

Molecular Mimicry in Primary Biliary Cirrhosis

Evidence for Biliary Epithelial Expression of a Molecule Cross-reactive with Pyruvate Dehydrogenase Complex-E2

Judy Van de Water,* Jill Turchany,* Patrick S. C. Leung,* Jack Lake,† Santiago Munoz,‡ Charles D. Surh,* Ross Coppel,|| Aftab Ansari,¹ Yasuni Nakanuma,** and M. Eric Gershwin*

*Division of Rheumatology, Allergy and Clinical Immunology, University of California at Davis, TB 192, School of Medicine, Davis, California 95616; †Division of Gastroenterology, Department of Medicine, University of California, San Francisco, California 94102;

‡Division of Gastroenterology, Department of Medicine, Jefferson Medical College, Philadelphia, Pennsylvania 19107; ||Walter and Eliza Hall Institute for Medical Research, Melbourne 3050, Australia; ¹Department of Pathology, Emory University School of Medicine, Atlanta, Georgia 30322; and **Department of Pathology, Kanazawa University, School of Medicine, Kanazawa 920, Japan

Abstract

Sera from patients with primary biliary cirrhosis (PBC) react with enzymes of the 2-oxo dehydrogenase pathways, particularly PDC-E2. These enzymes are present in all nucleated cells, yet autoimmune damage is confined to biliary epithelial cells. Using a panel of eight mouse monoclonal antibodies and a human combinatorial antibody specific for PDC-E2, we examined by indirect immunofluorescence and confocal microscopy sections of liver from patients with PBC, progressive sclerosing cholangitis, and hepatocarcinoma. The monoclonal antibodies gave typical mitochondrial immunofluorescence on biliary epithelium and on hepatocytes from patients with either PBC, progressive sclerosing cholangitis, or hepatocarcinoma. However, one of eight mouse monoclonal antibodies (C355.1) and the human combinatorial antibody reacted with great intensity and specificity with the luminal region of biliary epithelial cells from patients with PBC. Simultaneous examination of these sections with an antiisotype reagent for human IgA revealed high IgA staining in the luminal region of biliary epithelial cells in patients with PBC. IgG and IgA antibodies to PDC-E2 were detected in the bile of patients with PBC but not normal controls. We believe that this data may be interpreted as indicating that a molecule cross-reactive with PDC-E2 is expressed at high levels in the luminal region of biliary epithelial cells in PBC. (*J. Clin. Invest.* 1993. 91:2653–2664.) Key words: pyruvate dehydrogenase • autoantibodies • aberrant expression • IgA • confocal microscopy

Introduction

Primary biliary cirrhosis (PBC)¹ results from autoimmune mediated destruction of intrahepatic bile ducts with progres-

sive inflammatory scarring. The association of PBC with high titer autoantibodies to mitochondrial antigens has long been recognized and the possible significance has been discussed (1, 2). It has now been established that PBC sera react with a series of highly conserved intramitochondrial proteins, including the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2), the E2 subunit of the branched chain keto acid dehydrogenase complex, the E2 subunit of the α -ketoglutarate dehydrogenase complex, the E1 α and E1 β subunit of the pyruvate dehydrogenase complex, and protein X (3–6). Among this group of autoantigens, the major antimitochondrial antibody (AMA) activity is directed against the 74-kD antigen, PDC-E2.

It is not yet established what the original stimulus for autoantibody production is or why autoreactivity is directed at a highly conserved intracytoplasmic autoantigen and how immune chelators, such as antibody, may attack an intracellular target. Furthermore, it is unclear as to why the immune damage in PBC is tissue and organ specific being limited to intrahepatic biliary duct cells and not to any of the other myriad of cell types in various organs that express the same antigens. Finally, it is not known whether the observed responses to the mitochondrial autoantigens are in fact related to the disease process.

Earlier work from our laboratory and others by fluorescent microscopy has demonstrated that large quantities of material immunoreactive with antibodies to PDC-E2 are present in biliary epithelial cells of patients with PBC but not patients with progressive sclerosing cholangitis (PSC) or chronic active hepatitis (7–9). We have now taken advantage of a panel of eight affinity-purified mouse monoclonal antibodies directed to different regions of PDC-E2 (10), as well as a human combinatorial autoantibody derived from a lymph node from a patient with PBC (11), to examine the expression and localization of immunoreactive molecules in biliary epithelial cells. We report herein that an increase in staining in the bile duct epithelium (BDE) of patients with PBC is noted only with one of eight murine monoclonal antibodies directed against PDC-E2 and with the human combinatorial antibody LC 5. The intense signal seen with these two antibodies is heavily concentrated in the luminal region of the bile duct and shows a striking similarity to the pattern observed when biliary epithelium is probed with a polyclonal antibody to IgA. We believe that this altered staining suggests the existence of a molecule cross-reactive with PDC-E2 expressed specifically in PBC biliary epithelium and not in normal biliary epithelium. This molecule, whether encoded by human or viral DNA, would explain the highly directed and specific response to only one major epitope in PDC-E2. The data also raise intriguing questions as to how to proceed with further understanding the mechanism(s) of disease.

Address correspondence to M. Eric Gershwin, M.D., Division of Rheumatology/Allergy, University of California at Davis, TB 192, School of Medicine, Davis, CA 95616.

Received for publication 24 July 1992 and in revised form 21 January 1993.

1. Abbreviations used in this paper: AMA, antimitochondrial antibody; BDE, bile duct epithelium; PBC, primary biliary cirrhosis; PDC-E2, pyruvate dehydrogenase complex-E2, PSC, primary sclerosing cholangitis.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.

