2$ and $MgCl_2$ was autoclaved, as were separate 1 M stocks of those two salts; after cooling, the two salts were added and the buffer was stored at room temperature. Elution buffer (0.1 N HCl–1 mg of BSA/ml [pH 2.2]) was made by mixing water, a 50-mg/ml stock solution of BSA, and 0.4 N HCl (pH adjusted to 2.2 with glycine); the buffer was filter sterilized and stored at room temperature. Isopropylthio-β-D-galactoside (IPTG) was dissolved to 1 M, to make a stock solution, which was then filter sterilized and stored at 4°C. NAP buffer consisted of 80 mM NaCl and 50 mM $NH_4H_2PO_4$; the pH was adjusted to 7.0 with NH_4OH, and the buffer was autoclaved and stored in the refrigerator or at room temperature. NPP substrate was made just before use by adding 10 μl of 1 M $MgCl_2$ and 100 μl of 50-mg/ml p-nitrophenylphosphate (stored in aliquots at −20°C) to 10 ml of 1 M diethanolamine (pH adjusted to 9.8 with HCl). For NZY medium, 10 g of NZ amine A, 5 g of yeast extract, and 5 g of NaCl were dissolved in 1 liter of water, the pH was adjusted to 7.5 with NaOH, and the solution was autoclaved and stored at room temperature. This medium was also made at a 2× concentration for NZY plates. For the plates, 11 g of Bacto-agar in 500 ml of water was autoclaved in a 2-liter polypropylene flask; without cooling, 500 ml of 2× NZY medium at room temperature was added, followed by supplements such as tetracycline as required, the flask was swirled to mix its contents, and about 25 ml was poured into each 100-mm petri dish. TBS was 50 mM Tris-HCl (pH 7.5)–0.15 M NaCl, autoclaved and stored at room temperature. TBS-Tween was 0.5% (vol/vol) Tween 20 in TBS, autoclaved and stored at room temperature. A 20-mg/ml stock solution of tetracycline was made up as a 1:1 (vol/vol) mixture of filter-sterilized 40-mg/ml tetracycline and autoclaved glycerol (cooled before mixing) and was stored at −20°C. Tetracycline plates were NZY plates supplemented with 40 μg of tetracycline/ml. TTDBA was 1 mg of dialyzed BSA/ml–200 μg of NaN_3/ml in TBS-Tween.

Antibodies and conjugates. Affinity-purified, AP-conjugated goat antibodies specific for human and mouse immunoglobulins were purchased from Southern Biotechnology Associates Inc. (Birmingham, Ala.); they were supplied at unspecified concentrations in 25 mM Tris-HCl (pH 8.0)–0.05% NaN_3–50% glycerol and were stored at −20°C. AP-anti-hIg (catalogue number 2010-04) recognizes human immunoglobulins in general; AP-anti-hIgG (2040-04) recognizes human immunoglobulin G (IgG) of all subclasses; AP-anti-hIgM (2020-04) recognizes human IgM; AP-anti-mIgG2b (1090-04) recognizes mouse subclass IgG2b immunoglobulins. Anti-fd MAb is mouse IgG2b monoclonal antibody B62-FE2 specific for filamentous phage M13, fd, and f1 (47); it was purchased from Research Diagnostic Inc. (Flanders, N.J.) (catalogue number RDI-PRO61097), dissolved at 50 μg/ml in 0.5× phosphate-buffered saline (pH 7.4)–2.5 mg of BSA/ml–0.045% NaN_3–50% glycerol, and stored at −20°C.

Bacteria, vectors, and libraries. The bacterial host for all filamentous phage in this work was *E. coli* strain K91BlueKan. Its sex is Hfr Cavalli; it therefore deploys the F pilus, the attachment site for filamentous phage infection. Its chromosomal genotype is *thi lacZ*ΔM15 *lacY*::*mkh lacI*^{q} (80), where *mkh* is the "mini-Kan hopper" transposon (77), which confers kanamycin resistance on the cell.

All phage display vectors used in this work were derived from fd-tet (83) (GenBank accession number AF217317), a derivative of wild-type filamentous phage fd with the tetracycline resistance determinant of transposon Tn*10*. Vector fUSE5 (60) (GenBank accession number AF218364) displays its guest peptides on all five copies of the minor coat protein pIII at one tip of the virion; vector f88-4 (S. Choukri and G. P. Smith, unpublished data) (GenBank accession number AF218363) displays its guest peptides on 100 to 300 copies of the major coat protein pVIII; vector f8-1 (54) (GenBank accession number AF218734) displays its guest peptides on all 3,900 copies of pVIII; and vector f8-5 (V. A. Petrenko, personal communication), used only as an ELISA control in this work, differs from wild-type phage by the amino acid replacement E → D at position 4 of mature pVIII.

Phage display libraries f3-6mer (60) and f3-15mer (49) (GenBank accession numbers AF246446 and AF246445, respectively) were constructed in vector fUSE5; libraries f88-15mer (Choukri and Smith, unpublished) (GenBank accession number AF246448), f88-Cys0 to f88-Cys6 (G. P. Smith, unpublished data) (GenBank accession numbers AF246449 to AF246455, respectively), and f88-LX6 (4) (GenBank accession number AF246456) were constructed in vector f88-4; and library f8-8mer (54) (GenBank accession number AF246447) was constructed in vector f8-1. The general format of the random peptides displayed in these libraries is shown in Table 1. In the f88-15mer, f88-Cys0 to f88-Cys6, and f88-LX6 libraries, the random peptide is fused to a recombinant form of pVIII encoded by a second, artificial gene VIII, driven by the IPTG-inducible *tac* promoter/operator; about 150 of the pVIII subunits are encoded by this gene under fully induced conditions (1 mM IPTG), the remaining pVIII subunits deriving from the wild-type gene VIII that also resides in the f88-4 genome.

General methods for propagating, purifying, titering and affinity selecting peptide-displaying phage have been published previously (66, 80). The libraries will be called RPLs throughout this paper.

Human sera. Ten individual human serum samples (500 μl each) from patients with confirmed diagnoses of LD (positive sera) and ten samples (also 500 μl) from control donors with rheumatoid or psoriatic arthritis (negative sera) were

obtained from A. Steere (New England Medical Center, Boston, Mass.). Pooled serum samples from ostensibly healthy donors were purchased from Sigma Chemical Co. (catalogue number S-7023). Sera were stored at −20°C in closed containers to reduce the risk of dissemination of blood-borne pathogens. Except as noted, all manipulations with human sera were carried out in a biosafety hood; suitable measures were taken to protect workers and to contain any possible contamination during manipulations that had to be carried out outside the hood; all materials removed from the hood were immediately decontaminated by autoclaving or soaking in 10% household bleach.

