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Prediction of the Immunodominant Epitope of the Pyruvate Dehydrogenase Complex E2 in Primary Biliary Cirrhosis Using Phage Display

Merrill J. Rowley,1 Marita Scealy, James C. Whisstock, Jennifer A. Jois, Lakshmi C. Wijeyewickrema, and Ian R. Mackay

Primary biliary cirrhosis (PBC) is an autoimmune liver disease characterized by autoantibodies reactive with the pyruvate dehydrogenase complex. A conformational epitope has been mapped to aa 91–227 within the inner lipoyl domain of the E2 subunit (pyruvate dehydrogenase complex E2 (PDC-E2)). We have used phage display to further localize this epitope. A random heptapeptide library was screened using IgG from two patients with PBC, with negative selection using pooled normal IgG. Phage that contained peptide inserts (phagotopes) selected using PBC sera differed from those selected using IgG from patients with RA or polychondritis. Two motifs occurred only among the PBC-selected phagotopes; these were MH (13 sequences, 16 phagotopes) and FV (FVEHTRW, FVEIYSP, FVLPWRI). The phagotopes selected were tested for reactivity with anti-PDC-E2 affinity purified from four patients with PBC. Phagotopes that contained 1 of 15 different peptide sequences were reactive with one or more of these four anti-PDC-E2 preparations, whereas phagotopes that contained 1 of the remaining 28 sequences were negative. The peptides (FVLPWRI, MHLNTPP, MHLTQSP) encoded by three phagotopes that were strongly reactive with all four preparations of anti-PDC-E2 were synthesized. Each of the selected peptides, but not an irrelevant peptide, inhibited the reactivity by ELISA of affinity purified PBC serum with recombinant PDC-E2 and reduced the inhibition of the enzyme activity of PDC by a PBC serum. The peptide sequences, along with the known NMR structure of the inner lipoyl domain of PDC-E2, allow the prediction of nonsequential residues 131HM132 and 178FEV180 that contribute to a conformational epitope.

X-ray crystallographic studies have been used to examine the structure of epitopes for a number of different Ags (1). In most cases, the Abs react with conformational epitopes formed from assembled topographic determinants made up of residues brought into contact on the surface of the molecule during protein folding; such regions often contain T cell epitopes as well (2). These epitopes have an area of 650–900 Å2, and the affinity of Ag-Ab binding depends on the interaction of just a few contiguous but discontinuous amino acid residues on the surface of the Ag molecule (1). Identification of these reactive residues is fundamental to understanding the generation of the Ab response but conventional techniques of epitope mapping rarely define conformational epitopes more precisely than to within 100–200 aa. Although crystallographic studies to define critical residues for Ag binding have been performed for immune Abs, very little is known about the interactions of spontaneously occurring autoantibodies with cognate autotigens. In particular, critical residues for autoantibody binding have not been defined, although this is essential for an understanding of the nature of the autoimmune response and for identifying cross-reactivities that could be operative in examples of molecular mimicry.

Primary biliary cirrhosis (PBC)2 is an autoimmune liver disease characterized by autoantibodies (anti-M2) reactive with components of the pyruvate dehydrogenase complex (PDC) or of the related enzyme complexes, 2-oxoacid dehydrogenase and branched chain oxoacid dehydrogenase (3). PDC, a multienzyme complex \( M_r \ 8.5 \times 10^6 \), is located on the inner mitochondrial membrane and catalyzes the conversion of pyruvate to acetyl-CoA. The complex is composed of multiple copies of three enzymes that act in sequence, the pyruvate dehydrogenase E1, the dihydrolipoamide acetyltransferase E2, and the flavine adenine dinucleotide-dependent dihydrolipoamide dehydrogenase E3 (4). Most patients (>90%) with PBC have autoantibodies to PDC-E2.

