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Random phage mimotopes recognized by monoclonal antibodies against the pyruvate dehydrogenase complex-E2 (PDC-E2)

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ABSTRACT **Dihydrolipoamide acetyltransferase, the E2 component of the pyruvate dehydrogenase complex (PDC-E2), is the autoantigen most commonly recognized by autoantibodies in primary biliary cirrhosis (PBC). We identified a peptide mimotope(s) of PDC-E2 by screening a phage–epitope library expressing random dodecapeptides in the pIII coat protein of fd phage using C355.1, a murine monoclonal antibody (mAb)that recognizes a conformation-dependent epitope in the inner lipoyl domain of PDC-E2 and uniquely stains the apical region of bile duct epithelium (BDE) only in patients with PBC. Eight different sequences were identified in 36 phage clones. WMSYPDRTLRTS was present in 29 clones; WESYPFRVGTSL, APKTYVSVSGMV, LTYVSLQGRQGH, LDYVPLKHRHRH, AALWGVKVRHVS, KVLNRIMAGVRH and GNVALVSSRVNA were singly represented. Three common amino acid motifs (W-SYP, TYVS, and VRH) were shared among all peptide sequences. Competitive inhibition of the immunohistochemical staining of PBC BDE was performed by incubating the peptides WMSYPDRTLRTS, WESYPDRTLRTS, APKTYVSVSGMV, and AALWGVKVRHVS with either C355.1 or a second PDC-E2-specific mAb, C150.1. Both mAbs were originally generated to PDC-E2 but map to distinct regions of PDC-E2. Two of the peptides, although selected by reaction with C355.1, strongly inhibited the staining of BDE by C150.1, whereas the peptide APKTYVSVSGMV consistently inhibited the staining of C355.1 on biliary duct epithelium more strongly than the typical mitochondrial staining of hepatocytes. Rabbit sera raised against the peptide WMSYPDRTLRTS stained BDE of livers and isolated bile duct epithelial cells of PBC patients more intensively than controls. The rabbit sera stained all size ducts in normals, but only small/medium-sized ductules in PBC livers. These studies provide evidence that the antigen present in BDE is a molecular mimic of PDC-E2, and not PDC-E2 itself.**

A central issue in the pathogenesis of the autoimmune disease is the role played by the autoantigen in breaking of tolerance and the nature of the autoimmune response (1). In primary biliary cirrhosis (PBC), a chronic autoimmune liver disease characterized by deterioration of interlobular bile ducts resulting in cirrhosis (2), sera contain autoantibodies that recognize components of the mitochondrial 2-oxo-acid dehydrogenase enzyme complexes. These complexes include dihydrolipoamide acetyltransferase, the E2 subunit of pyruvate dehydrogenase complex (PDC-E2), the branched chain 2-oxoacid dehydrogenase complex, the 2-oxoglutarate dehydrogenase complex, PDC-E1α, PDC-E1β, and protein-X (3–8). Of these, PDC-E2 is the immunodominant autoantigen recognized by over 90% of patients with PBC and the inner lipoyl domain, a functional site of the PDC-E2 complex, is the major epitope (9). A number of PDC-E2 specific murine monoclonal antibodies (mAbs) have been generated and most of these antibodies map to the inner lipoyl domain of human PDC-E2 (10) indicating the dominant immunogenic nature of this region. In immunofluorescent confocal microscopic studies, one of these mAbs, C355.1, gave very intense and distinctive luminal staining of bile duct epithelium (BDE) in liver sections from patients with PBC, but not in BDE derived from normals or patients with other liver diseases (11). In contrast, the other mAbs produced a typical cytoplasmic mitochondrial staining pattern that was similar in tissues from both PBC patients and control subjects (12). Thus, a key question in PBC relates to the identity of the molecule recognized by C355.1, its relationship to PDC-E2, and whether it may be a molecular mimic involved in causation of the disease.

To derive a set of reagents to further define this molecule, we have panned a random peptide library expressing dodecapeptides on the phage surface (13, 14) with the PDC-E2 specific mAb C355.1. Reactive peptide bearing phage were then purified and the dodecapeptide sequence determined. The specificity of peptide mimotopes was determined by examining the ability of the encoded peptide to inhibit the binding of various anti-PDC-E2 specific mAbs to liver tissue sections. Rabbit sera raised against one of the peptides was used to examine the subcellular location of the mimotope by immunoelectronmicroscopy.

MATERIALS AND METHODS

Random Phage–Epitope Library. A random phage–epitope library (fAFF ON-159.2) expressing a dodecapeptide at the N terminus of pIII proteins of fd phage affinity vector (fAFF1) was generated as described (15). ON-159.2 has 3×10^8 recombinants.