S/D treatment. All sera were subjected to a scaled-down adaptation of the solvent-detergent (S/D) treatment commonly used to kill enveloped viruses in serum that will serve as a source of human blood proteins (33). A 200-μl portion of each serum sample was mixed with 4 μl of a 1:1 (vol/vol) mixture of Triton X-100 and tri-(*n*-butyl)-phosphate in a 1.5-ml screw-cap microcentrifuge tube by continuous inversion at 30°C for 4 h (tubes were enclosed in a larger vessel during rotation outside the biosafety hood). To extract the solvent and detergent and clear insoluble material, the samples were filtered through Micro-Spin C_{18} filter devices (Alltech, Deerfield, Ill.; catalogue number 31251; no longer available) that had been previously activated with 700 μl of 100% ethanol and equilibrated with 700 μl of ImmunoPure (G) binding buffer (referred to below as binding buffer; Pierce Chemical Co., Rockford, Ill.). Samples that were to be processed for isolation of IgG were diluted with 500 μl of the binding buffer prior to filtration; other serum samples were filtered undiluted and stored at −20°C.

A scaled-up version of the above procedure was used to process 6 ml of pooled normal human serum samples in preparation for isolation of IgG; for this purpose, a 25-mm C_{18} Novo-Clean syringe filter (Alltech catalogue number 705350) was substituted for the Micro-Spin C_{18} filter device.

Bio-IgG. Twenty 2-ml disposable chromatography columns were packed with 500-μl beds of UltraLink protein G chromatography beads (Pierce) in binding buffer (described above), and mounted in an autoclavable rack inside the biosafety hood. Each S/D-treated serum sample (700 μl, including the 500 μl of binding buffer) was loaded onto one of the columns and allowed to drain in, after which the column was washed eight times with 500 μl of binding buffer. The columns were then mounted over 10- by 75-mm polypropylene tubes containing 220 μl of neutralizing buffer (1 M NaH_2PO_4 [pH adjusted to 8.0 with NaOH]), and the IgG fraction was eluted into the tubes in three 733-μl portions of ImmunoPure elution buffer (Pierce). The neutralized eluates were drawn up into 3-ml syringes through ¾-inch 22-gauge needles, injected into 3-ml Slide-A-Lyzer cassettes (molecular weight cutoff, 10 kDa; Pierce), and dialyzed in the cold against three changes of 0.1 M $NaHCO_3$ (unadjusted pH 8.6) (dialysis was performed outside the hood in sealed polypropylene jars, and precautions were taken to avoid contamination of the outside of the vessels).

The 20 protein G columns were regenerated by passing through, in succession, three 2-ml portions of the ImmunoPure elution buffer, two 2-ml portions of 1% sodium dodecyl sulfate in TBS, three 2-ml portions of water, and one 2-ml portion of 0.02% NaN_3 in water. The chromatography gel (assumed to have been adequately decontaminated by the regeneration process) was removed from the columns and stored at 4°C for further use.

When dialysis of the IgG samples was complete, the cassettes were returned to the biosafety hood. A water-soluble biotinylating reagent (sulfo-NHS-LC-Biotin; Pierce catalogue number 21335) was dissolved at a concentration of 3.43 mM in 6 mM sodium acetate (pH adjusted to 6.0 with acetic acid). A 150-μl portion was immediately injected into each dialysis cassette with a 1-ml syringe through a ¾-inch 22-gauge needle and was mixed with the IgG sample by repeated inversion of the cassette. The final volume inside the cassettes was nominally 2.57 ml, and the final concentrations of the reactants were nominally 200 μM biotinylating reagent and 5 to 7 μM IgG in 0.1 M $NaHCO_3$. The cassettes were sealed in small plastic bags and incubated for 2 h at room temperature. To quench and remove the remaining biotinylating reagent, the cassettes were dialyzed in the cold against three changes of TBS. The biotinylated IgG (Bio-IgG) was concentrated by placing the cassettes in small plastic bags with Slide-A-Lyzer concentrating solution (Pierce) for 2 h at room temperature, during which time the volume inside the cassettes dropped to less than 500 μl. The concentrating solution was washed away from the outside of the cassettes, and the samples of Bio-IgG were withdrawn through ¾-inch 23-gauge needles into 1-ml syringes and transferred into 1.5-ml screw-cap microcentrifuge tubes. TBS was added to bring the volume to 600 μl, followed by 600 μl of glycerol; after thorough mixing, the Bio-IgG samples were stored at −20°C.

Bio-IgG concentrations were estimated using a Coomassie protein assay reagent (Pierce) in the wells of a 96-well microplate read on a plate reader at 595 nm; bovine IgG at known concentrations served as standards. The final concentrations of Bio-IgG turned out to be 300 to 1,000 μg/ml.

A scaled-up adaptation of the above procedure was used to prepare Bio-IgG from 6 ml of pooled normal human serum (see "S/D treatment" above) (yield, 53 mg); this preparation will be called normal human Bio-IgG. The biotinylation level was estimated at 7 to 8 biotin groups per IgG molecule (data not shown); presumably the biotinylation level was roughly the same for the 20 small-scale Bio-IgGs from the individual positive and negative sera.

Absorption with phage carrier. A 120-μg sample of each Bio-IgG was diluted to 1 ml in D-PBS in a 1.5-ml screw-cap microcentrifuge tube. Then 50 μl of cross-linked wild-type fd phage (65) at a concentration of 5.2×10^{13} virions/ml in TBS was added, and the suspension was rotated overnight at 4°C. The cross-linked phage were removed by centrifugation at 13,000 × *g* for 5 min in a microcentrifuge and transfer of the supernatant to a fresh 1.5-ml microcentrifuge tube. The absorption procedure was repeated three more times with additional 50-μl portions of cross-linked phage. The fd-absorbed Bio-IgGs were stored at −20°C.

Depleting RPLs of peptides that bind major non-disease-specific subspecificities by precipitation from the solution phase. Portions (375 μl) of the RPLs at 10^{14} virions/ml in TBS were mixed with 350 μl of 4.56-mg/ml normal human Bio-IgG in TBS in screw-cap 1.5-ml microcentrifuge tubes. After rotation overnight at 4°C, 80 μl of streptavidin at 10 mg/ml in water was added to each tube, creating an insoluble aggregate that contained all the streptavidin, all the Bio-IgG, and any phage whose displayed peptide happened to bind one of the subspecificities in the IgG (the amount of streptavidin required to coaggregate all the streptavidin and all the Bio-IgG had been determined in preliminary experiments). The aggregates were removed by centrifugation for 5 min at 13,000 × *g* in a microcentrifuge and transfer of the supernatants to fresh 1.5-ml tubes. Reconstitution experiments indicated that this procedure removed at least 90% of a phage clone with high affinity for a subspecificity constituting only about 0.003% of the total IgG population (data not shown).