The major epitope within PDC-E2 has been mapped to the region associated with the inner lipoyl domain wherein a lysine residue binds the lipoic acid cofactor for the enzyme, and there is a minor epitope associated with the outer lipoyl domain (5, 6). Using recombinant polypeptides, the inner lipoyl domain epitope was mapped to aa 91–227 (5); a short synthetic peptide representing aa 167–186 that included the inner lysine residue 173 (K173) absorbed most reactivity with PBC sera by ELISA, albeit only at a serum dilution of 1:80,000 (5). On the other hand, studies by Koike et al. (7) using purified porcine PDC and PDC-E2, or a 37-kDa trypsin fragment that represented the two lipoyl domains of PDC-E2, showed that there was a very clear loss of autoantigenicity during fragmentation of the PDC-E2. Moreover, although a short synthetic peptide (aa165–178) central to the inner lipoyl domain slightly inhibited the binding of PBC sera to solid-phase PDC by ELISA, the level of inhibition with 25 µg of peptide was much less than that obtained by 0.005 µg of PDC. As further evidence that

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2 Abbreviations used in this paper: PBC, primary biliary cirrhosis; PDC, pyruvate dehydrogenase complex; RA, rheumatoid arthritis.
the reactive epitope is not precisely defined by a short peptide central to the inner lipoyl domain, immunoreactivity of PDC-E2 may not require the lipolic acid cofactor, since enzymatic delipolylation or delipolysis did not change the reactivity of purified PDC or PDC-E2 with PBC sera (7), although a peptide 167–184 was more reactive after delipolysis (8). Thus, although the potent reactivity of PBC sera by immunoblottting on recombinant PDC-E2 is suggestive of a sequential epitope, it is more likely that there is an undefined conformational epitope residing within the peptide sequence 91–227 of PDC-E2.

A new approach to the identification of nonlinear epitopes is Ab probing of phage-displayed peptide libraries which can reveal conformational epitopes and also linear mimotopes that mimic the shape of conformational epitopes (9). Conformational epitopes have been identified using mAbs (10–12) and also using polyclonal Abs in immune sera from both animals and humans (13, 14). However, there have been few definitive studies on polyclonal autoantibodies in human sera using phage display libraries. The very high levels of Abs to PDC-E2 in PBC, and the ready availability of recombinant PDC-E2, indicate that phage display technology should be applicable to identification of mimotopes of PDC-E2 that are reactive with autoantibodies. We describe the isolation of phage that contain peptide inserts (phagotopes) using individual sera from two PBC patients and the identification of particular phagotopes that react with affinity-purified PDC-E2 from four patients. This has enabled a prediction of the major conformational epitope for PDC-E2 in PBC based on comparison of the reactive sequences derived by phage library screening with the known NMR structure of the inner lipoyl domain.

Materials and Methods

**Sera**

The sera were used from four patients with PBC (PBC 1–4) diagnosed according to published criteria (15). All patients had high titers of Abs to PDC-E2 and other related Ags as determined by immunoblottting on bovine heart mitochondria (16). One patient, PBC1, also had type I diabetes and her serum contained autoantibodies to glutamic acid dehydrogenase. Control sera were derived from patients with rheumatoid arthritis (RA;1 subject) and relapsing polychondritis (1 subject), selected by reason of very high serum levels of an irrelevant autoantibody to type II collagen, and healthy laboratory staff (10 subjects). The 10 normal sera were pooled to provide IgG for negative selection during biopanning. IgG was prepared from the sera by affinity purification on protein A-Sepharose (Pharmacia, Uppsala, Sweden).

**Preparation of affinity-purified Abs using recombinant PDC-E2**

A cDNA fragment of human PDC-E2 that represented nt 1095–2207 corresponding to aa 109–470 was ligated into pGEX and expressed as a GST fusion protein (17). This fusion protein contains only the inner lipoyl domain but reacts strongly and specifically with PBC sera (17). Abs to PDC-E2 were prepared by affinity purification on recombinant protein (16).

**Phage-displayed random peptide library**

The Ph.D-7 random heptapeptide library was purchased from New England Biolabs (Beverly, MA). The library contained 2 × 10^10 PFU/ml with a complexity of 2 × 10^10 transformatants. The displayed heptapeptides were expressed at the amino terminus of the pIII coat protein of the filamentous coliphage M13, and the library was used at a concentration of 8 × 10^10 PFU/ml. The library was stored in TBS (pH 7.4) with 50% glycerol. Phage was propagated in *Escherichia coli* strain ER2537, which was provided with the library kit as a noncompetent glycerol stock. The DNA inserts from individual nonselected colonies were sequenced from the library to confirm that they contained random peptide sequences. All phages selected contained inserts, although two of the phages had the amber stop codon (TAG) that is expressed as glutamine in this library. All 20 aa codons were represented in the random sequences close to their expected frequencies based on the genetic code.