Affinity Selection of the Phage Library. C355.1 was purified from the culture supernatant using protein G (Pierce); 1 mg of the mAb was biotinylated with a protein biotinylation kit (Amersham), and subsequently used to screen a phage–epitope library, as described (16), with slight modifications. Phage (10^{11}) were incubated with 200 μl of streptavidin–agarose beads for 6 hr at 4°C and microcentrifuged for 5 min to remove the beads and streptavidin-bound nonspecific phage. The harvested phage suspension was incubated with 20 μl of biotinylated C355.1 (7 μg) overnight at 4°C. Fresh streptavidin–agarose beads (200 μl) was added to the phage and antibody mixture. After 1 hr at 4°C, the mixture was

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Abbreviations: BDE, bile duct epithelium; PDC-E2, the E2 component of the pyruvate dehydrogenase; PBC, primary biliary cirrhosis; RT, room temperature.

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microcentrifuged for 5 min, and the supernatant was aspirated. The streptavidin–agarose beads and attached phage were washed 10 times with TBS (50 mM Tris·HCl/150 mM NaCl, pH 7.5) containing 0.5% Tween 20 at 5-min intervals. The phage were eluted with 200 μl of elution buffer (0.1 M HCl, pH adjusted to 2.2 with glycine, containing 0.1% BSA) at reverse transcription (RT) for 10 min and microcentrifuged for 5 min Twenty microliters of 2 M Tris was added immediately into the eluted sample. Amplification of the eluted phage was performed as described (17). The amplified phage stock was used in three rounds of affinity selection as described above. Percentage yield was determined by calculating the number of eluted phage/number of input phage × 100.

Determination of Specificity of Isolated Phage. The specificity of isolated phage clones was analyzed by ELISA. Briefly, 100 μl of 10 μg/ml biotinylated C355.1 in coating buffer (0.1 M $NaHCO_3$, pH 9.6) was added to microtiter plates and incubated overnight at 4°C. Individual *Escherichia coli* colonies from the third round of affinity purification were grown overnight and microcentrifuged for 5 min. After blocking the wells of the microtiter plates with 1% BSA solution, 100 μl of *E. coli* culture supernatant containing phage particles was added to each well and incubated for 1 hr at RT. The wells were washed four times with PBS/Tween, and a 1:5000 dilution of rabbit anti-phage sera (18) added to the microtiter plates. After 1 hr incubation at RT, the wells were washed with PBS/Tween and 1:3000 dilution of horse radish peroxidase conjugated goat–rabbit IgG (Caltag, South San Francisco, CA) was added for 1 hr at RT. The microtiter plates were then developed with 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (Sigma) (10).

DNA Sequencing. Among 108 individual *E. coli* clones isolated after 3 cycles of affinity selection, 36 clones showing specific reactivity to mAb C355.1 by ELISA were selected and their DNA inserts sequenced from a 3′ primer (5′-CGA TCT AAA GTT TTG TCG TCT-3′) using standard methods (18, 19).

Determination of IC_{50} of Synthetic Peptides. Based upon the isolated peptide motifs, five synthetic peptides, WMSYPDRTLRTS, WESYPDRTLRTS, AALWGVKVRHVS, APKTYVSVSGMV, and KEANNRNEKSSE, of >80% purity were purchased from Bio-Synthesis (Lewisville, TX) and PeptidoGenic (Livermore, CA), and the inhibitory constant of these peptides with 100 ng of purified C355.1 was measured by competitive inhibition ELISA (20).

Immunohistochemistry. A mAb C150.1 specific for the PDC-E2 outer lipoyl domain was generated previously as described (10). A predetermined optimal dilution of the antibodies for use in the staining process was used throughout. Liver sections were prepared from paraffin embedded liver biopsies from three patients with PBC. Sections were treated as described (11). The mAbs were mixed with 1 mM of each synthetic peptide (WMSYPDRTLRTS, WESYPDRTLRTS, AALWGVKVRHVS, APKTYVSVSGMV or an irrelevant peptide, KEANNRNEKSSE, as a negative control) and incubated for 2 hr at 37°C. The sections were washed briefly with TBS and incubated with the antibody/peptide mixtures for 1 hr at RT and then treated as described (11). A culture supernatant from an irrelevant murine hybridoma clone not specific to PDC-E2 was used as negative controls. The slides were dried and coverslipped with Slo-Fade (Molecular Probes) and analyzed by a Bio-Rad MRC 600 laser confocal microscope (11, 21). Combinations of mAbs and peptides were tested against all three liver biopsies.

Generation of Rabbit Sera Against Synthetic Peptide. Synthetic peptide WMSYPDRTLRTS was conjugated with the Inject Supercarrier EDC system (Pierce) according to the manufacturer's instructions, conjugated with alum and used to immunize rabbits intradermally three times. This peptide was chosen based on the isolation frequency for this sequence from the peptide library screening.