Depleting RPLs of peptides that bind major non-disease-specific subspecificities: solid-phase step. The depleted RPLs were subjected to two rounds of scaled-up mock affinity selection, using normal human Bio-IgG instead of positive Bio-IgG as the selector molecules. Twelve 150-mm polystyrene petri dishes (Fisher Scientific, Pittsburgh, Pa.) (catalogue number 8-757-14) were coated overnight with 40 ml of 10-μg/ml streptavidin in 0.1 M $NaHCO_3$ (unadjusted pH about 8.6), and another 12 were coated overnight with 40 ml of 10-μg/ml neutravidin (Pierce) in the same buffer. The dishes were emptied, blocked by filling them to brimming with blocking solution (see "Solutions and preparations" above) and incubating for 2 h at room temperature, and washed six times with TBS-Tween. Each dish was filled with 30 ml of 25-μg/ml normal human Bio-IgG in TTDBA and rocked overnight at 4°C in a humid plastic box. After six washes with TBS-Tween, the dishes were filled with 16 ml of TBS-Tween. The neutravidin-coated dishes were stored in a humid plastic box at 4°C. Meanwhile, biotin was added to the streptavidin-coated dishes to a final concentration of 10 μM, and the dishes were incubated for an additional 1 h at room temperature. Each of the depleted phage libraries (see above) (nominally 805 μl) was added to one of the streptavidin-coated dishes (without removing the solution already in the dish), and the dishes were rocked overnight at 4°C in a humid plastic box. Biotin was then added to the 12 neutravidin-coated dishes to a final concentration of 10 μM; the dishes were incubated for 1 h at room temperature, emptied, filled with the phage solution from the streptavidin-coated dishes, and rocked overnight at 4°C in a humid plastic box. The phage solutions collected from these dishes are called "depleted" libraries and served as the inputs for affinity selection; they were stored at 4°C. The physical particle concentrations, determined spectrophotometrically (15), ranged from 6.8×10^{11} to 3.2×10^{12} virions/ml.

Affinity selection. This paragraph outlines the overall plan of affinity selection; experimental details are given below. Each of the 12 depleted RPLs was subjected to three successive rounds of affinity selection, using Bio-IgGs from eight of the positive sera as the immobilized receptor molecules; these eight sera and their corresponding Bio-IgGs are called the "selector" sera and Bio-IgGs, respectively. Selector Bio-IgGs were exhaustively absorbed to remove antibodies (if any) against the phage carrier (see "Absorption with phage carrier" above); even at a low level, anticarrier antibodies can completely undermine affinity selection. In the first round, each of the eight selector Bio-IgGs was used to affinity select phage from each of the 12 depleted libraries, giving 96 first-round sublibraries. In the second round, three or four selector Bio-IgGs were used separately to affinity select phage from each of the 96 first-round sublibraries, giving 336 second-round sublibraries. The Bio-IgGs for the second round were chosen so that all 28 pairwise combinations of the eight selector Bio-IgGs were used in one order or the other to select phage from each of the 12 original depleted libraries (Fig. 1). For the third round of affinity selection, the two selector Bio-IgGs used for each of the 336 second-round sublibraries were mixed in equal amounts and used to affinity select peptides from that same sublibrary;

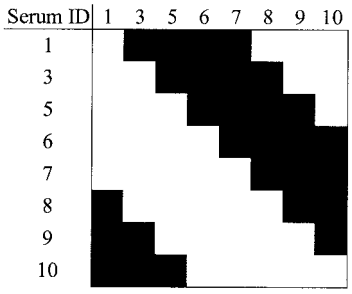

FIG. 1. Pairs of selector Bio-IgGs used to select phage from each RPL. Serum ID for selectors used in the first round of selection are listed down the leftmost column; serum ID for selectors used in the second round are listed across the top row. The 28 ordered pairs of selector Bio-IgGs are indicated by the filled cells in the table. Two of the positive sera from Lyme arthritis patients (ID 2 and 4) were not used in affinity selection.

this yielded 336 third-round sublibraries, which were analyzed for binding properties and served as the source of candidate diagnostic peptides.

First-round affinity selections were carried out in four polystyrene 24-well dishes (not treated for tissue culture; Falcon catalogue number 1147; Becton Dickinson and Co., Lincoln Park, N.J.). Wells were coated with streptavidin (10 μg/ml in 0.1 M $NaCO_3$; 200 μl/well), blocked, and washed with TBS/Tween as described above. The appropriate selector Bio-IgGs (2.5 μg) in 200 μl of TTDBA were added to the wells, and the dishes were incubated for 2 h at 4°C on a rocker to allow Bio-IgG molecules to be captured by the immobilized streptavidin. Biotin (20 μl of a 1 mM dilution) was added to each well and allowed to block unoccupied biotin-binding sites for 1 h at room temperature. Wells were washed six times with TBS-Tween and loaded with 500 μl of the appropriate depleted RPL (see above) diluted in TBS-Tween to a concentration of 10^{12} virions/ml (the libraries that were already slightly less concentrated than this were used undiluted). Dishes were rocked overnight at 4°C in a humid plastic box and washed 10 times with TBS-Tween to remove unbound phage. Bound phage were eluted in 100 μl of elution buffer (see "Solutions and preparations" above) for 10 min at room temperature on a rocker, the eluates were pipetted from the wells into tubes containing 18.5 μl of 1 M Tris-HCl (pH 9.1), and the tubes were immediately vortexed to allow the Tris buffer to neutralize the acid in the elution buffer. These were the 96 first-round sublibraries.

The first-round sublibraries were pipetted into sterile 1- by 3-in. polypropylene culture tubes with plastic caps, each of which then received 100 μl of K91BlueKan cells that had been freshly starved (80) and suspended in NAP buffer. After 10 min at room temperature to allow attachment of phage to bacterial host cells, 10 ml of NZY medium containing tetracycline at a concentration of 0.2 μg/ml was added, and the tubes were shaken for 45 min at 37°C (the subinhibitory concentration of tetracycline induces expression of the tetracycline resistance gene in the phage vector). Additional tetracycline was added to each culture to a final concentration of 20 μg/ml; for sublibraries in the f88-4 vector, IPTG was also added to a final concentration of 1 mM to induce expression of the recombinant pVIII displaying the guest peptide. A 200-μl portion of each culture was spread on a tetracycline plate, and the 12 input depleted RPLs were titered in parallel by standard procedures (80), allowing the yield to be calculated by side-by-side comparison of input and output for each of the 96 selections. The calculated yields were 2×10^{-6} to 2×10^{-5} percent—typical background yields observed after a single round of affinity selection.