**Biopanning and isolation of phage**

The library was screened by biopanning using 100 μg of IgG mixed overnight at 4°C with 2 μl of the phagemid library in 500 μl of PBS (pH 7.3) containing 1 mg/ml BSA (PBS-BSA). Phages that bound to the IgG were isolated using magnetic beads coated with anti-human Ig (Chemicon International, Temecula, CA), washed 10 times in PBS-BSA, eluted from the magnetic beads with 1 mg/ml BSA, 0.1 M HCl (pH 2.2) adjusted with glycine, and then neutralized with 1 M Tris-HCl (pH 9). After this first round of “positive” selection, there was a “negative” selection step in which phages not specifically reactive with PBC sera were removed using 100 μg of IgG prepared from pooled normal sera. Then the phages were amplified by infecting *E. coli* ER2537 cells in the logarithmic phase of growth and allowing them to grow at 37°C with shaking for 4.5 h. Phages from different culture were obtained by twice centrifuging the supernatant twice by centrifugation, 13,000 × g for 5 min. Phage particles were precipitated with 4% polyethylene glycol 6000 in 0.5 M NaCl, centrifuged at 13,000 × g for 15 min, and the pellets were resuspended in 1 ml TBS. The amplified eluate was enriched by a further two rounds of positive selection, negative selection, and amplification. Phages were purified from single colonies after the third round of biopanning and were used for DNA sequencing, and tested for reactivity with affinity-purified anti-PDC-E2 by capture ELISA.

Each biopanning involved three rounds of positive selection with IgG from one patient. In all, there were performed four separate biopannings using IgG from two patients with PBC: one with RA and one with relapsing polychondritis.

**DNA sequencing**

Single-stranded DNA was prepared by phenol extraction as described by Sambrook et al. (18) and sequenced using the Sequenase version 2.0 T7 DNA polymerase sequencing kit (United States Biochemical, Cleveland, OH). The M13 (-28 gII) primer sequencing primer supplied in the library kit was used and α-35S-dATP Redivue (Amersham International, Little Chalfont, U.K.) was used as label. Sequencing was conducted using dGTP, and ambiguous sequences were resequenced using the nucleotide analogue dITP to minimize secondary structure formation.

**Capture ELISA**

Phages were tested for reactivity with Abs to PDC-E2 by capture ELISA in which 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated with 100 μl/well of affinity-purified anti-PDC-E2 in sterile PBS (pH 7.3) overnight at 4°C in a humidified chamber. The plates were blocked for 2 h at room temperature with 200 μl/well of 1% skimmed milk powder in 0.05% Tween 20 in PBS and washed three times with 0.005% Tween 20 in PBS (pH 7.4). Plates were blocked with 100 μl of purified phage diluted 1:20 in sterile PBS were added to the wells and held overnight at 4°C. The plates were blocked and washed as before, and 100 μl of a 1:2000 dilution of an Ab to M13 raised in sheep (Pharmacia Biotech, Uppsala, Sweden) in 1% skimmed milk powder in 0.05% Tween 20 in PBS was added to each well. IgG was detected using HRP-conjugated anti-sheep/goat Ig (Silenus, Hawthorn, Australia) with 0.5 mg/ml 2,2'-azino-bis-(3-ethylbenzathiazoline-6-sulfonic acid) (Boehringer Mannheim, Mannheim, Germany) in 0.03 M citric acid, 0.04 M NaHPO₄, and 0.003% H₂O₂ (pH 4) as substrate. A result greater than the mean + 2 SDs of the A₄15 readings obtained for eight different preparations of phage without inserts on the same plate was considered positive.