Table 1. Epitope sequence of phages isolated by a third round of affinity selection with biotinylated C355.1

Epitope*	Binding,† O.D. 405 nm	No. phages identified
WMSYPDRTLRTS	745 ± 8	29
WESYPFRVGTSL	521 ± 2	1
APK**TYVS**VSGMV	936 ± 8	1
L**TYVS**LQGRQGH	470 ± 1	1
LD**YV**PLKHRHRH	486 ± 3	1
AALWGVK**VRHVS**	811 ± 9	1
KVLNRIMAG**VRH**	430 ± 4	1
GN**VALVS**SRVNA	461 ± 2	1

*Amino acid motifs are denoted by boldface letters.
†Binding data were obtained by ELISA as described and the results are expressed by the absorbance at 405 nm × 1000 (mean ± SD) of duplicated data after subscription of background readings.

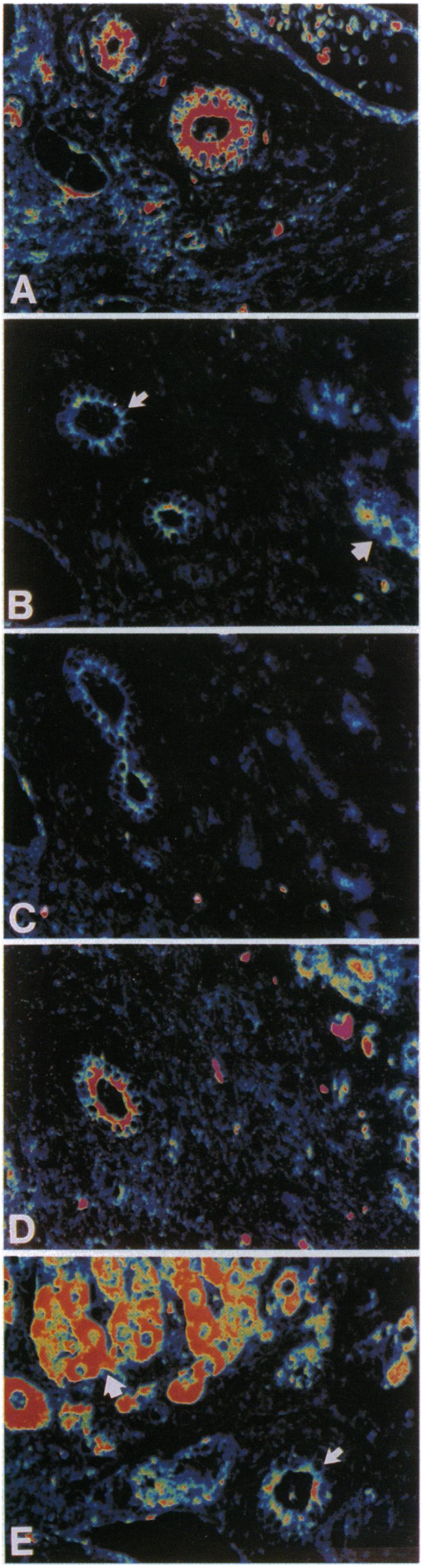
Immunohistochemical Staining of Liver Sections and Purified Biliary Epithelial Cells by Rabbit Sera Against Synthetic Peptide WMSYPDRTLRTS. Normal liver (n = 3) was obtained from adult allografts reduced in volume for pediatric liver transplant recipients. PBC liver (n = 3) was obtained from hepatectomy specimens removed from patients undergoing liver allografting. One-centimeter cubes of liver were snap-frozen in liquid nitrogen and stored at −70°C. Cryostat sections (5 μm) were fixed in acetone for 5 min at room temperature. Sera were diluted in TBS (pH 7.6). Sections were incubated sequentially with 10% normal swine serum (30 min) and rabbit anti-serum or pre-immune serum (1 hr). After washing (3 × 5 min), the sections were incubated for 1 hr with alkaline phosphatase-conjugated swine anti-rabbit (1:40, Dako). Sites of antibody binding were visualized with fast-red substrate (1 mg/ml in 0.1 M TBS, pH 8.2/0.8% levamisole/0.4% naphthol AS-MX phosphate/3% dimethyl formamide, all Sigma). Similarly, reactivity of the pre-immune sera were studied on serial sections.