0021-9738/93/06/2653/12 \$2.00

Volume 91, June 1993, 2653–2664

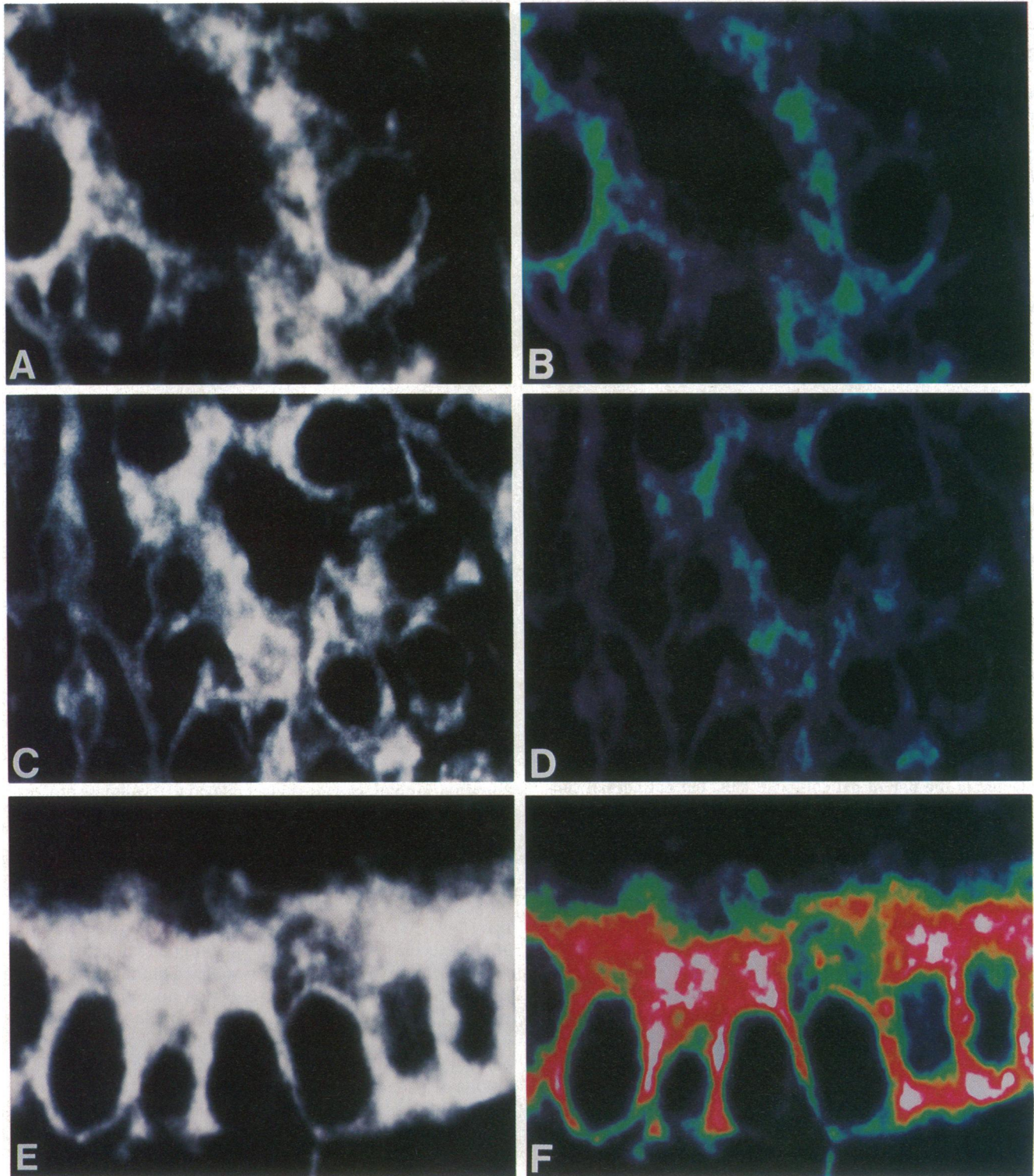


Figure 1. Confocal micrographs of PBC liver sections stained with the murine monoclonal antibodies. In *A*, the mAb C315 shows a typical mitochondrial pattern in the BDE of the PBC liver. In *B*, the PBC BDE from *A* was examined using Lutz color intensity banding. The Lutz color banding range is from dark blue (lowest) to white (highest). Note the overall blue and green (second lowest level) staining. In *C*, mAb S184 was used to again demonstrate the typical mitochondrial pattern. In *D*, the section of BDE of *B* was examined using Lutz color intensity banding. The overall staining with S184 is restricted to the low range or blue with very little green. In contrast, there was intense homogeneous luminal staining in *E* with mAb C355.1. In *F*, the section of 1E was examined using Lutz color intensity banding. Note the overall red pattern (second highest intensity level) with several white (highest level) focal points (all $\times 200$; zoom 8).

Methods

Sources of tissue. Liver specimens were obtained from 25 patients with PBC, 12 patients with PSC, and two patients with hepatocarcinoma. All specimens were obtained from the University of California Medical Center at Davis and at San Francisco. The diagnosis of all patients was based on established disease criteria and confirmed by histologic review by an independent observer (7). Additionally, 22 bile specimens were obtained for study, including 16 specimens from patients with PBC and six normal controls. The bile was collected at the time of transplantation and frozen at -70°C until used.

Monoclonal antibodies. We have previously reported the development and characterization of PDC-E2 specific murine monoclonal antibodies (10). Eight of these antibodies were used to study the localization of PDC-E2 in liver by scanning confocal imaging microscopy. These eight monoclonal antibodies, coined C355.1, S184, C315, C339, C545, C150, C157, and C192, have previously been described in detail and their mapping described (10). These reagents map to four different regions of PDC-E2 when studied by ELISA with overlapping recombinant fragments. All produce intense immunofluorescence of mitochondria when used to stain Hep-2 cells. In addition to the eight murine monoclonal antibodies, we used a human Fab monoclonal antibody termed LC 5 derived from a PBC lymph node combinatorial library. Lymph node mRNA was extracted, amplified, subcloned, and expressed as described (11). This Fab recombinant antibody reacts with PDC-E2 and protein X but not branched-chain ketoacid dehydrogenase, α -ketoglutarate-E2, or PDC-E1 α by immunoblotting. It is also specific for PDC-E2 by ELISA and maps to the inner lipoyl domain (11). As additional controls for all antibody studies, an irrelevant murine monoclonal antibody to thymocytes (SAG-3) (7) and a polyclonal antibody to a common mitochondrial enzyme, pyruvate decarboxylase, were used.

Confocal microscopy. After collection, tissues were fixed in 8% neutral buffered formalin and paraffin imbedded; 6- μm sections were cut and mounted to prevent tissue detachment. The sections were deparaffinized by heating overnight at 60°C followed by two 5-min changes of xylenes, two 5-min changes of 100% ethanol, two 3-min changes of 95% ethanol, and one 3-min change in 70% ethanol followed by two 5-min changes in PBS. Sections were blocked with 0.1% gelatin/PBS for 1 h, and then individual sections were incubated overnight at 4°C with (a) SAG-3, an irrelevant antibody (1:10); (b) affinity-purified mAbs C355.1, S184, C315, C339, C545, C150, C157, and C192 (standardized for each mAb on HEp2 cells); (c) rabbit anti-pyruvate carboxylase (a generous gift from Dr. M. S. Patel, Case Western Reserve University, Cleveland, OH) as a positive mitochondrial control (1:10); (d) rabbit anti-human IgA diluted 1:100 (Sigma Chemical Co., St. Louis, MO); and (e) biotinylated human combinatorial LC 5-specific for PDC-E2 (11) diluted 1:4. After incubation, the slides were washed in three changes of PBS followed by a 30-min incubation with previously determined optimum titers of either rhodamine-conjugated goat F(ab')₂ anti-mouse Ig or goat anti-rabbit Ig (both of which were human absorbed), or streptavidin-rhodamine (in the case of LC 5) (all from Caltag, South San Francisco, CA), for 30 min at room temperature. After three washes in PBS, the sections were coverslipped using (Slo-Fade; Molecular Probes, Inc., Eugene, OR) and viewed by using a laser confocal microscope (MRC 600; Bio-Rad Labs, Richmond, CA) with a GHS filter block and a no. 1 neutral density filter. To determine whether the mAb C355 and the combinatorial antibody LC 5 react with the same molecule, an inhibition immunohistochemistry assay was performed. The slides were treated as above except that the serial sections were first incubated with LC 5 for 2 h at room temperature followed by C355 overnight at 4°C . After washing, the slides were incubated with rhodamine-conjugated goat F(ab')₂ anti-mouse Ig and visualized as above. The resulting confocal images were analyzed using an SOM program integrated with the Bio-Rad confocal system. In addition to the irrelevant monoclonal controls, further controls were included by omitting either the primary or secondary reagents.

Western blot analysis. To determine the presence of autoantibod-

ies, as well as their isotypes to the 2-oxo-dehydrogenase enzymes in bile, an immunoblot was performed using beef heart mitochondria substrate (6) and bile fluid as the probe. Briefly, 40 μg of the mitochondria preparation was boiled in SDS-sample buffer containing 2-mercaptoethanol and separated in a 10% SDS-PAGE gel (6). The gels were then blotted onto nitrocellulose. The blotted proteins were then probed with bile from 16 patients with PBC and six non-PBC controls diluted 1:100 in 2.5% milk/PBS for 1 h. After washing three times, the blots were incubated with 0.5 $\mu\text{Ci/ml}$ ^{125}I -anti-human Ig, washed again, and autoradiographed overnight. To illustrate the reactivity of the mAbs C355, C315, S184, and LC 5 to mitochondria, a Western blot was performed as above on beef heart mitochondria using the mAbs diluted 1:20 and the combinatorial antibody LC 5 at 1:10. The secondary antibodies used were goat anti-mouse peroxidase conjugate (Tago, Burlingame, CA) diluted 1:1,000 and goat anti-human F(ab')₂ (Jackson ImmunoResearch, West Grove, PA) diluted 1:1,000. The blots were visualized using chemiluminescence (ECL; Amersham Corp., Arlington Heights, IL).

Isotyping of bile autoantibodies. Isotypes of the PDC-E2 specific antibodies present in the bile were determined by ELISA using polystyrene microtiter plates coated overnight with porcine PDC (Sigma Chemical Co.) at a concentration of 10 $\mu\text{g/well}$ at 4°C . The plates were then washed and blocked for 1 h at room temperature with 1% BSA. Bile fluid was diluted 1:50 in PBS/BSA and 0.1 ml incubated in microtiter wells for 1 h at room temperature. After washing three times, a predetermined optimum concentration of a heavy chain-specific goat anti-human IgG, IgA, and IgM (Sigma Chemical Co.) and goat anti-human Ig peroxidase conjugate (Tago) were added and incubated for 30 min at room temperature. After washing, the isotyping plates were incubated with optimally titered rabbit anti-goat Ig peroxidase conjugate (Cappel, Inc., Durham, NC) for 30 min. The reaction was visualized using 2,2'-Azinobis (3-ethylbenzthiazolinesulfonic acid) (Sigma Chemical Co.), stopped after 10 min with 10% SDS, and read at 450 nm. As controls for all assays, bile from normal controls, serum from three patients with PBC and serum from three normal volunteers, were studied concurrently.