**Peptide ELISA**

Peptides representing the sequences in the three most reactive phagotopes in the capture ELISA (FVLPWRI, MHLNTPP, and MHLTQSP) and an irrelevant control peptide CIAPKRHNSAC were purchased from Chiron Technologies (Melbourne, Australia). The peptides were synthesized with an N-terminal free amine and a C-terminal free acid and were of 90–95% purity as assessed by HPLC and mass spectrometry. The reactivity of PBC sera with the peptides was tested by ELISA. Microtiter plates were coated with 20 μg/ml of peptide in 50 mM carbonate buffer (pH 9.6) or PBS (pH 7.4) and held overnight at 4°C. Plates were washed, blocked with 3% BSA in PBS, and reacted with doubling dilutions from 1:200 of sera from PBC patients or controls overnight at 4°C. Bound IgG was detected using HRP-conjugated anti-human IgG (Silenus) with 0.5 mg/ml 2,2’-azino-bis-(3-ethylbenzathiazoline-6-sulfonic acid) as substrate. A result greater than the mean + 2 SDs of the A₄15 readings obtained for eight different preparations of phage was considered positive.

**Peptide inhibition of reactivity of PBC sera with rPDC-E2**

The ability of the phage peptides to inhibit the reactivity of a PBC serum with rPDC-E2 was tested in a direct ELISA. Microtiter plates were coated with 1 μg/ml of PDC-E2 in PBS (pH 7.4) overnight at 4°C. Serum from
PBC2 at a final dilution of 1:100,000 was mixed with dilutions of peptide inhibitors, preincubated for 1 h at room temperature, transferred to the rPDC-E2-coated plate, and left overnight at 4°C before development as described above. The serum dilution (1:100,000) was chosen to give an A450 in the ELISA of ~0.5 in the absence of inhibitor. The peptides were tested at 1, 2, 5, and 25 μg/ml, and rPDC-E2 was used as a positive control at 10 μg/ml. Each dilution was tested in quadruplicate and compared with the results of 16 wells that contained no inhibitor. The enzyme inhibitory activity of PBC sera was measured in a microtiter plate assay as described previously (19). To test the ability of the peptides to interfere with the strong enzyme inhibitory function of anti-PDC-E2, 2 μl of a highly inhibitory serum diluted 1:20 in PBS was preincubated for 1 h at room temperature in a total volume of 60 μl with 4 μg of rPDC-E2, 50 μg of peptide, or the equivalent volume of peptide diluent. The enzyme reaction was initiated by the addition of 50 m of the enzyme mixture containing PDC from porcine heart (Sigma, St. Louis, MO) in 50 mM phosphate buffer (pH 7.4) containing 2% BSA and 10 mM DTT, and 100 μl of a reaction mixture containing the essential cofactors for the reaction, coenzyme A, and the NAD analogue thio-NAD. Absorbance was read immediately at 405 nm and then at minute intervals for 10 min (19). The rate of change of absorbance was calculated, and the percent inhibition was tested by capture ELISA for reactivity with preparations of affinity-purified anti-PDC-E2 from four different subjects with PBC.

Results

Phagotopes isolated by biopanning with polyclonal sera

The peptide inserts in phagotopes isolated after three rounds of biopanning using IgG from each of the two PBC sera were sequenced (Table I). Sequences were obtained for 27 phagotopes derived from PBC1 serum and 28 derived from PBC2 serum. In each case, six phagotopes did not have DNA inserts. The sequences obtained were compared with those of inserts in phagotopes obtained by biopanning with serum from the patient with RA (11 sequences) and the patient with relapsing polychondritis (11 sequences), and from the biopanning itself in the absence of IgG, using magnetic beads coated with anti-human IgG (18 sequences) and unselected sequences from the library (20 sequences) (Table I).