To analyze the staining pattern of the rabbit sera on bile duct epithelial cells, biliary epithelial cells were prepared from normal (n = 3) and PBC (n = 3) liver by immunomagnetic separation (22). The freshly isolated cells were plated onto 1-cm diameter glass coverslips in medium containing 10% fetal bovine serum (23) and allowed to adhere for 48 hr. Antibody binding to the exterior aspect of the cells was studied. Blocking serum and antibodies were diluted in Ham's F-12 tissue culture medium (GIBCO). Biliary epithelial cells were incubated sequentially with 10% blocking serum (30 min, 4°C) and primary anti-serum or pre-immune serum (30 min, 4°C). For electron microscopy the Biliary epithelial cells were washed and incubated with biotinylated second antibody (1:100, 30 min at 4°C). Following further washes the cells were fixed in 2.5% glutaraldehyde (30 min at 4°C) and sites of

Table 2. Selective inhibition of binding by synthetic dodecapeptides of PDC-E2-specific mAbs to bile duct epithelial cells from patients with PBC

Synthetic peptides	mAbs, percent inhibition	
	C355.1	C150.1 (control)
WMSYPDRTLRTS	85 ± 12*	55 ± 5
WESYPDRTLRTS	95 ± 15	25 ± 4
AALWGVKVRHVS	23 ± 19	74 ± 24
APKTYVSVSGMV	90 ± 11	28 ± 14
KEANNRNEKSSE*	0	0

*Average pixel value was derived from a 200-μm area of cytoplasm of BDE and measured in three patients with PBC. Percentage of staining inhibition by peptides was determined by confocal microscopy and calculated by [(average pixel value of negative peptide − average pixel value of a synthetic peptide)/average pixel value of negative peptide] × 100. Data showed mean ± SD.
*An irrelevant synthetic peptide used as a negative control.



antibody binding were visualized using streptavidin–peroxidase and 3,3′-diaminobenzidine substrate as described (24).

RESULTS

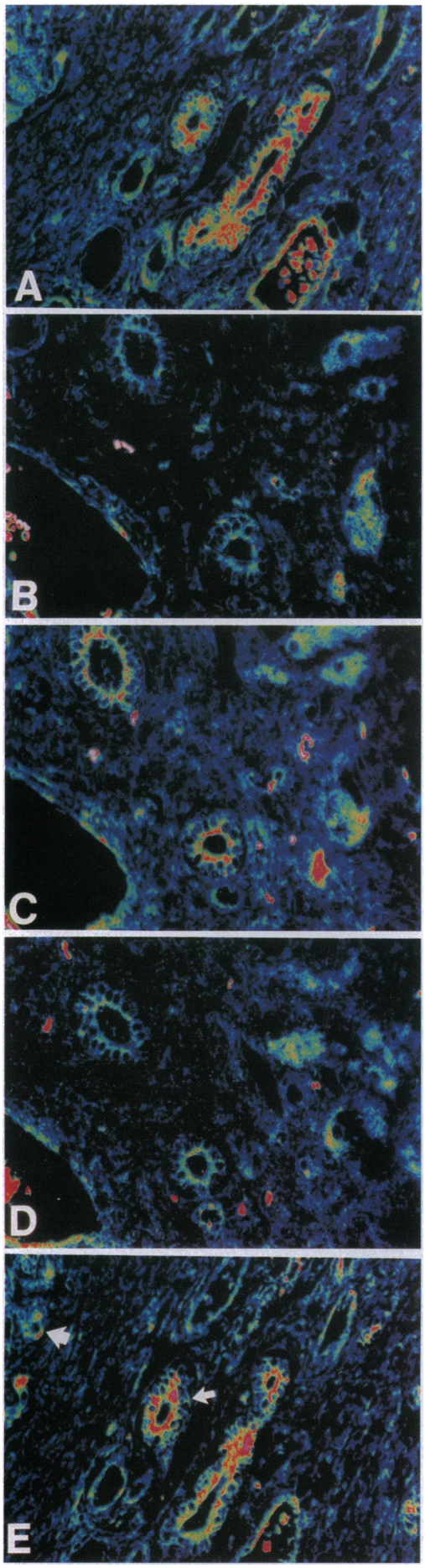
Isolation of Phage by Affinity Selection and Determination of Binding Sequences. We set out to identify the peptide epitopes that reacted with C355.1 by screening a dodecapeptide random peptide library (ON-159.2) of approximately 10^{11} phage, by affinity purification on biotinylated C355.1 coupled to streptavidin–agarose beads. The percentage yield of phage clones was calculated as (number of eluted phage/number of applied phage) × 100. After the third round of affinity purification the percentage yield of phage clones was 0.1% in the C355.1 screening, whereas that of the negative control (without biotinylated C355.1) was about 7×10^{-4}% (data not shown).