Results

Confocal microscopy. All eight murine monoclonal antibodies produced typical and equivalent mitochondrial staining on hepatocytes and biliary epithelium in sections from patients with hepatocellular carcinoma and primary sclerosing cholangitis. Livers from 25 patients with PBC were concurrently examined with the same panel of murine monoclonal antibodies. Only 9 of the 25 PBC livers still had recognizable bile ducts. Interestingly, one of the eight murine monoclonal reagents, C355.1, produced intense staining on or near the luminal surface of biliary epithelial cells. The other seven antibodies reacted only with mitochondria (Fig. 1). This intense luminal staining pattern with C355.1 was seen in biliary epithelium in all of the PBC livers with remaining bile ducts but not in the surrounding hepatocytes nor in cells in sections from patients with the two other liver diseases studied (Figs. 2 and 3). This unique pattern of luminal staining of PBC biliary epithelium was also found using LC 5, a human combinatorial antibody directed against PDC-E2 (Fig. 4). It is important to emphasize that while the seven additional murine monoclonal antibodies did not react in the region of the surface of bile duct epithelial cells, they did stain mitochondria in a manner similar to PBC sera and C355.1 (Table I). As an additional control for these observations, an antibody to pyruvate decarboxylase, a common mitochondrial enzyme, was also studied. This latter reagent stained hepatocytes and biliary epithelium in a typical mitochondrial pattern, but did not stain the luminal region of biliary epithelium. We simultaneously examined the distribution

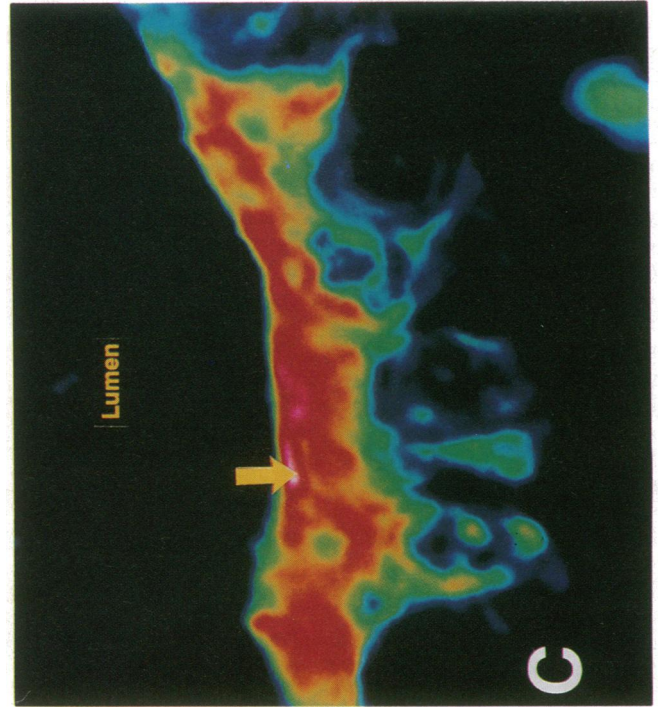
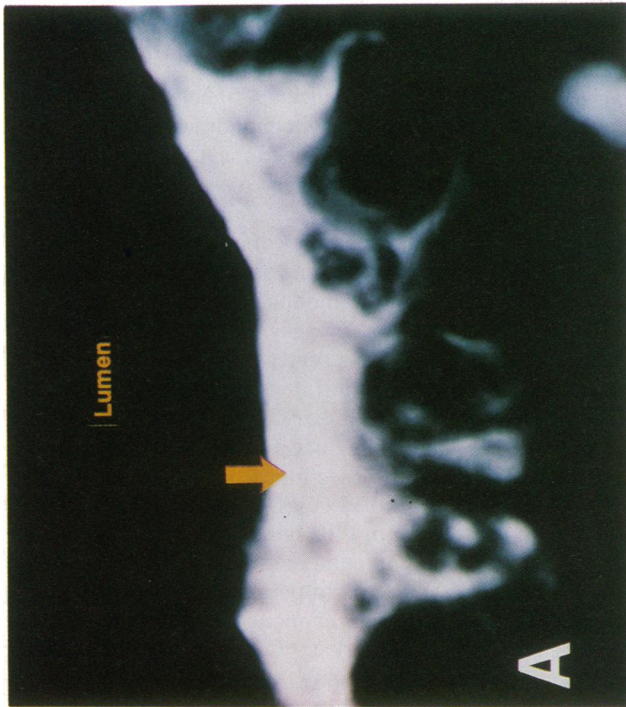
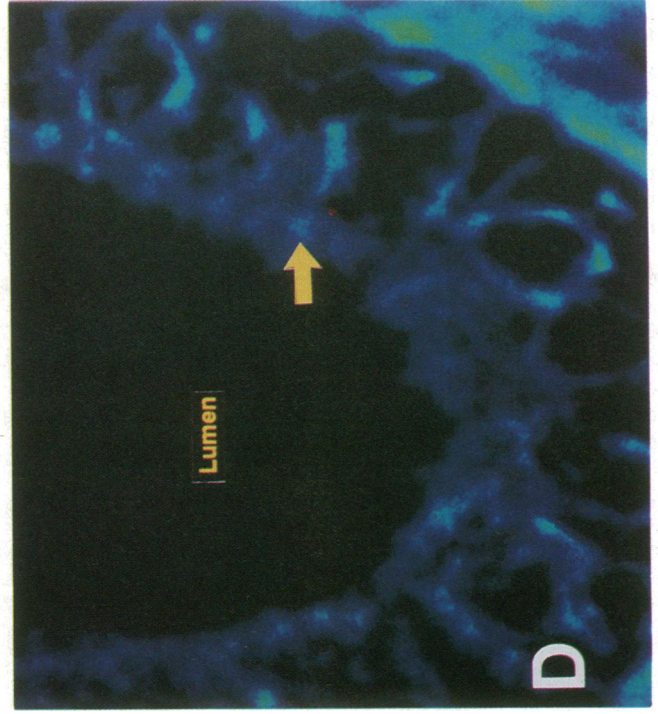
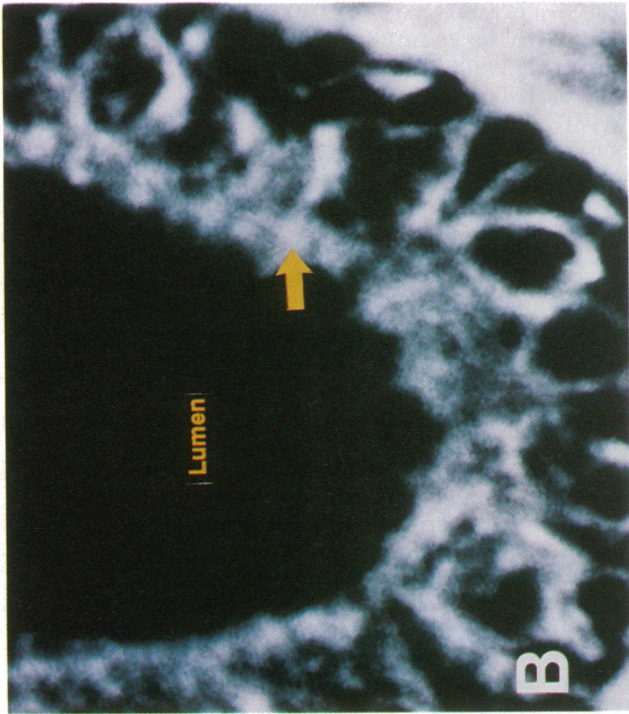


Figure 2. For figure legend see page 2659.