The sequences derived with the PBC sera showed distinct similarities and most differed clearly from sequences derived from all other sources. This was reflected by the differing frequency of each amino acid in the isolated peptides, when a comparison was made between the frequencies of particular amino acid in peptides from nonselected phagotopes, peptides derived from PBC sera, peptides from the control sera, and peptides derived after biopanning with beads only. Using beads only, tryptophan (W), asparagine (N), and tyrosine (Y) were each increased 5–6-fold and histidine (H) and lysine (K) more than 2-fold. Although the frequency of these particular amino acids tended to be increased in peptides selected when Abs were used for biopanning, there were, in addition, selective decreases in frequency of particular amino acids according to the source of the Ab. For PBC sera, several motifs were distinguishable. Of the 43 different sequences isolated, 8 sequences that were derived from 11 phagotopes (isolated from biopannings with the two PBC sera) contained the motif H-aromatic-HRPxx, and 13 sequences derived from 16 phagotopes began with MH. There were two sequences, including one isolated with IgG from each PBC serum, that began with FVE (FVEHTRW, FVEIYSP), and another one sequence was FVLIPWRI. Of another two sequences (AGFNVPW, HHKYWHR) that were isolated using each PBC serum, HHKYWHR was considered to be a “nuisance” peptide since it resembled a sequence HHKYWWYR previously identified from biopanning with beads coated with anti-mouse Ig but no selecting Ab (20).

Sequences from phagotopes positive by capture ELISA

All phagotopes derived with IgG from either PBC1 or PBC2 were tested by capture ELISA for reactivity with preparations of affinity-purified anti-PDC-E2 from four different subjects with PBC.

| Number of times each sequence was isolated. | Table I. Peptide sequences from phagotopes isolated by biopanning with two PBC sera (PBC1 and PBC2), sera from patients with RA and relapsing polychondritis (RP), and isolated by biopanning with anti-human IgG beads alone |
Phagotopes that contained 1 of 15 different peptide sequences were reactive with 1 or more of the 4 affinity-purified preparations of anti-PDC-E2, whereas phagotopes that contained 28 different sequences were negative with all 4 Ab preparations (Table II). Of the phagotopes that were positive, most reacted with more than one of the preparations of anti-PDC-E2, and 4 phagotopes with sequences FVLPWRI, MHLNTPP, and MHLTQSP were reactive with all 4 of the antisera. The reactive phagotopes included 7 of 13 in which the peptide included the motif MH, all of the three with the motif FV, and that containing the peptide AGFNVPW that was isolated from both biopanions.

Reactivity of synthetic peptides with PBC sera

The peptides FVLPWRI, MHLNTPP, and MHLTQSP that were displayed on the three phagotopes that were strongly reactive with all preparations of anti-PDC-E2 (OD > 1) and an irrelevant control peptide CIAPKRHNSAC were synthesized and tested for reactivity with PBC sera. None of the 4 peptides reacted with any of the PBC or control sera by direct ELISA under the conditions tested. However, each of the three selected peptides, but not the control peptide, inhibited the reactivity of the PBC sera with rPDC-E2 as determined by ELISA. The most inhibitory peptide was FVLPWRI, which gave complete inhibition (100% ± 2%, mean ± SD) at 25 μg/ml, as did 10 μg/ml of rPDC-E2 as the positive control (99% ± 2%), whereas the negative control peptide CIAPKRHNSAC gave 5 ± 3% inhibition at the highest concentration. The two similar peptides, MHLTQSP and MHLNTPP, were also inhibitory at 25 μg/ml, although to a lesser extent, 70 ± 3% and 30 ± 11%.

Effect of peptides on enzyme inhibition

Fig. 1 shows the PDC enzyme activity under varying conditions, based on changes with time in readings at A405 representing production of thio-NADH. The reaction rate in the absence of any Ab (inset) was 0.013 ± 0.0006 (mean ± SD) per min and 0.00016 ± 0.000046 in the presence of serum from PBC2, which represented 99% inhibition of the enzyme activity. Addition of an irrelevant peptide had no significant effect on the rate of reaction, whereas the addition of rPDC-E2 and each of the phage-selected peptides MHLTQSP, MHLNTPP, and FVLPWRI was counterinhibitory in significantly restoring the rate of reaction over the 10-min period of observation. Thus, the rates of reaction after addition of PDC-E2 or the peptides MHLTQSP, MHLNTPP, and FVLPWRI were 0.003 ± 0.00013, 0.0026 ± 0.00010, 0.0021 ± 0.00013, and 0.0014 ± 0.0002, respectively, compared with 0.0005 ± 0.00014 with the control peptide, which represented percent inhibition of 73, 80, 84, 89, and 96%. The addition of rPDC-E2 led to the greatest restoration of reactivity, although the molar concentration of the rPDC-E2 was ~1000 times lower than that of any of the peptides.