After three cycles of affinity purification, 108 individually isolated phage clones were tested by ELISA for binding to biotinylated C355.1. Thirty-six of the clones bound strongly to biotinylated C355.1 and were selected for further characterization. Table 1 shows the frequencies of peptide sequences deduced from the DNA sequence and the strength of signal in a C355.1 binding ELISA. Eight different dodecapeptide sequences appeared in the 36 clones. Twenty-nine clones were found to have an identical peptide sequence, WMSYPDRTLRTS, implying either preferential affinity selection of this peptide, or overrepresentation of the phage that contained this peptide insert in the library. Peptide sequences of WESYPFRVGTSL, APKTYVSVSGMV, LTYVSLQGRQGH, LDYVPLKHRHRH, AALWGVKVRHVS, KVLNRIMAGVRH, and GNVALVSSRVNA were found in one clone each. A W-SYP [where (−) represents variable amino acid(s)] motif was found in two of the sequences, including the most commonly isolated peptide sequence. The sharing of TYVS and VRH motifs was also seen with lower representation among the isolated peptides.

Inhibition of mAb Staining of Bile Duct Epithelial Cells by Synthetic Peptides. Confocal microscopy was used to quantitate the degree of inhibition of mAb C355.1 and C150.1 staining on biliary epithelium by the synthetic peptides. The synthetic peptides differentially inhibited the staining of biliary epithelium by these mAbs. Apical staining of the BDE with C355.1 was inhibited by the peptides WMSYPDRTLRTS (85%), WESYPDRTLRTS (95%), and APKTYVSVSGMV (90%) (Fig. 1, Table 2), whereas AALWGVKVRHVS did not show significant inhibition (23%) when compared with the negative control peptide. The degree of staining was quantitated by image analysis and confirmed the inhibition of staining by the three peptides (data not shown).

The staining results were in contrast to the data obtained in a competitive ELISA to purified recombinant PDC-E2 inner lipoyl domain (aa 128–227). The peptides, WMSYPDRTLRTS, WESYPDRTLRTS, and APKTYVSVSGMV inhibited the binding of C355.1 to PDC-E2 inner lipoyl domain fusion protein at an IC_{50} of 2.5 μM, whereas AALWGVKVRHVS inhibited the binding at an IC_{50} of 0.4 μM (data not shown). Based upon these IC_{50} values, it was expected that the peptide AALWGVKVRHVS would show the best inhibitory effect on C355.1 staining. However, this peptide produced the least

FIG. 1. Confocal micrographs demonstrating inhibition by synthetic peptides of immunohistochemical staining in bile duct epithelial cells of serial liver sections from a patient with PBC using C355.1. Aliquots of C355.1 were mixed with 1 mM of an irrelevant negative control peptide KEANNRNEKSSE (*A*), WMSYPDRTLRTS (*B*), WESYPDRTLRTS (*C*), AALWGVKVRHVS (*D*), and APKTYVSVSGMV (*E*) for 2 hr at 37°C before applying to the slides. Note that a substantial reduction in C355.1 staining intensity was obtained by WMSYPDRTLRTS (*B*), WESYPDRTLRTS (*C*), and APKTYVSVSGMV (*E*) compared with negative control peptide (*A*). Of particular interest, note that the peptide APKTYVSVSGMV markedly reduced the C355.1 staining in bile duct epithelial cells (*E*, small arrow) but not hepatocytes (*E*, large arrow). This is in contrast to the inhibition seen with peptide WMSYPDRTLRTS (*B*), where staining in both the bile duct (small arrow) and the hepatocytes (large arrow) are equally reduced. (×145, zoom 2.0).



amount of apical staining inhibition of all of the peptides tested. Of great interest was the peptide APKTYVSVSGMV, which consistently inhibited the C355.1 apical staining of biliary epithelium, but had no effect on the staining of hepatocytes in all three patients with PBC (Fig. 1 and Table 2).

Mitochondrial staining of the liver with the control mAb C150.1, which maps to the outer lipoyl domain of PDC-E2 (10), was strongly inhibited by the peptides WMSYPDRTLRTS (55%) and AALWGVKVRHVS (74%) (Fig. 2, Table 2). Replacement of Met with Gln greatly reduced the inhibitory effect of the synthetic peptide on C150.1 staining from 55 to 25%. This finding, in combination with the C355.1 data above, suggests that WMSYPDRTLRTS may correspond to the peptide mimotope of the outer lipoyl domain of PDC-E2.