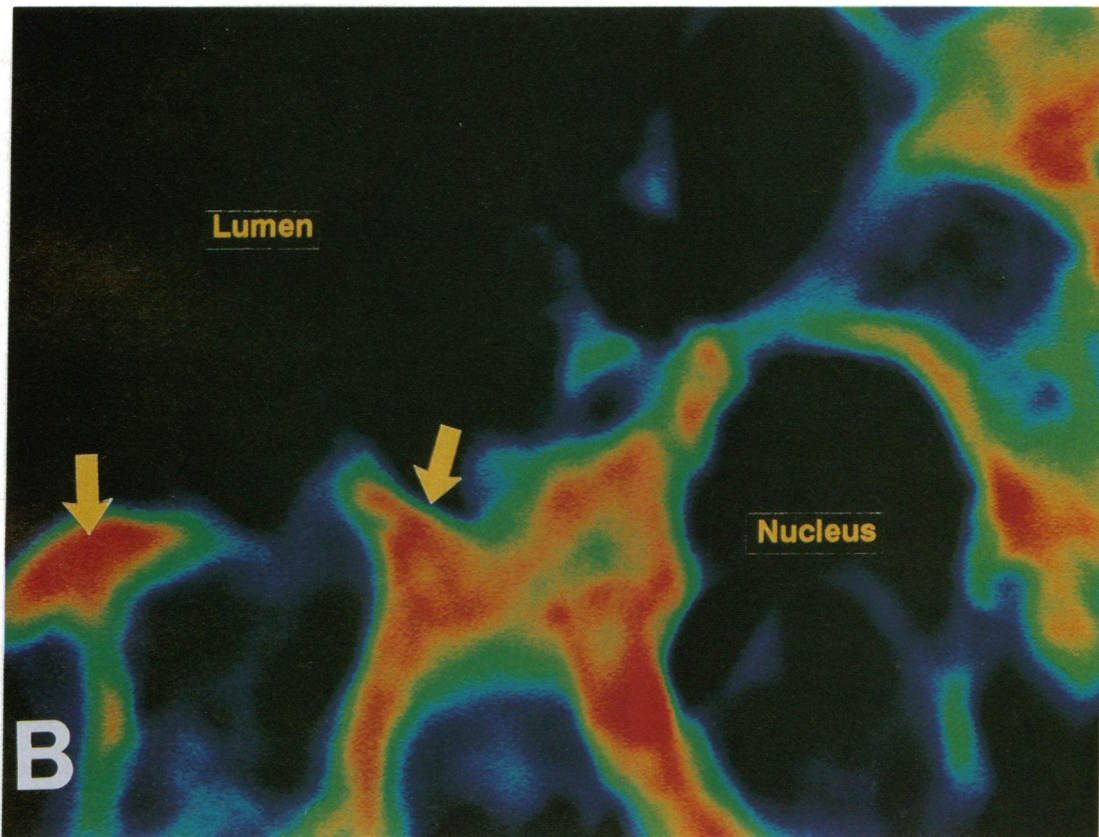
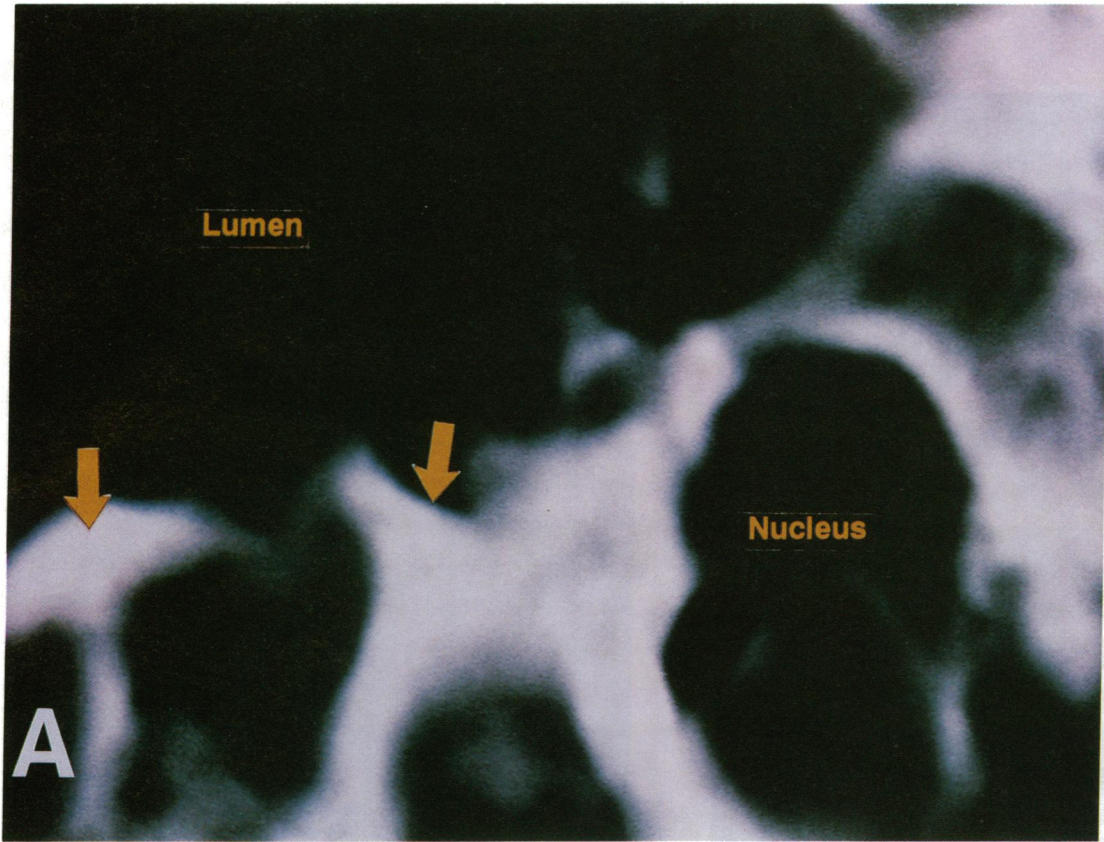


Figure 3. For figure legend see page 2659.

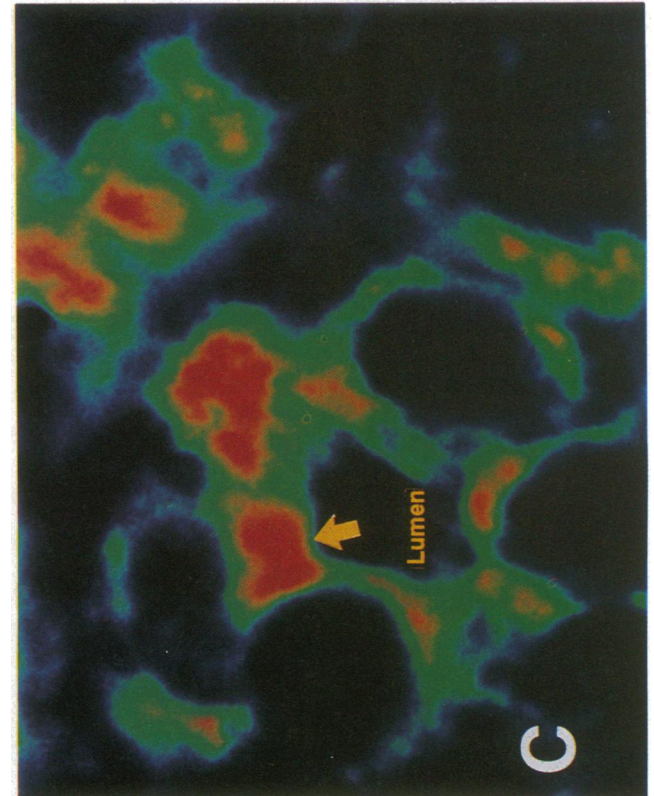
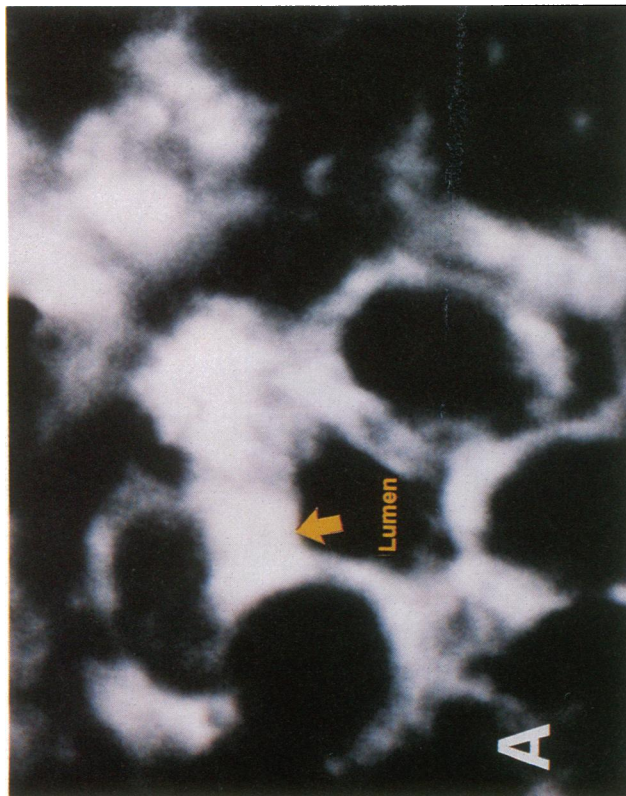
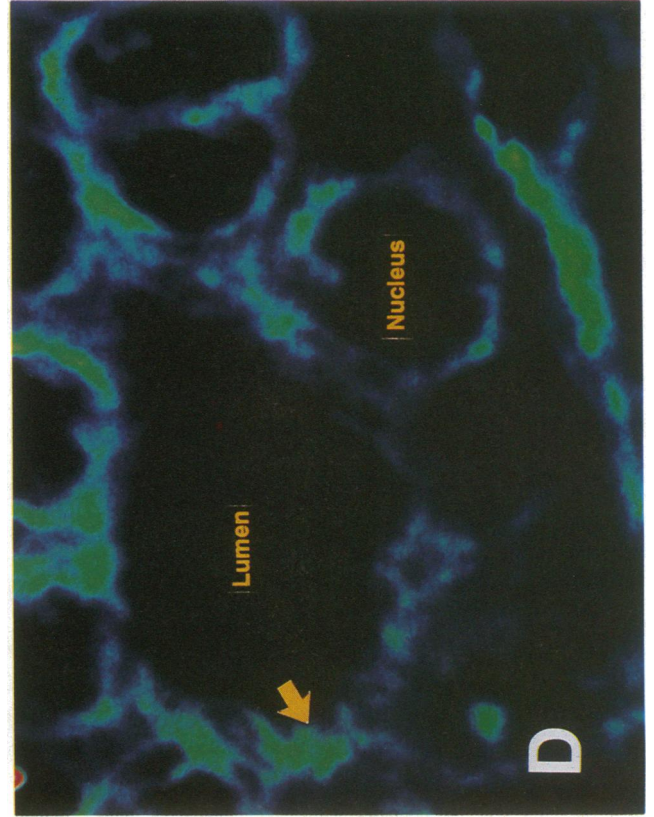
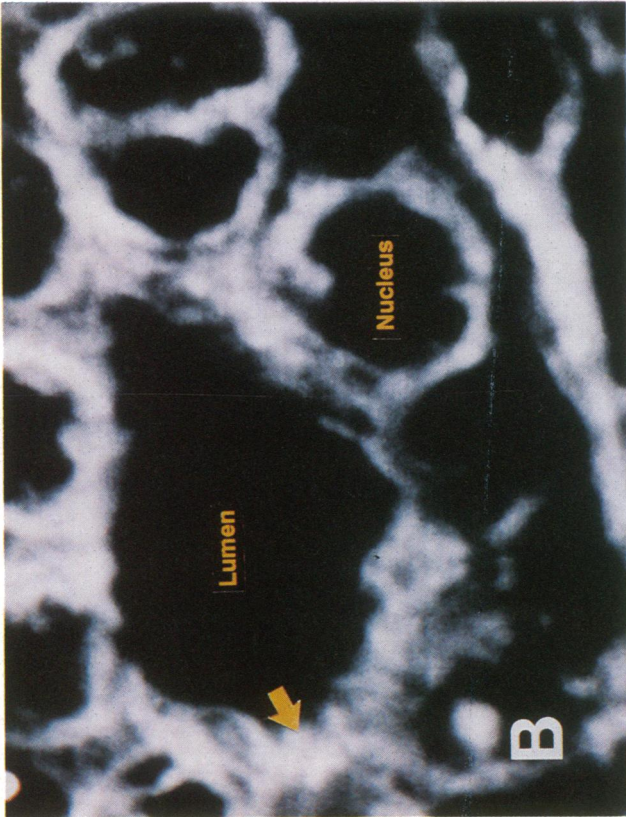