Discussion

In the present study, we have used Ab screening of a phage-displayed library of random heptapeptides expressed in the pIII coat
protein of bacteriophage to derive peptide mimotopes of PDC-E2. Our strategy was to use polyclonal IgG from patients with PBC in two separate sequences of three rounds of positive selection using an individual IgG and negative selection using IgG from pooled normal sera. This allowed the development of panels of “individual specific” phagotopes containing insert sequences that could be compared with sequences obtained using sera from other diseases.

We tested the phagotopes for reactivity with four preparations of affinity-purified anti-PDC-E2 from PBC sera and so identified a set of reactive phagotopes with sequences that were reactive by ELISA with affinity-purified anti-PDC-E2. Since similar sequences were derived by biopanning with IgG from two different patients with PBC, and the derived phagotopes reacted unequivocally with affinity-purified anti-PDC-E2 from four patients with PBC, we suggest that there is a single major conformational and discontinuous epitope within PDC-E2 that is recognized by most PBC sera.

Not all of the phagotopes derived by biopanning with PBC sera were reactive with anti-PDC-E2. This is understandable, given that polyclonal IgG includes a myriad of Abs so that, even after extensive rounds of negative selection, the phagotopes isolated will include many that react with other Abs that are either specific or nonspecific to the disease. The difficulty in discriminating between sequences that are specific or nonspecific for any given disease (or for Abs that accompany that disease) has greatly hampered the use of phage-displayed technology with polyclonal sera. In the present study, disease-specific phagotopes were identified using a capture ELISA in which plates were coated with affinity-purified anti-PDC-E2. However, for other autoantibodies, affinity-purified Abs may not be readily available. Such limitations have been offset by our utilization of an alignment algorithm PILEUP (21) to group peptide sequences into clusters according to their physicochemical relatedness (20). Using PILEUP, it was found that PBC-derived phagotopes that contained sequences that were reactive by ELISA with affinity-purified anti-PDC-E2 aggregated into disease-specific clusters, whereas other disease-specific clusters were unreactive (20). It is likely that some of the PBC-specific sequences derived from the phage library will represent mimotopes for autoantibodies other than anti-PDC-E2, particularly mimotopes of conformational epitopes of the related molecules 2-oxoglutarate dehydrogenase complex-E2 and branched chain oxoacid dehydrogenase-E2, or various PBC-associated nuclear autoantigens. In addition, some of the nondisease-specific phagotopes sequences that we derived would include either nuisance peptides intrinsic to the biopanning
process itself or mimotopes of bacterial or viral Ags for which Abs were absent from the particular pool of IgG used for negative selection. Examples include HHKYWHR that may react with beads used for biopanning in the absence of any selecting Ab, and sequences with the motif H-aromatic-HRPxx, as judged by the study of Davies et al. (20); in that study, there was a branch of the guide tree that contained a cluster of sequences with the motif HHRPxx derived from sera of patients with type I diabetes as well as PBC sera.

Our interpretation of the epitope structure for anti-PDC-E2 has been facilitated by the derivation of an NMR structure for aa 131–233 of human PDC-E2 that encompasses the major immunodominant epitope(s) (22) (Fig. 2a). Hitherto epitope mapping with PBC sera has linked reactivity to the highly conserved amino acids surrounding the lipoyl-lysine K173, particularly linear peptides 167–183 (AEIETD KATIGFEVQEGYL) (5, 8) or 165–178 (LLAEIETDKATIGF) (7). However, the lipoyl-lysine K173 may not contribute to the epitope as judged by the marked scarcity of lysines among the selected phagotopes. Moreover, lipoylation or delipoylation of PDC-E2 does not affect its antigenicity (7), although this may not be the case for Abs reactive with PDC-E2 from E. coli (6). Finally, although the amino acids of the previously identified “linear” epitope (5, 7, 8) are predominantly surface exposed, most are actually not on the face of the molecule that contains the lipoyl-lysine K173 as judged by the structural model (Fig. 2a). Accordingly there are good grounds for redefining the major immunodominant region of PDC-E2.