Meanwhile, the 96 culture tubes were shaken overnight at 37°C, and phage were partially purified from culture supernatant by two polyethylene glycol (PEG) precipitations (80), the final precipitates being dissolved in 1 ml of TBS to give a final physical particle concentration of about 10^{12} virions/ml. These amplified first-round sublibraries provided the inputs to the second round of affinity selection.

The second round was carried out in four 96-well ELISA dishes, using essentially the same procedure as that used for the first round. A key difference was that neutravidin was substituted for streptavidin to capture Bio-IgG molecules in order to reduce the possibility of selecting streptavidin-binding peptides. The wells were coated with neutravidin (400 ng/well in 40 μl of 0.1 M $NaCO_3$), filled to brimming with blocking solution, incubated at least 2 h at room temperature, and washed five times with TBS-Tween on an automatic plate washer. Selector Bio-IgGs (250 ng in 25 μl of TTDBA) were added to appropriate wells and allowed to react with the immobilized neutravidin at least 2 h at 4°C on a rocker in a humid plastic box. Wells were washed five times with TBS-Tween on an automatic plate washer, filled with 20 μl of 0.1 mM biotin in TTDBA, and incubated at 4°C for 1 h. Without removing the biotin solution already in the wells, 20-μl portions of the amplified first-round sublibraries (3×10^{10} to 7×10^{10} virions) were added to the appropriate wells, and the dishes were incubated overnight at 4°C in a plastic humid box. Unbound phage were removed by washing with TBS-Tween in an automatic plate washer, and bound phage were eluted as described for the first round, yielding 336 second-round sublibraries. These sublibraries were amplified as described above for the first-round sublibraries, except that the volumes of eluate and of starved cells were both reduced to 50 μl, the culture volume was reduced to 5 ml, and phage were partially purified from only 1 ml of the culture supernatant so that they could be entirely processed in 1.5-ml microcentrifuge tubes. The final precipitates were dissolved in 100 μl of TBS and had nominal final physical particle concentrations of about 5×10^{12} virions/ml; these were the amplified second-round sublibraries. The yields for the second round of affinity selection were measured at 5×10^{-4} to 1.7×10^{-3} percent.

The third round of affinity selection was like the second round with two modifications. First, streptavidin was substituted for neutravidin. Second, the two selector Bio-IgGs used in the first two rounds were mixed at a concentration of 4 μg/ml each in 25 μl of TTDBA and reacted with the streptavidin-coated wells. Yields fell mostly between 0.001 and 0.01%, though yields from the f88-Cys6 and f8-8mer libraries were generally higher. As in the second round, the amplified sublibrary phage were partially purified from 1 ml of culture supernatant; the final precipitates were dissolved in 500 μl of TBS and archived in 96 deep-well plates (USA/Scientific Plastics, Ocala, Fla.; catalogue number 7553-9600). Meanwhile, the remaining 4 ml of the culture supernatants were screened by the phage capture ELISA described below and also served as the source for the individual candidate diagnostic peptides.

Phage capture ELISA. Wells of 96-well ELISA dishes were coated overnight at 4°C with 500 ng of streptavidin in 50 μl of 0.1 M $NaHCO_3$ (unadjusted pH 8.6); washed five times with TBS-Tween on an automatic plate washer; blocked by being filled to brimming with 5% dialyzed BSA and incubating for 1 h at room temperature; washed again; reacted with 100 ng of the test Bio-IgG in 50 μl of TTDBA for 2 h at room temperature; washed again; reacted with 50 μl of unprocessed culture supernatant containing phage at a physical particle concentration of about 3×10^{11} virions/ml for 2 h at room temperature; washed again; reacted 2 h at room temperature with 5 ng of anti-fd MAb freshly diluted in 50 μl of TTDBA; washed again; reacted for 2 h at room temperature with 50 μl of a fresh 1:2,000 dilution of AP-conjugated anti-mIgG2b in TTDBA; washed again; filled with 90 μl of NPP substrate; and read on a kinetic plate reader as described previously (80). The slope of yellow color development, measured in terms of change in optical density per 1,000 min (mOD/min), was taken as the ELISA signal (these units will be roughly but not exactly comparable from one plate reader to another). The ELISA signal gauges the number of phage captured from the culture supernatant by the immobilized Bio-IgG. Several other combinations of detection reagents were tried, but they gave much higher background-to-signal ratios than the procedure described above.

Antibody capture ELISA. Phage clones were propagated in 200-ml cultures, and virions were prepared at high purity as described previously (79). Wells of ELISA dishes were coated for at least 4 h at 4°C with 2×10^{10} virions in 50 μl of TBS; washed with TBS-Tween on a plate washer; reacted for at least 2 h at 4°C with 50 μl of individual serum samples (treated as described under "S/D treatment" above, but otherwise unprocessed) diluted 1:1,600 in TTDBA; washed; reacted for 1.5 h at room temperature with 50 μl of AP-conjugated goat antibodies to human immunoglobulins (AP-anti-hIg, AP-anti-hIgG, or AP-anti-hIgM) diluted 1:2,500 in TTDBA; washed again; and developed with NPP substrate as described under "Phage capture ELISA" above. For each serum, the ELISA signal with a control phage that does not bear a guest peptide (vector f8-5) was subtracted from the signals with peptide-bearing test phage to correct for the serum's background reactivity with the filamentous phage carrier; these background reactivities were all much lower than the positive signals reported in this article.

RESULTS

RPLs. The source of diagnostic peptides was 12 large RPLs, each consisting of a mixture of filamentous phage displaying random peptides on their surfaces. The salient features of the libraries, including the general structure of the displayed peptides, are summarized in Table 1. The libraries differ in the

TABLE 1. Characteristics of RPLs

Library	No. of clones[a]	Vector	Displayed peptide[b]	Conformational constraint	Copy no.[c]	Host coat protein[d]
f3-6mer	2×10^8	fUSE5	$ADGAX_6$	None	5	pIII
f3-15mer	2.5×10^8	fUSE5	$ADGAX_{15}$	None	5	pIII
f88-15mer	2×10^9	f88-4	X_{15}	None	~150	pVIII
f88-Cys0	5.6×10^8	f88-4	AX_5CCX_5	Disulfide	~150	pVIII
f88-Cys1	2.8×10^9	f88-4	AX_5CXCX_5	Disulfide	~150	pVIII
f88-Cys2	5.5×10^7	f88-4	$AX_5CX_2CX_5$	Disulfide	~150	pVIII
f88-Cys3	5.2×10^7	f88-4	$AX_5CX_3CX_4$	Disulfide	~150	pVIII
f88-Cys4	1.7×10^8	f88-4	$AX_4CX_4CX_5$	Disulfide	~150	pVIII
f88-Cys5	5.9×10^8	f88-4	$AX_4CX_5CX_4$	Disulfide	~150	pVIII
f88-Cys6	2.7×10^8	f88-4	$AX_4CX_6CX_4$	Disulfide	~150	pVIII
f88-LX6	10^{10}	f88-4	XCX_6CX	Disulfide	~150	pVIII
f8-8mer	2×10^9	f8-1	AX_8	Close packing	3,900	pVIII