Figure 4. For figure legend see page 2659.

Figure 2. Confocal micrographs of PBC (*A* and *C*) and PSC (*B* and *D*) liver sections stained with monoclonal antibody C355.1. In (*A*), the PBC BDE demonstrates intense homogeneous luminal staining (*arrow*). In *B*, the PSC BDE, studied at the same laser intensity for standardization, demonstrates a normal granular pattern of mitochondrial staining in the cytoplasm. In *C*, the section of PBC BDE of *A* was examined using Lutz color intensity banding. The Lutz color banding range is from dark blue (lowest) to white (highest). Note the overall red staining (second highest intensity level) with one area (*arrow*) showing the highest intensity possible (white) near the luminal surface. In contrast, in *D*, the section of PSC BDE of *B* was examined using Lutz color intensity banding. Note that the overall staining in PSC is restricted to the low range or blue (all $\times 200$; zoom factor 6).

Figure 3. Using high power ($\times 400$; zoom 8), a liver section from a patient with PBC was stained and studied by confocal microscopy with C355.1. In *A*, note the intense staining throughout BDE cells, but especially concentrated toward the luminal surface. In *B*, the intensity is again represented using Lutz color intensity banding. Note that the red (*left arrow*), yellow, and green (*right arrow*) areas are concentrated at the luminal surface.

Figure 4. Confocal micrographs of PBC (*A* and *D*) and PSC (*C* and *D*) liver sections stained with the human combinatorial antibody LC 5. In *A*, the PBC BDE demonstrates intense luminal staining (*arrow*). In *B*, the PSC BDE studied at the same laser intensity for standardization, demonstrates a normal granular pattern of mitochondrial staining. In *C*, the section of PBC BDE of Fig. 3 *A* was examined using Lutz color intensity banding. Note the intense staining in red and yellow (*arrow*). In contrast, in *D*, the section of PSC BDE of Fig. 3 *B*, was examined using Lutz color intensity banding. Note that the overall staining in PSC to the blue/green spectrum is restricted ($\times 200$; zoom 8).

of IgA in the biliary epithelium. The pattern of staining directed against IgA was similar to that seen with C355.1 and LC 5, with staining throughout the cell, but heaviest at the luminal surface region (Fig. 5).

In an effort to derive an objective observation of the degree of staining, a whole-cell histogram analysis was performed in detail on two representative liver sections from two patients with PBC and two patients with PSC using the MacIntosh Image program with a 10-color band restriction. Using this program, the values obtained are represented as the number of pixels at a given pixel intensity level (color band); the maximum staining intensity possible is 255 pixels. As demonstrated in Fig. 6, the histogram analysis of biliary epithelial cells from patients with PBC stained with C355.1 demonstrated high intensity luminal region staining. In contrast, histogram analysis of biliary epithelial cells from patients with PSC demonstrated staining primarily in the cytoplasmic region. Similarly, when biliary epithelial cells from patients with PBC were stained with the human combinatorial antibody, a dramatic increase in pixel intensity was noted in the luminal area in patients with PBC but not PSC (Fig. 7). When these same sections were stained for IgA, there was a significantly increased signal in the biliary epithelial cells of patients with PBC, whereas the biliary

epithelial cells of patients with PSC had an expected moderate pixel intensity level (data not shown).

To determine whether C355 and LC 5 both react with the same molecule in bile duct epithelium, an immunohistochemistry inhibition assay was performed. When serial sections were preincubated with LC 5 followed by C355, a virtual absence of staining was observed when compared to C355 alone (Fig. 8). This reduction was also observed in the mitochondrial staining of hepatocytes.

Autoantibodies in bile. 16 of 16 bile samples, diluted 1:100, from patients with PBC demonstrated strong activity against both PDC-E2 and occasionally protein X when probed against beef heart mitochondria (Fig. 9). In contrast, six normal bile samples run in parallel failed to show detectable bands. Using ELISA the isotypes of these AMA included IgG and IgA (Table II). There was little IgM anti-PDC-E2 detected in bile fluid. Positive control sera from patients with PBC gave expected values with IgM, IgG, and IgA anti-PDC-E2 activity; negative sera controls gave background values.

Mitochondrial reactivity of mAbs. Western blots were performed using beef heart mitochondria to determine the reactivity of the mouse monoclonal antibodies C355, C315, S184, and the human combinatorial antibody LC 5. All four antibodies

Table I. Altered Staining Pattern of Bile Duct Epithelial Cells Detected with Monoclonal Antibodies to PDC-E2*

Group	C355.1 [‡]	S184	C315	C339	C545	C150	C157	C192	LC5
Primary biliary cirrhosis									
Mitochondrial staining [§]	9/9	8/9	9/9	9/9	9/9	9/9	9/9	9/9	9/9
Luminal surface staining	9/9 [¶]	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9
Primary sclerosing cholangitis									
Mitochondrial staining	7/12	4/12	7/12	8/12	8/12	8/12	8/12	8/12	8/12
Luminal surface staining	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12
Hepatocarcinoma									
Mitochondrial staining	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2
Luminal surface staining	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2

* Antibodies to pyruvate decarboxylase stain specimens only in a typical mitochondrial pattern without evidence of staining at the luminal surface; [‡] number of positive/total; [§] typical mitochondrial staining (e.g., normal AMA-type); ^{||} intense staining at the luminal surface of the bile duct epithelial cells independent of the normal mitochondrial staining within the cytoplasm; [¶] 25 PBC specimens were examined but only nine had visible bile ducts. All of the controls had bile ducts.