Reaction of Rabbit Anti-WMSYPDRTLRTS Anti-Serum with Biliary Epithelium. We set out to determine the staining pattern of anti-peptide antibodies raised against the most frequently isolated mimotope sequence on BDE using three different assays: immunohistocytochemistry, confocal microscopy, and immunoelectron microscopy. In immunohistochemical studies, rabbit anti-WMSYPDRTLRTS anti-sera bound with high intensity to biliary epithelium in histological sections of liver from patients with PBC (Fig. 3*B*). Staining of BDE was not present in serial sections in which pre-immune serum was used instead of immune serum (Fig. 3*A*). Confocal studies showed that in liver from normal controls, the antibodies did react moderately with bile ducts and ductules of all sizes (Fig. 3*C*). However, in liver sections from patients with PBC, areas of much higher intensity staining were seen in some ducts (Fig. 3*D*). On closer examination, these highest intensity staining regions were found to be medium-sized bile ducts (Fig. 3*E*). Marginal ductules and larger septal ducts were only weakly stained (Fig. 3 *D* and *E*). No clear association of antibody binding with the plasma membrane of BDE was seen in confocal studies, although such a measurement is at the limits of resolution of the instrument.

To more accurately determine the subcellular distribution of anti-WMSYPDRTLRTS binding to BDE, immunoelectron-microscopy was performed on purified isolated BDE cells. Purified BDE stained with anti-WMSYPDRTLRTS showed intense staining on PBC BDE (Fig. 4*B*). The preimmune serum and BDE from normals showed only insignificant nonspecific background staining (Fig. 4 *A* and *C*).

DISCUSSION

The mechanism that leads to the generation of high titer autoantibodies characteristic of autoimmune diseases remains unknown. Studies in mixed connective tissue disease and Sjögren syndrome have shown that the antibodies recognize many epitopes on their respective autoantigens (25, 26). This finding has been interpreted as suggesting that the process of autoantibody formation in these diseases is driven by the presence of self-antigen. The case is clearly different in primary biliary cirrhosis in which the observation of a single extended autoepitope suggests that a process of molecular mimicry may be involved. Previous studies (11, 12) have shown that mAb C355.1 produces an unusual luminal staining pattern on biliary epithelium unlike the conventional mitochondrial cytoplasmic staining pattern seen with other mAbs specific to PDC-E2. This prompted

FIG. 2. Confocal micrographs showing inhibition by synthetic peptides of immunohistochemical staining in bile duct epithelial cells of serial liver sections from a patient with PBC using the control mAb C150.1. Aliquots of C150.1 were mixed with 1 mM of an irrelevant negative control peptide KEANNRNEKSSE (*A*), WMSYPDRTLRTS (*B*), WESYPDRTLRTS (*C*), AALWGVKVRHVS (*D*), and APKTYVSVSGMV (*E*) for 2 hr at 37°C before applying to the slides. Note that a substantial reduction in C150.1 staining intensity was obtained by WMSYPDRTLRTS (*B*) and AALWGVKVRHVS (*D*). Of particular interest, note that little reduction in staining was observed with the peptide APKTYVSVSGMV (*E*) in either the BDE (small arrow) or the hepatocytes (large arrow) when compared with C355.1 (Fig. 1*E*). (×145, zoom 2.0.)