[a] For all libraries but f3-6mer, the number of primary clones is also the number of different displayed peptides.
[b] Each sequence starts at the N terminus of the coat protein (just after the signal peptidase cleavage site) and extends through the last randomized amino acid. X, randomized amino acids; A, C, D, or G, invariant amino acids.
[c] Estimated number of copies of the peptide displayed per virion.
[d] Host protein to which the displayed peptide is genetically fused: either pIII (5 subunits in a ring at one tip of the virion) or pVIII (3,900 subunits arranged to form the tubular covering of the virion).

format of the display and in the presence of conformational constraints. In the f88-Cys0 to f88-Cys6 and f88-LX6 libraries, the constraint is a disulfide bond between a pair of invariant cysteines in an otherwise random sequence; the spacing between the cysteines ranges from 0 to 6 amino acids. The f8-8mer library is also considered to be constrained. Here, the random peptide is displayed on all 3,900 copies of the major coat protein, pVIII; the random peptide is held close to the surface of the phage particle, where interactions with that surface can impose a particular conformation on it. In the other three libraries, the displayed peptide has no purposely engineered constraints. All 12 libraries are "all-purpose," in that they display random peptides (encoded by degenerate synthetic oligonucleotides) that do not derive from any particular pathogen and are therefore applicable to any infectious disease.

Positive and negative sera. Twenty individual human serum samples were obtained from A. Steere (New England Medical Center). Ten of these (serum identification numbers [ID] 1 to 10) were positive sera from LD patients, and the other 10 were negative sera from control donors with rheumatoid arthritis (ID 11 to 15) or psoriatic arthritis (ID 16 to 20). Of the 10 LD patients, six (ID 5 to 10) had erythema migrans followed within weeks by signs and symptoms of early disseminated infection. During this period, two of the six had migratory joint pain (ID 5 and 9), two had carditis with various degrees of AV nodal block (ID 8 and 10), and two had lymphocytic meningitis (ID 5 and 6). The other four LD patients (ID 1 to 4) had erythema migrans followed months to years later by intermittent attacks of joint swelling and pain in knee joints, a late manifestation of the disorder called Lyme arthritis. All 10 LD patients met the Centers for Disease Control and Prevention (CDC) criteria for diagnosis of LD (78): they had characteristic clinical manifestations of the disorder and a positive antibody response to *B. burgdorferi* by ELISA and immunoblotting, interpreted according to CDC and Association of State and Territorial Public Health Laboratory Directors criteria (7). Conversely, the 10 control donors had negative serological tests for *B. burgdorferi*. Pooled sera from normal donors served as a generic negative control serum, which will be called normal human sera. The sera were processed to reduce the risk of infection by bloodborne pathogens, and their IgG fractions were isolated and biotinylated (see Materials and Methods); each serum sample was thus represented by the Bio-IgG derived from it.

The antibodies in these sera are actually complex populations of thousands of subspecificities, each recognizing an epitope on a foreign antigen the subjects have been exposed to in their lifetimes. The population in positive sera will include some disease-related subspecificities that have been elicited by natural epitopes on the pathogen. But of course all sera, both positive and negative, will contain numerous background subspecificities against epitopes that have nothing to do with the disease. In order to be diagnostically useful, a peptide must be recognized by disease-related subspecificities in some of the positive sera, but not by background subspecificities in any of the negative sera.

Affinity selection of candidate peptides from RPLs. Bio-IgGs from positive sera were used as "selectors" to affinity select phage-displayed peptides that bind subspecificities within the IgG population (see Materials and Methods). The positive Bio-IgGs were used in pairs: one Bio-IgG served as the selector in the first round of affinity selection, the other served as the selector in the second round, and a mixture of both served as the selector in the third round. This procedure should enrich for peptides that bind at least two positive sera—arguably a minimal standard for a useful diagnostic peptide. It is entirely possible that a single peptide epitope will come to dominate the selected population after this stringent selection series. In the hope of preserving a diversity of peptide epitopes, therefore, peptides were selected from the 12 RPLs separately, using all 28 pairwise combinations of eight positive Bio-IgGs as selectors (see "Affinity selection" above) (Bio-IgGs from two Lyme arthritis patients, ID 2 and 4, were not used for selection but were used for subsequent screening). The result of these 336 ($= 12 \times 28$) parallel selection series is a collection of 336 "sublibraries," each comprising a selected subset of the phage-displayed peptides present in one of the 12 initial libraries—possibly even a single peptide.

Although the foregoing selection scheme is designed to strongly favor peptides that bind at least two positive Bio-IgGs,

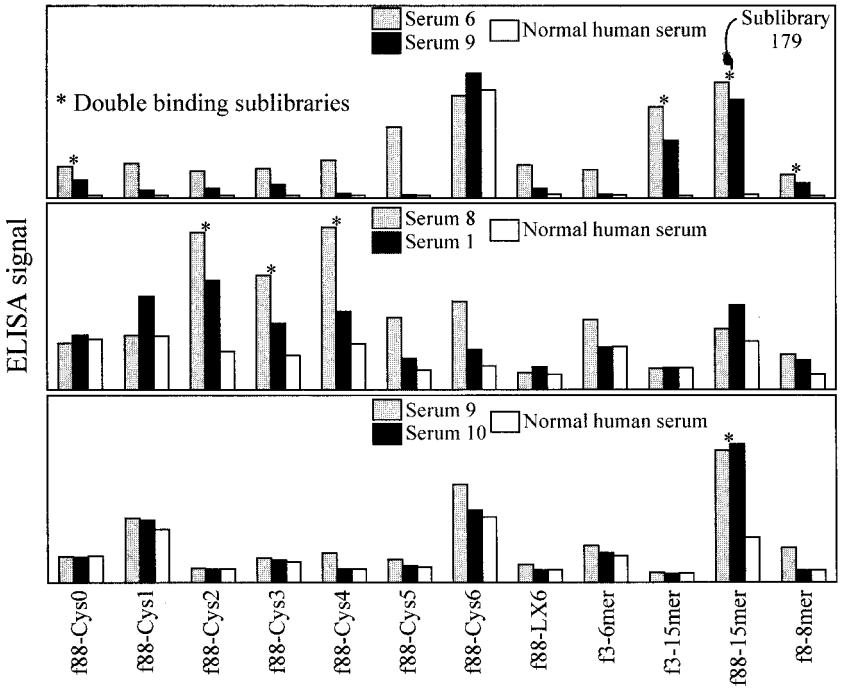

FIG. 2. Survey of 36 sublibraries by phage capture ELISA. Each of the three panels shows results for the 12 sublibraries affinity selected with a given pair of selector Bio-IgGs. Shaded and solid bars, ELISA signals with the first and second selector Bio-IgGs, respectively; open bars, ELISA signals with the pooled normal human Bio-IgG. The original libraries (Table 1) from which the sublibraries were selected are shown along the bottom. For all three panels, full scale on the ordinate axis corresponds to an ELISA signal of 60 mOD/min. Asterisks mark sublibraries classified as double binders (see the text), including, as an example, sublibrary 179.