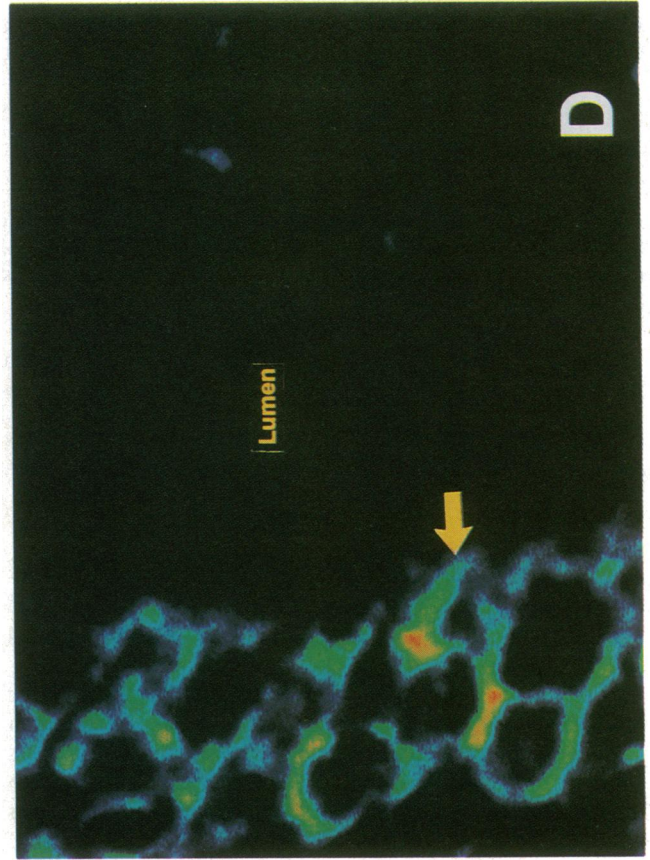
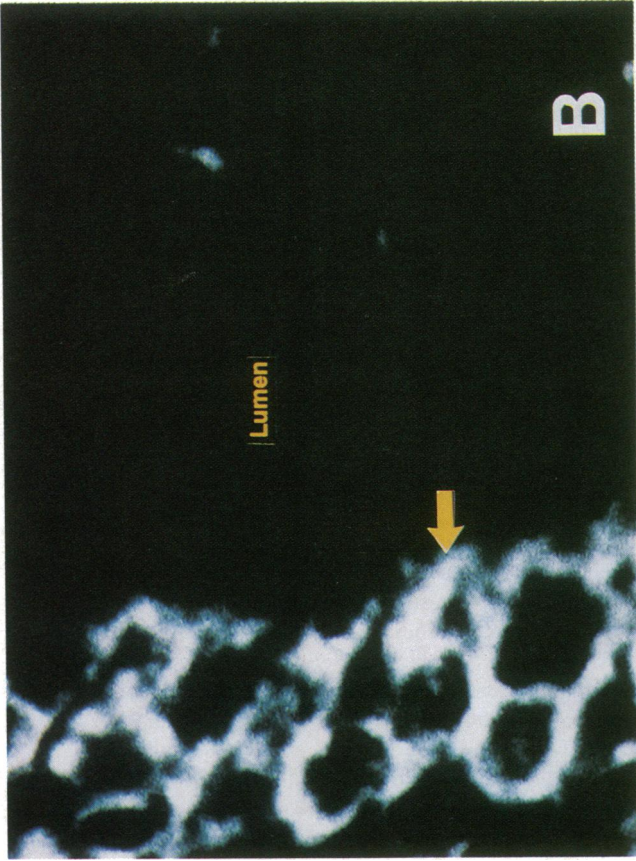
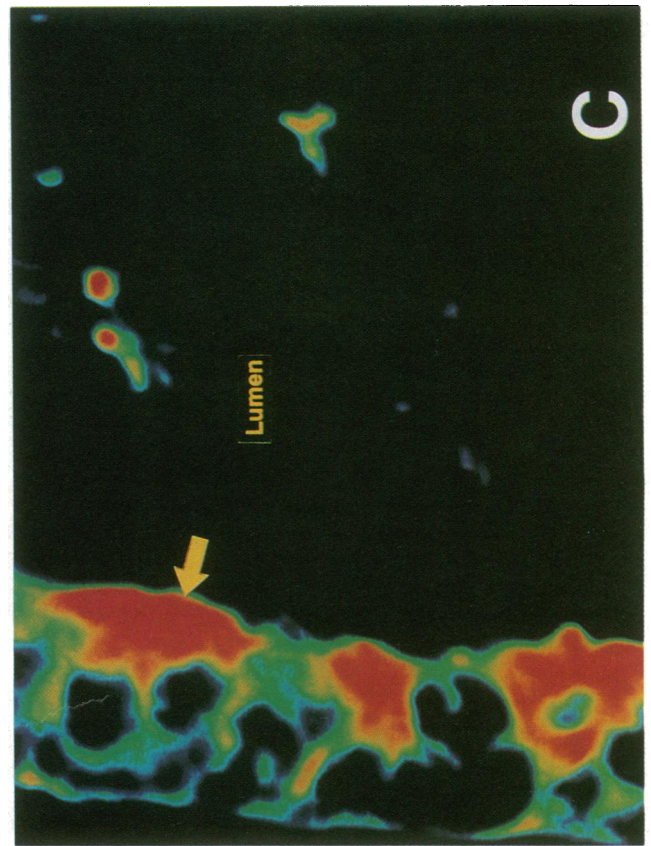
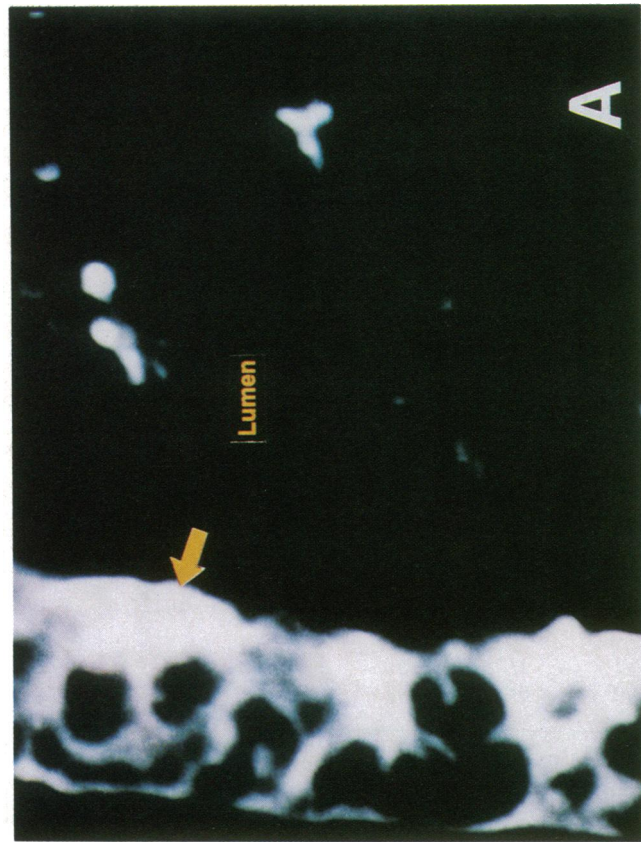


Figure 5. For legend see next page.

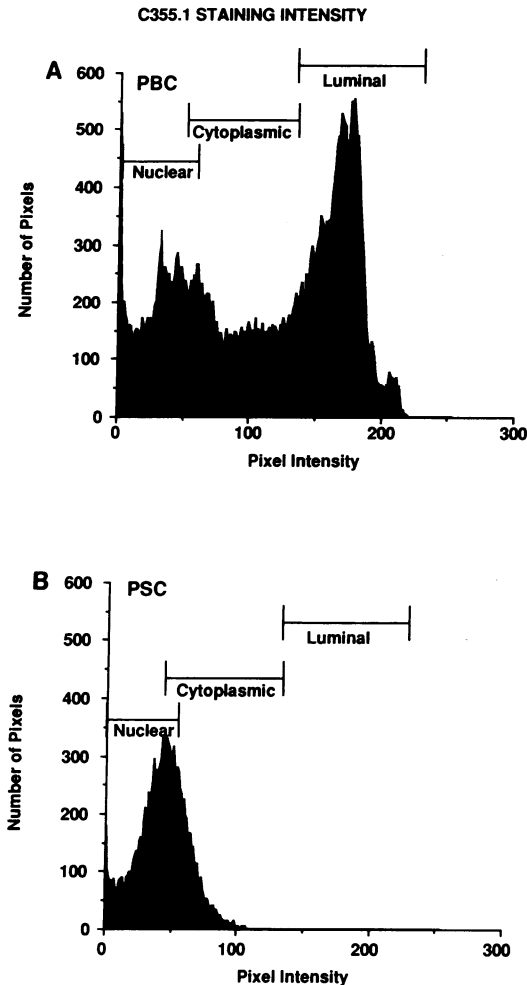


Figure 6. Histogram analysis of color intensity banding for PBC and PSC liver sections of BDE cells stained with C355.1. The y axis is the number of pixels in the area analyzed of designated pixel (color) intensity. These color (pixel) determinations correspond to the color banding patterns used in Figs. 1–4. The nuclear, cytoplasmic, and luminal regions are designated by the labeled bars. Thus, with a maximum color intensity of 255, the PBC histogram (A) shows a much greater pixel density > 100 in the luminal area with > 500 pixels in the high intensity range. In contrast, the PSC histogram has ~ 350 pixels at a much lower intensity range of 50 in the nuclear/cytoplasmic area.