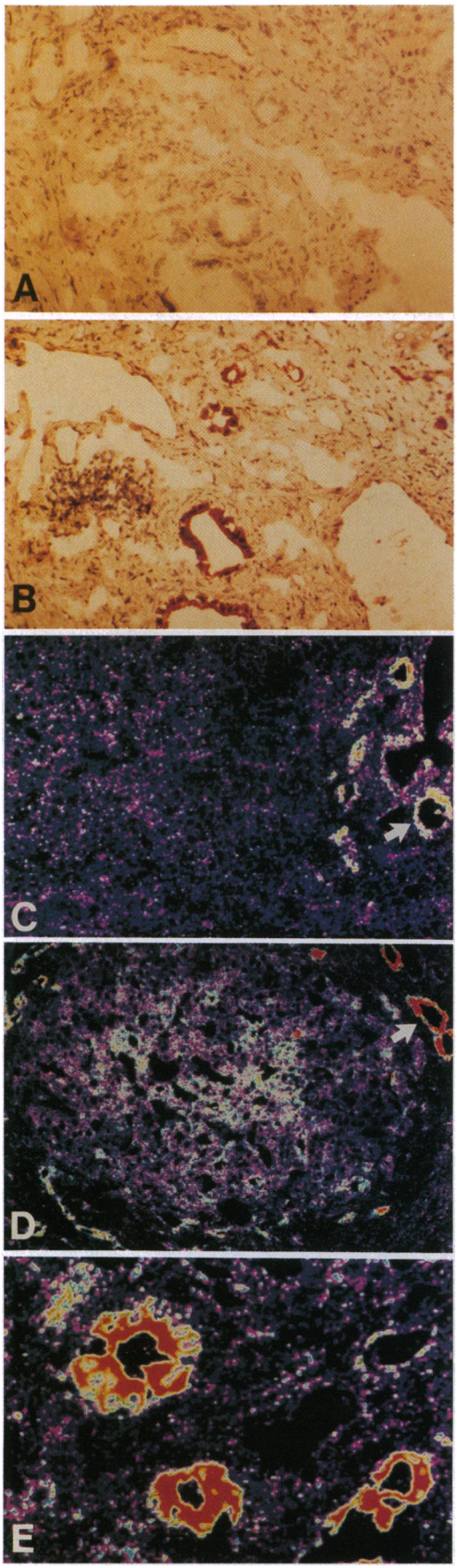


FIG. 3. Micrographs of immunohistochemical staining of bile duct epithelial cells of liver sections. PBC liver stained with pre-immune rabbit serum (*A*), PBC liver stained with rabbit anti-WMSYPDRTLRTS (*B*). (*C*) Confocal micrograph showing the relative intensity of rabbit anti-WMSYPDRTLRTS binding to biliary epithelium and an adjacent hepatocyte lobule in normal liver. Uniform staining is present on all ducts. (*D*) Confocal micrograph showing the relative intensity of the same antibody bound to biliary epithelium in PBC liver. Note that the higher intensity of staining is present on medium-sized ducts relative to the marginal ductules (*A–D*, ×75). (*E*) A higher magnification confocal micrograph showing the subcellular distribution of anti-WMSYPDRTLRTS on medium-sized bile ducts in PBC liver. The highest intensities are intracellular as denoted by the red color intensity (*E*, ×145).

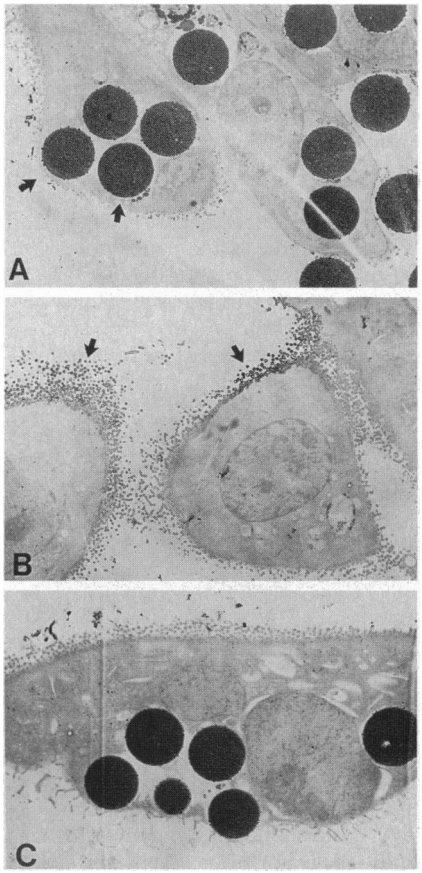


FIG. 4. Transmission electron micrographs showing the distribution of antibody binding to purified BDE cells. (*A*) PBC BDE cells stained with pre-immune rabbit serum. Note that magnetic beads were used to isolate the cells (arrows). (*B*) PBC BDE cells stained with rabbit anti-WMSYPDRTLRTS. Note the specific staining of the plasma membrane as illustrated by the arrows. (*C*) BDE cells from a normal control liver were negative when stained with rabbit anti-WMSYPDRTLRTS (×1800).

us to screen a random epitope library with the mAb 355.1, in search of a peptide sequence that more closely resembled the primary sequence of a possible crossreactive molecule.

Suggested examples of molecular mimicry include U1-snRNP, M1 matrix protein of influenza B viruses, Sm antigen, Epstein–Barr virus nuclear antigen 1, La protein and the M6 protein of *Streptococcus pyrogenes* (27). However, the demonstration of sequence homology or induction of crossreactive antibodies only suggests an association, but does not prove an etiological relationship between the molecular mimics and the clinical disease. In any case, the suggestion is that the initial immune response is elicited by extrinsic molecules originating from either infectious agents or by homologous cellular components resulting in primary tissue damage. Release of cryptic crossreactive host proteins from cells into the extracellular environment is followed by immune processing and induction of further immune responses (28, 29). The response is now directed against host molecules and a true autoimmune state supervenes, one that persists even after elimination of the original triggering molecules. Unexplained

factors in this scenario include the ability of the original agent to induce a response that is anti-self, and why self-tolerance fails to function. Computer homology searches have documented numerous examples of shared sequences between microbial proteins and host proteins and, clearly, powerful mechanisms are operating to prevent the induction of crossreactive responses. In the case of the most commonly recognized peptide sequence, WMSYPDRTLRTS, there are no identical sequences in the database, and the most similar sequences have at least five mismatches. Interestingly, one of these is the gene product of the bundle-forming pilin gene of enteropathogenic *E. coli.*

Molecular mimicry as a cause of PBC has been previously suggested because of the highly conserved structure of PDC-E2 between bacteria, yeast, and mammals. The conservation is particularly strong at the lipoic acid binding site in the inner lipoyl domain of PDC-E2. An alternative mechanism for molecular mimicry in PBC has been suggested by Burroughs *et al.* (35) based on the assumption that the lipoyl domain is also an epitope for T cells. They noted the similarity in sequence between the HLA-DRα molecule and the lipoic acid binding site and hypothesized this as a possible triggering factor in disease by as yet unknown mechanisms (27).