there is no guarantee that any individual peptide within a sublibrary population is recognized by a disease-related subspecificity. The peptides obtained from affinity selection were therefore surveyed in three stages with the entire panel of positive and negative sera to identify those with a diagnostically useful pattern of reactivity, as described below.

Screening whole-sublibrary populations and individual clones by phage capture ELISA. As the first stage in surveying the selected peptides, each of the 336 sublibrary populations was tested en masse—without being resolved into individual peptide-bearing phage clones—for the ability to bind the two selector Bio-IgGs used to select it; as a negative control, the sublibraries were also tested for reactivity with normal human Bio-IgG from a pool of sera from healthy donors. Sublibraries showing good reactivity with the two selector Bio-IgGs and low reactivity with normal human Bio-IgG were considered the most promising lodes to mine for diagnostic peptides.

This first stage of the survey was accomplished using a phage capture ELISA (see Materials and Methods), whose inputs were unprocessed culture supernatants from propagating the sublibrary populations (see Materials and Methods). By obviating the need to partially purify the peptide-bearing phage before they could be assayed, this method greatly expedited the survey. The signal-to-noise ratio is relatively low in this assay, but still high enough to identify promising sublibraries.

Results for 36 of the 336 sublibraries are shown as an example in Fig. 2. Each panel gives ELISA signals for the 12 sublibraries selected with a given pair of selector sera. Sublibraries classified as "double binders" reacted well with both selector Bio-IgGs and much less with normal human Bio-IgG. Among the double binders is sublibrary 179, selected from the f88-15 library using Bio-IgGs from positive sera 6 and 9 as the selectors.

Altogether 21 of the 336 sublibraries were classified as double binders. Three to five individual peptide-bearing phage clones from each of these sublibraries (more from a few sublibraries; 99 clones altogether) were propagated using standard methods (see Materials and Methods) and tested by phage capture ELISA with the 10 individual positive Bio-IgGs and (as a negative control) with normal human Bio-IgG. Results for seven clones from sublibrary 179 are shown as an example in Fig. 3. As is evident in the graph, clones 6, 7, and 9 show little reactivity with normal human Bio-IgG but strong reactivity with several of the positive Bio-IgGs—not only the selector Bio-IgGs (those from sera 6 and 9), but also at least two other Bio-IgGs (those from sera 3 and 5). This survey identified 22 individual peptides with good reactivity with the selector Bio-IgGs and little or no reactivity with normal human Bio-IgG. These promising peptides were studied further by sequence analysis and antibody capture ELISA, as described below.

Selected peptides fall into eight sequence motifs. The 22 promising peptides identified above, along with four other peptides identified in various pilot experiments, were sequenced by determining the peptide coding sequence in the phage DNA (80). The 26 clones turned out to represent 17 different amino acid sequences, which could be classified into eight motifs, labeled A to H in Table 2. None of them convincingly matches any of the hypothetical proteins of *B. burgdorferi* (The Institute for Genomic Research website [http://www.tigr.org/tdb/CMR/gbb/htmls/SplashPage.html]). This does not mean that they do

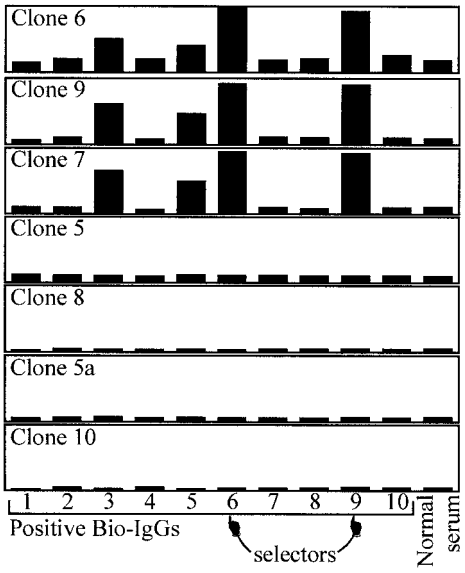

FIG. 3. Survey of seven individual phage clones from sublibrary 179 by phage capture ELISA. Bars, ELISA signals for each clone tested with each of the 10 positive Bio-IgGs and with normal human Bio-IgG, as listed along the bottom; the Bio-IgGs used as selectors for sublibrary 179 are indicated. For all seven panels, full scale on the ordinate axis corresponds to an ELISA signal of 42 mOD/min.

not correspond to pathogen epitopes, however. Antibodies elicited by both proteinaceous and nonproteinaceous epitopes frequently select peptides from RPLs that bind the antibody tightly and specifically but do not align with the eliciting epitope at the amino acid sequence level; such peptides are called "mimotopes" (25, 26).

Table 2 shows the library of origin and the pair of positive Bio-IgGs used to select each peptide. The distribution of motifs among the 336 sublibraries vindicates the selection strategy outlined above. As anticipated, many individual sublibraries were dominated by a single motif; for instance, clones 6, 7, and 9 from sublibrary 179 (Fig. 3) all belong to motif A (Table 2, footnote a). This indicates stringent selection for a single "fittest" motif and accounts for the fact that the serum reaction pattern of a bulk sublibrary population is frequently a good guide to the reaction patterns of its individual member clones. Nevertheless, by and large, different initial libraries yielded different motifs (except motif F, which was obtained from both the f88-Cys2 and f88-Cys3 libraries), and different pairs of selector sera could select different motifs from the same initial library (motifs A and B from the f88-15mer library; motifs D and E from the f88-LX6 library). Thus, diversity was maintained even in the face of stringent selection. An alternative strategy for accomplishing much the same goal using DNA hybridization has been published (2).