We note that there are two particular paired residues, FV and MH, that featured among the phagotope sequences with frequent reactivity with affinity-purified anti-PDC-E2, and that the inner lipoyl domain contains only one histidine within the linear sequence F131 HMQVLL137. The NMR structure of the lipoyl domain shows that this single histidine and the adjacent methionine are surface exposed, and therefore, these are in juxtaposition to phenyalanine (F178) and valine (V180) which are also surface exposed. These four amino acids are too far apart in the structure to form part of an epitope that includes the lipoyl-lysine (K173) (Fig. 2a). However, F178 and V180 are part of the previously described linear epitope comprising aa 167–183 (5). The amino acids surrounding the region of juxtaposition of M133, H132, F178, and V180 within the ~20 Å diameter of a putative Ab epitope include proline (P), leucine (L), glutamine (Q), glutamate (E), tyrosine (Y), and serine (S), each of which was well represented among the reactive phagotopes. Accordingly, this region could readily be proposed as the major immunodominant epitope, although it excludes the lipoyl-lysine K173. As further evidence in support of this proposed epitope structure, we can cite sequence differences between the inner and outer lipoyl domains, noting the infrequent reactivity of the outer lipoyl domain with PBC sera. Thus, the PHMQL of the inner lipoyl domain is represented by PHQK in the outer domain, and the sequence GFEV in the inner domain is represented by GFES in the outer domain (Fig. 2b). The characteristically potent inhibition of PDC enzyme activity by Abs to the PDC-E2 epitope would be explained according to our epitope structure by steric interference with the swinging movement of the lipoyl domain, since the epitope is close to the flexible regions that link lipoyl domains and the E3-binding domain.

Alternatively, given that phagotopes were selected using polyclonal IgG, the peptides selected could represent more than one epitope rather than regions of the immunodominant epitope for anti-PDC-E2. However, the observation that the peptide MHLTQSP was 70% inhibitory for the reactivity of a PBC serum with rPDC-E2 by ELISA, and that the peptide FVLPWRI was totally inhibitory for the reaction with the same serum suggest that the epitope is a complex topographic determinant formed from at least two separate regions of the linear sequence, H132 and M133, and F178 and V180, and that each of these residues are critical for Ab binding. Moreover, this is the region at which enzyme inhibitory Abs bind, since each of the peptides tested markedly reduced the enzyme inhibitory effect of a PBC serum.

Phage display has not previously been used to identify the PDC-E2 epitope using polyclonal Abs; a random dodecapeptide phage-displayed library was screened with a mouse mAb C355.1 to the inner lipoyl domain of PDC-E2 (23). In that study, among 36 phagotopes selected, 3 common amino acid motifs were identified: SYP, TYVS, and VRH. It is notable that these amino acids occur within our putative epitope and that the actual sequence SYP occurs in the same epitope region (SYPPHM) of the inner but not the outer lipoyl domain of PDC-E2. The mAb C355.1 reacted not only with PDC-E2, but also with the luminal surface of bile duct epithelium only from patients with PDC, and not from normal subjects or patients with other liver diseases (23). This reactivity was similar to that of human combinatorial Abs specific to the inner lipoyl domain of PDC-E2, and the peptides identified by phage display using C355.1 inhibited immunohistological staining of human combinatorial Abs to PBC biliary epithelial cells (24). The colocalization of the epitope isolated with C355.1 and the epitope defined using polyclonal anti-PDC-E2 in the present study suggest that this epitope is relevant to the pathogenesis of PBC.

In conclusion, we have, for the first time, amalgamated findings from Ab screening of a phage library with the known structure of an immunodominant region of an autoantigenic molecule to locate precisely a conformational epitope, in this case the epitope for anti-PDC-E2, which is the major autoantibody in PBC. These findings should give new impetus to the search for mimicking sequences among Ags of microbial provenance that could be implicated in the pathogenesis of PBC and could stimulate similar studies with other well-defined autoantigen-Ab systems.

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