showed strong 74-kD (PDC-E2) staining with variable reactivity to the breakdown products of PDC-E2 and the 56-kD protein X (Fig. 10). Reactivity to these bands could be removed upon absorption with either recombinant or purified PDC-E2 (data not shown). These results indicate that all of the antibodies show similar recognition of specific mitochondrial proteins. This is in contrast to their staining pattern of bile duct epithelium in patients with PBC. The specificity of the antibodies for

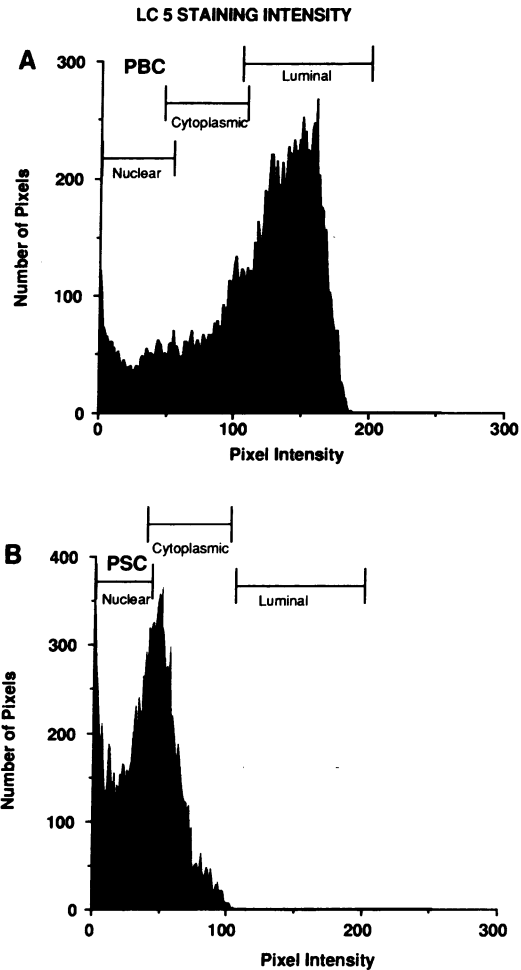


Figure 7. Histogram analysis of color intensity staining banding for PBC and PSC liver section of BDE cells stained with LC 5. Note the luminal region in PBC BDE is much more intense than PSC BDE (see Fig. 6 legend).

mitochondria in cells under normal conditions is further substantiated by the specific staining of the mitochondria of HEP2 cells with no staining of the nucleus, nuclear membrane, or cell membrane (10, 11).

Discussion

In primary biliary cirrhosis, as in several other autoimmune diseases, the autoantigen is an intracellularly located molecule, present in every cell in the body; yet the disease is tissue-specific, targeted to the biliary ductular tissue of the liver. A serologic response to the 2-oxo-acid dehydrogenase complexes is highly specific for PBC, found at relatively high titers in 90–100% of all patients, and rarely in other diseases (12, 13). How-

Figure 5. Confocal micrographs of PBC (A and C) and PSC (B and D) liver sections stained with anti-IgA. In A, the PBC BDE demonstrates intense luminal and cytoplasmic staining (arrow). In B, the PSC BDE, studied at the same laser intensity for standardization, demonstrated an expected diffuse strong cytoplasmic staining. In C, the section of PBC BDE of Fig. 4 A was examined using Lutz color intensity banding. Note the very strong red areas of high intensity staining, especially near the lumen (arrow). In contrast, in D, the section of PSC BDE of Fig. 4 C was examined using Lutz color intensity banding. Note that the PSC BDE has only a few red and yellow cytoplasmic areas confined to the perinuclear cytoplasm ($\times 200$; zoom 6).

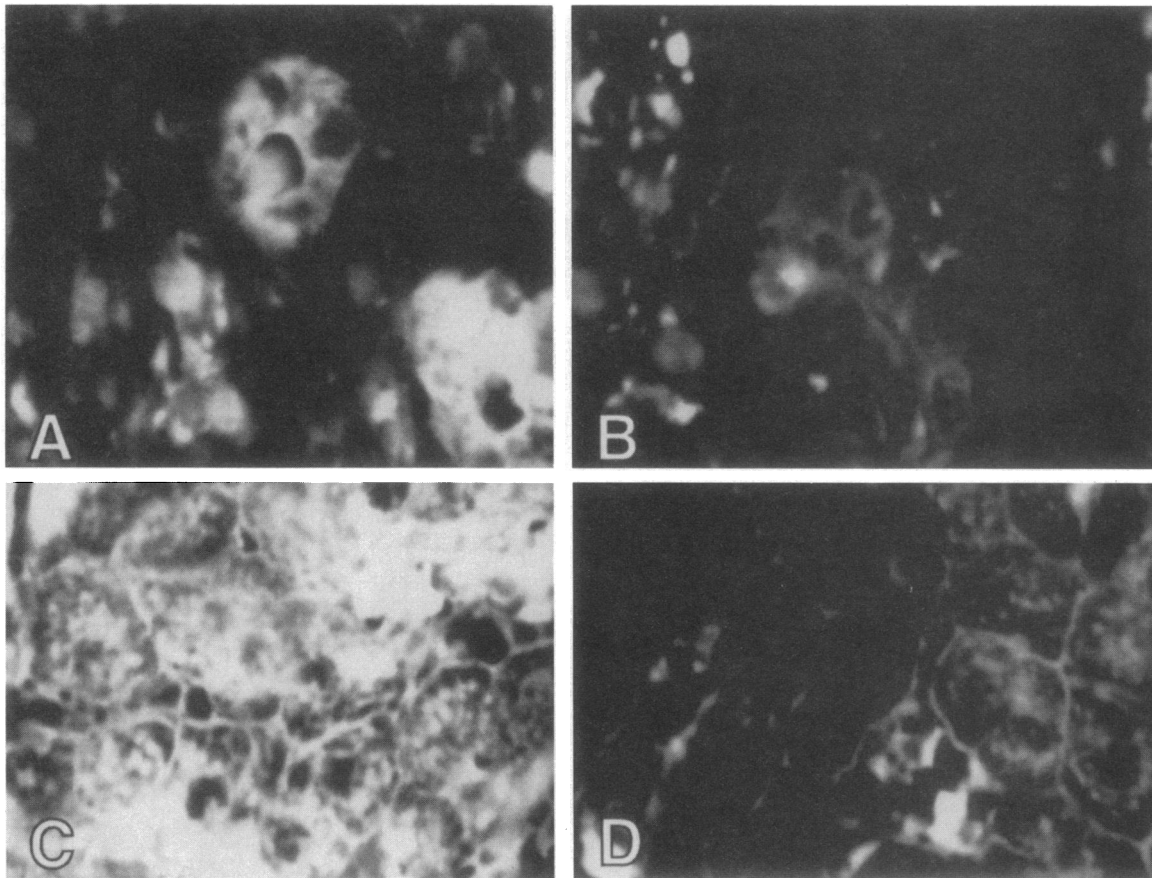


Figure 8. Confocal micrographs of PBC serial liver sections from an immunohistochemistry inhibition assay. In *A*, BDE from a patient with PBC was stained with C355.1. Note the intense staining pattern characteristic for this antibody. In *B*, a serial section showing the same duct preincubated with LC 5 followed by C355.1. Note the virtual absence of staining after blocking with LC 5. The same phenomenon can be observed in the mitochondrial staining as seen in hepatocytes in *C* and *D* from the same PBC liver sections. In *C*, the tissue was stained with C355.1. In *D*, the serial section of *C* was blocked with LC 5 and significantly reduced the mitochondrial staining (all $\times 200$; zoom 4).

ever it is not understood why a mitochondrial antigen, physically sheltered from the immune system by two membrane barriers, should elicit an immune response or, once the immunity is established, how this "self-reactive" immunity causes tissue damage when the antigen is intracellular.