Screening peptides with the entire panel of positive and negative sera by antibody capture ELISA. Of the 17 peptides shown in Table 2, 12 were chosen for further study (motifs D, E, and G were dropped because these peptides showed little or no reactivity with Bio-IgGs other than their respective selectors). The reactivities of all 10 positive and all 10 negative sera for each of these peptides were surveyed using antibody capture ELISA. In this assay, it is antigen, not antibody, that is immobilized to the surface of the plastic well; the test antibody is added to the antigen-coated well in the form of whole serum, and antibody molecules that are captured by the immobilized antigen and remain bound after extensive washing are detected with appropriate secondary reagents (see Materials and Methods). Antibody capture ELISA requires purification of the antigen but can accommodate unprocessed serum; it is therefore suitable for surveying large numbers of serum samples with a limited number of antigens. Antibody capture ELISA is the method predominantly used in actual serodiagnostic tests.

The ELISA signals in Fig. 4 were developed with an enzyme-linked second antibody specific for human IgG (AP-anti-hIgG). It is evident from Fig. 4 that the peptides with a given motif have very similar patterns of reactivity, while peptides with different motifs have very different patterns. Thus, motifs reflect real functional groupings, not just an arbitrary labeling.

All peptides are much more reactive with some of the positive sera than with any of the negative sera. Furthermore, the highly reactive sera include not only the selector sera used to select the peptide in the first place, but also positive sera that played no role in its isolation. This reaction pattern strongly suggests that the peptides are recognized by authentic disease-related subspecificities characteristic of LD patients, not idiosyncratic, non-disease-related subspecificities that happen to be shared by the two selector sera.

It is noteworthy in Fig. 4 that all peptides react strongly with sera from LD patients in multiple diagnostic categories, a

TABLE 2. Sequences and origins of selected peptides

Peptide sequence[a]	No. of clones[b]	Selector serum[c]		Motif	Library[d]
		First	Second		
GNNSVSKEKPPSLNWPp	1	6	9	A	f88-15mer
TELKLAPPVLNAPPL	2	6	9	A	f88-15mer
KAHPPLLLNSPRDVPL	1	6	9	A	f88-15mer
YPKESPPRLNAPWYQ	1	9	10	A	f88-15mer
ESKLTPPPLNPIRVV	2	5	9	A	f88-15mer
KPRDTLPPPLNRPPP	3	8	9	A	f88-15mer
VPVDAPHAGTKPHSA	2	10	1	B	f88-15mer
PKSSCTQNPILCAILS	1	7	10	C	f88-Cys6
SCPEGSKLCI	1	7	9	D	f88-LX6
TCPEGAKLCD	1	7	9	D	f88-LX6
SCAEGAKYCL	1	7	9	D	f88-LX6
ACVAGDATCK	1	1	10	E	f88-LX6
EKRFACKPLCNTPA	2	8	1	F	f88-Cys3
QKDFACKHCKLPSP	2	8	1	F	f88-Cys2
RPDRLCPCVDPRE	1	8	1	G	f88-Cys2
QRSECSTSKCFVRK	2	8	1, 10[e]	H	f88-Cys4
YREACTNGKCFVLK	2	8	1, 10[e]	H	f88-Cys4

[a] The amino acids shown correspond to the random peptides in Table 1, except that the invariant Ala (A) at the N termini of the f88-Cys0 to f88-Cys6 peptides is omitted. Shared amino acids that define motifs A, D, F, and H are underlined. The proline residue at the end of the first peptide (lowercase "p") is actually an invariant flanking residue but is included because it aligns with the consensus sequence of motif A. Clones 7 and 9 from sublibrary 179 (Fig. 3) are identical and display the second peptide; clone 6 from sublibrary 179 displays the sixth peptide.

[b] Number of clones displaying the indicated peptide among the analyzed phage.

[c] Serum sources of the Bio-IgGs used as selectors for the first and second rounds of affinity selection.

[d] Initial RPL (Table 1) from which each peptide was selected.

[e] Each of these peptides was selected once using Bio-IgG from serum 8 as the first selector and Bio-IgG from serum 1 as the second, and also once using serum 8 Bio-IgG as the first selector and Bio-IgG from serum 10 as the second.

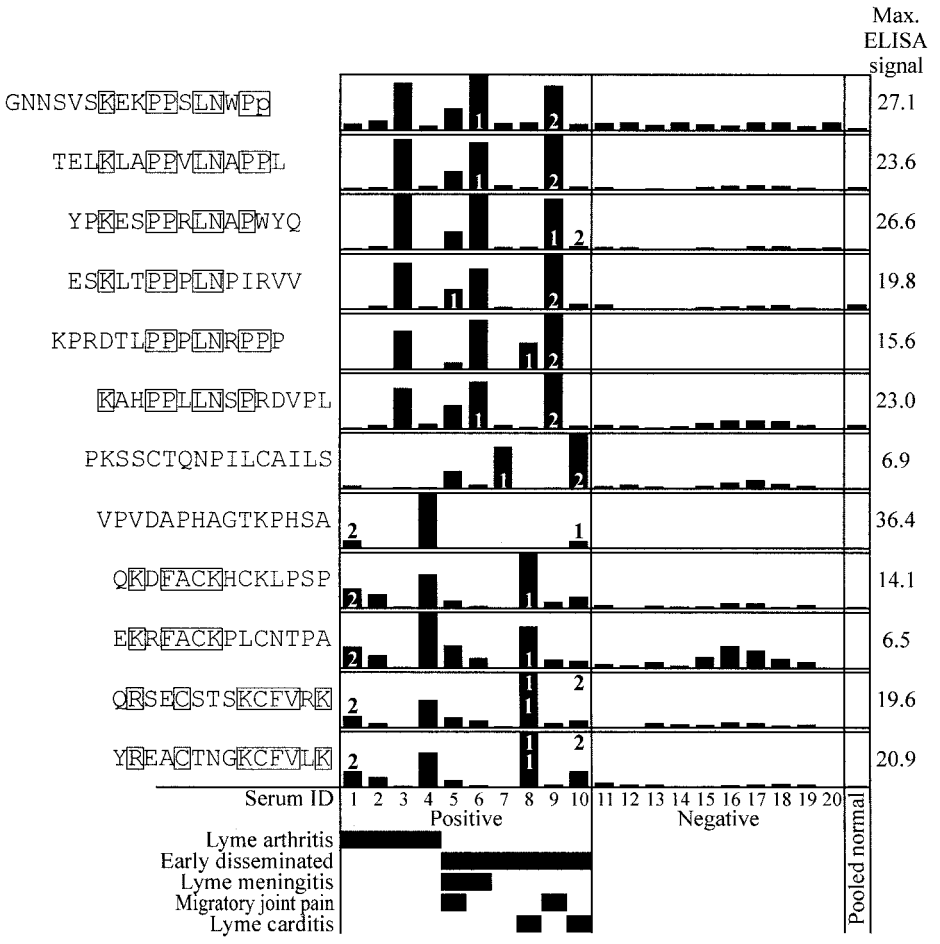

FIG. 4. Reactivities of 12 phage-displayed peptides with positive and negative sera as determined by antibody capture ELISA. In each panel, the ELISA signals have been normalized to the maximum signal for that peptide, which is given on the right. The numbers 1 and 2 indicate the sera whose Bio-IgGs served as selectors in the first and second rounds of affinity selection, respectively. For the last two peptides, which emerged in two different sublibraries, two pairs of selectors are indicated in separate rows. Motif residues are boxed.