In this study, we identified three putative amino acid motifs (W-SYP, TYVS, and VRH) shared among peptide sequences recognized by C355.1. In the case of linear epitopes of molecular mimics (31), many autoepitopes are not continuous primary sequence peptides but are conformation-dependent (32, 33). A random peptide library may provide not only linear epitopes but also "conformational mimotopes." The amino acid residues of the conformational epitope may be unrelated to the original sequences that compose the epitope. For example, a hexapeptide that mimics a conformation-dependent binding site of acetyl choline receptor, the major autoantigen in myasthenia gravis, was found to have no homology to the sequence of the AcChoR (34).

A competitive immunohistochemical assay, using synthetic peptides and a mAb with a highly specific PBC BDE staining pattern (C355.1), as well as a control PDC-E2-specific mAb (C150.1), which maps to the outer lipoyl domain, was used to demonstrate that the peptides differentially inhibited the binding of these two mAbs to biliary epithelium. It is interesting that the WMSYPDRTLRTS peptide, that appeared most frequently in our phage clones, inhibits the binding of both C150.1 and C355.1 to PBC BDE cells. This suggests that this peptide acts more efficiently as a mimotope for the inner lipoyl domain of PDC-E2. It is possible that C355.1 and C150.1 may share some degree of structural similarity in their paratope, so that the combining sites can be occupied by a single peptide mimotope, but perhaps with different affinities. However, when a single amino acid substitution was made between the peptides WMSYPDRTLRTS and WESYPDRTLRTS, there was a profound difference in staining inhibition between C355.1 (95%) and C150.1 (25%) suggesting that the glutamic acid substitution enhances the resemblance of the mimotope structure to the inner lipoyl domain of PDC-E2.

Interestingly, the peptide AALWGVKVRHVS effectively inhibited the binding of C355.1 to a purified PDC-E2 inner lipoyl domain fusion protein (aa 128–227) by ELISA at an IC_{50} value of 0.4 μM (data not shown) comparable to the IC_{50} of peptides in a previous report (35). However, it did not block the C355.1 staining on biliary epithelium. The opposite was noted when C150.1 was incubated with AALWGVKVRHVS, where there was a 74% inhibition of BDE staining. These data suggest that the peptide AALWGVKVRHVS acts as a mimotope for the PDC-E2 inner lipoyl domain and may in fact be more representative of the outer lipoyl domain. In contrast, the peptide APKTYVSVSGMV exhibited an extremely interesting pattern of C355.1 staining inhibition. The contrast between the bile duct epithelial cell and hepatocyte staining inhibition was quite remarkable. These differences suggest that C355.1 appears to recognize a molecule whose location is restricted more to biliary epithelium than hepatocytes. This hypothesis is further substantiated by the lack of inhibition of C150.1 staining with APKTYVSVSGMV. The differential blocking effect by the peptide APKTYVSVSGMV on C355.1 staining of biliary epithelium and hepatocytes was reproducible in liver sections from all three patients with PBC. Therefore, the peptide APKTYVSVSGMV may be a mimotope of a crossreactive molecule that exists primarily in the biliary epithelium of patients with PBC and is recognized by C355.1 (11, 12). Based on the above immunohistochemical data, it is plausible to conclude that although C355.1 recognizes the inner lipoyl domain of PDC-E2, it also has an additional specificity that is uniquely different from the control mAb.

The distribution of rabbit anti-WMSYPDRTLRTS binding to sections of the liver was similar to the previously observed distribution of anti-PDC-E2 antibodies (11, 12, 24). Highest intensity of binding was associated with medium-sized interlobular ducts, the ducts primarily involved in PBC. The subcellular distribution of the antigen recognized by the antiserum appeared, by confocal microscopy of tissue sections, to be intracellular. We have found previously that it is not possible, using confocal microscopy of tissue sections, to confidently identify membrane antigens that also have a strong intracellular component. However, we were able to show by electron microscopy that rabbit anti-WMSYPDRTLRTS recognizes a molecule on the exterior of BDE cells in PBC but not in normals. Although the ultimate nature and biochemical identity of the molecule that is responsible for the increased luminal staining of BDE in PBC patients is still unknown, this study has generated powerful reagents to further investigate the identity of this putative PDC-E2 mimic molecule.

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