propitious reaction pattern for serodiagnostic epitopes. Such broadly reactive peptides are favored by the discovery process. This is most obviously so when the selector sera come from patients in different diagnostic categories. But even if the selector sera come from patients in the same category, subsequent screening by phage capture ELISA will identify peptides that bind serum antibodies from other categories. It is possible, therefore, that these peptides have a reaction pattern that is not typical of disease-related epitopes in general.

All positive sera react with at least some of the peptides. Indeed, four peptides give good coverage of all 10 positive sera with little reactivity to negative sera: YPKESPPRLNAPWYQ (motif A), PKSSCTQNPILCAILS (motif C), QKDFACKHC KLPSP (motif F), and YREACTNGKCFVLK (motif H).

The antibody capture ELISA was repeated using different enzyme-linked second antibodies to detect the captured primary antibodies: second antibodies specific for human IgM and for human immunoglobulins of all isotypes (AP-anti-hIgM and AP-anti-hIg, respectively). The second antibody specific for immunoglobulins of all isotypes gave ELISA signals that closely parallel those shown in Fig. 4 (data not shown). In contrast, none of the positive or negative sera showed significant IgM reactivity with any of the peptides, with one exception: one of the early disseminated-LD serum samples (serum ID 9) had definite IgM reactivity to all six motif A peptides (which also reacted with IgG from this serum sample), as well as to the motif C peptide (which did not react with IgG from this serum sample). The generally low IgM reactivity of the positive sera was not unexpected, since evidently the LD donors all mounted IgG responses to pathogen epitopes. Perhaps if serum 9 had been obtained later in the course of the disease, its IgM reactivity would have decayed and its IgG response to motif C would have emerged.

DISCUSSION

The reaction patterns of the peptides discovered in this project augur well for their value as diagnostic antigens. An ELISA using four of the peptides in separate wells distinguishes all positive sera from all negative sera in the panel, and would be far cheaper and simpler than Western blot analysis. It is likely that a one-well assay using a mixture of peptides would succeed as well, though inevitably some diagnostic power would be lost in thus reducing the number of dimen-

sions of signal information. There is no need for extensive processing of the samples, since unprocessed sera yield excellent results in antibody capture ELISAs (Fig. 4). Background reactivity with negative sera is very low, and it is entirely possible that background can be reduced further by synthesizing the peptides chemically and coupling them covalently to the reactive surface in the absence of carrier or other chemically complex components. The peptides are presumably adaptable to formats other than ELISA.

A true assessment of the peptides' value requires that they be surveyed with a large new panel of positive and negative sera that were not used to identify them in the first place. Such a survey will undoubtedly turn up false negatives (positive sera that fail to react with any of the peptides) and false positives (negative sera that react with one of the peptides). These sera can serve as the basis of a "remedial" discovery program that is specifically focused on deficiencies in the original set of peptides. Thus, antibodies from the false-negative sera are used as selectors to affinity select new peptides, which are screened for reactivity to both false-positive and false-negative sera (along with other positive and negative sera). This process should rapidly converge on a set of peptides with particularly high diagnostic value, enhancing the prospect of a definitive ELISA that could replace current Western blot analysis.

Early diagnosis of LD is an important goal, since antibiotic therapy soon after infection can prevent serious and sometimes irreversible sequelae. The earliest humoral response to a new foreign antigen generally manifests itself in the form of IgM antibodies, and an effective serodiagnostic test will undoubtedly encompass this isotype by use of appropriate second antibodies or other secondary reagents. The selector molecules used in the discovery program described here were not IgM antibodies from patients in the early stage of LD, however, but IgG antibodies from patients in whom LD was already well established. The reason for this choice of selectors was the assumption that many or most IgM responses to an epitope develop eventually into IgG responses to the same epitope. Indeed, the response of the donor of serum 9 to the motif A epitope may have been in transition from IgM to IgG, since antibodies of both isotypes bound the motif A peptides. IgG antibodies, which are generally of higher affinity and higher prevalence in the serum, may thus be more effective selectors for epitopes recognized by early IgM antibodies than the IgM antibodies themselves. Nevertheless, Western blots of pathogen extracts show an evolving pattern of reactivity as disease progresses, suggesting that there may be specific epitopes that are recognized by IgM antibodies soon after infection but not by IgG antibodies afterwards. This possibility would be corroborated if sera manifesting a definite early IgM response as evidenced by Western blot analysis generally fail to show IgM reactivity with the IgG-selected peptides. In that case, a remedial selection program that uses early IgM antibodies as the selectors might identify peptides that are particularly effective for early diagnosis of the disease. There is a limit to the improvement of early diagnosis, of course, since no serodiagnostic test, whatever molecules serve as antigens, can hope to detect an antibody response that has yet to emerge.

Although LD is caused by a well-known pathogen whose antigenic structure has been studied at length, nowhere was this advance knowledge used in the discovery process reported here. Nevertheless, that process succeeded in identifying diagnostic peptides, using all-purpose RPLs and a simple generic selection protocol that is applicable without change to almost any infectious disease. The only disease-specific resource exploited was a panel of positive and negative sera, a resource that is readily available for most infectious diseases—even emerging diseases for which the pathogen has yet to be identified. Indeed, peptides obtained through epitope discovery, being putative mimics of authentic pathogen epitopes, might provide a valuable new gateway to research into the pathogen. In particular, they can be used to affinity purify monospecific antipathogen antibodies from patient sera, and these antibodies can in turn be used as probes to help identify the pathogen or otherwise illuminate the disease process. We therefore regard the work reported here not only as an advance in LD diagnosis in particular, but also as proof of the principle of a new approach to diagnosis of great power and generality.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant R42 A141776 from the U.S. National Institutes of Health and by a special grant from Kosan Biosciences, Inc.

We are grateful to Robert Davis and Natalia Petrenko for excellent technical assistance, to Allen Steere (New England Medical Center) for serum samples and relevant medical histories of the donors, and to Daniel Santi (Kosan Biosciences, Inc.) for advice.

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