Previous studies using rabbit polyclonal sera to PDC-E2 have demonstrated strong staining of biliary epithelium in PBC, as well as increased staining in a subset of macrophages in portal lymph nodes. In contrast, staining with antibodies to E1 and E3 produced only characteristic mitochondrial reactivity in both PBC and control tissue (7, 8). In this study, we have confirmed these observations and have extended it by the use of a panel of monoclonal antibodies to PDC-E2. Importantly, the increased reactivity in PBC biliary epithelium is seen with only two monoclonal reagents, the murine monoclonal antibody C355.1 and the human combinatorial antibody LC 5. Moreover, there is significant overlap of the epitopes recognized by C355.1 and LC 5 as demonstrated by the inhibitory effect of LC 5 on C355.1 staining of bile duct epithelium. The remaining monoclonal antibodies gave only a typical mitochondrial pattern of staining that was similar in all tissues examined, despite the fact that the intensity of these antibodies on Western blot and HEP-2 cells is identical to C355 and often greater than LC 5. This suggests that the increased staining is

not caused by a molecule immunologically identical to the form of PDC-E2 present in mitochondria.

What then is the target molecule that is responsible for the increased staining? It does not seem likely that the staining is of a processed or otherwise modified form of PDC-E2 as several of the murine monoclonal antibodies have been shown to map to regions very close to the binding site of C355.1. Staining could be caused by a modified form of PDC-E2 that lacks all other epitopes except that recognized by C355.1. However, such grossly truncated products of a longer molecule are generally highly unstable and are usually rapidly removed from the cell by ubiquitin tagging and subsequent proteolysis (14). An alternative explanation is that the reactive material is caused by the presence of another molecule that shares a single cross-reactive epitope with PDC-E2. Further work will be required to show whether such a molecule may be a normal cellular component of biliary tissue that is usually expressed at very low levels, or alternatively, a host molecule aberrantly expressed in biliary cells, or even a neoantigen of viral origin. It is unlikely that this molecule is a lipid as the sections are devoid of lipids after the xylenes and alcohol treatment during processing.

Importantly, the existence of such a molecule creates a compelling scenario in which the initial events in the pathogenesis of PBC would be a tissue-specific overexpression of a novel

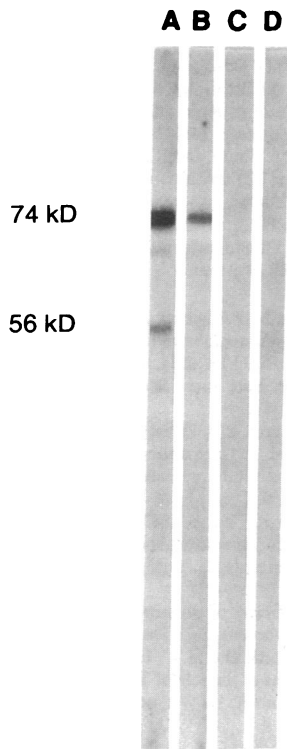


Figure 9. Beef heart mitochondria were separated by PAGE, blotted, and probed with bile from patients with PBC (lanes A and B) or patients with PSC (lanes C and D). In A, the bile reacts with the 74-kD PDC-E2 and the 56-kD protein X. In contrast, in lanes C and D, PSC bile is nonreactive.

substance with consequent presentation by MHC proteins on the tissue surface. Biliary epithelium normally only expresses class I but some studies have identified the presence of class II antigens on PBC biliary epithelium (15); this would allow induction of helper CD4⁺ cells that provide help for induction of CD8⁺ cytotoxic cells. It is also possible that this neoantigen may be surface expressed. High level expression of the neoantigen would lead to association with class I MHC antigens; reactivity against the biliary specific neoantigen, perhaps by CD8⁺ cytotoxic lymphocytes, would lead to cell lysis and release of mitochondrial products. Presentation of released proteins to lymphocytes primed to the cross-reactive PDC-E2 could result in presentation of physically associated proteins such as other components of the PDC complex and synthesis of a range of autoantibodies. Recent studies (16) have shown that with cytochrome *c*, self tolerance can be broken when B cells are stimulated with a foreign antigen that also binds to a self protein. These activated B cells can then present the self proteins to T cells, thus causing the generation of autoreactive T cells. Immune damage would be confined to the biliary tract because effective cytotoxic responses are aimed at the cells expressing the initiating antigen. It remains to be established by what means the biliary epithelium is able to take over the function of

Table II. Isotype Specificity of Bile PDC-E2*

Group	n	IgM	IgG	IgA
PBC	16	0.059±0.002 [‡]	0.161±0.012 [‡]	0.172±0.005 [‡]
Control	6	0.010±0.004	0.015±0.002	0.015±0.003

* Mean optical density±SD [‡] P < 0.01, Student's *t* test.

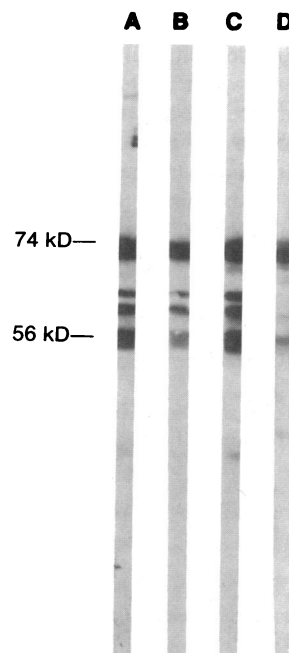


Figure 10. Beef heart mitochondria were separated by PAGE, blotted, and probed with the monoclonal antibodies. (Lane A) C355.1 gave a strong signal at 74 kD (PDC-E2) and 56 kD (protein X). Lane B shows similar staining with mAb C315, as does lane C when treated with mAb S184. Lane D was incubated with the combinatorial antibody LC 5 showing a strong 74-kD band with a less intense 56-kD band.

a "professional antigen presenting cell" in a way that allows provision of a second signal.

It is of further interest that the bile of patients with PBC contain IgG and IgA anti-PDC-E2 antibodies. The human bile duct circulatory system is distinct from many species in that IgA is transported to the lumen via a secretory component found only on the bile duct epithelial cells. In contrast, in rodents and many other mammals, IgA is transported to the lumen via hepatocytes (17). Thus, IgA is transported from the apex to the luminal surface of the bile duct epithelial cell. Moreover, in man, IgA is produced locally within the liver and extrahepatic biliary tissues, thus providing local liver immunity (18). Whether during its transport autoreactive IgA is capable of reacting with proteins in the biliary cell cytoplasm is unknown. The PDC-specific IgA may then, during its normal course from the apex to the lumen, recognize the neoantigen or PDC-E2 as it is being produced and/or transported in the cytosol of the biliary duct epithelial cell. Thus, if the IgA anti-PDC-E2 binds to PDC-E2 either before it reaches the mitochondrial receptor or on the mitochondrion itself, it may prevent uptake into the inner membrane. Eventually, the lack of PDC-E2 transported into the mitochondria would cause metabolic dysfunction, finally resulting in cell death.

There are additional organ systems containing ductal epithelial cells in man that belong to the secretory immune system; e.g., the salivary glands, intestinal mucosa, lacrimal, bronchia, and renal tubular epithelium. Recent studies involving patients with PBC and Sjögren's syndrome indicate evidence of focal sialadenitis in 93% of PBC patients examined, although the serum levels of anti-SS-B/La antibodies were only detected in 38% of patients with PBC (19). Thus, there appears to be a high incidence of salivary ductal infiltration and destruction in patients with PBC similar to that found in the liver. In studies by Domar et al. of intestinal alkaline phosphatase isoenzyme levels in patients with cholestatic liver disease, PBC was the only disease with significantly elevated levels of the

intestine-specific alkaline phosphatase (20) thereby indicating possible pathology in the intestine. In addition, Culp et al. report that in one study, 66% of patients with PBC had keratoconjunctivitis sicca (21). When combined, the above data suggests that the ductal pathology noted in the liver of patients with PBC may not be confined exclusively to the bile duct epithelium. It would be of interest to examine other ductal epithelial systems of patients with PBC for PDC-E2 expression.

Acknowledgments

We appreciate the expertise of Sanghoon Cha and the valuable secretarial assistance of Nikki Phipps.

This work was supported by National Institutes of Health (grant DK-39588) and by the Liver Tissue Procurement and Distribution System (grant 1-DK-6-2274).

References

1. Gershwin, M. E., R. L. Coppel, and I. R. Mackay. 1988. Primary biliary cirrhosis and mitochondrial autoantigens. Insights from molecular biology. *Hepatology* 8:147-151.
2. Van de Water, J., C. D. Surh, P. S. C. Leung, S. M. Krams, D. Fregeau, P. Davis, R. Coppel, I. R. Mackay, and M. E. Gershwin. 1989. Molecular definitions, autoepitopes, and enzymatic activities of the mitochondrial autoantigens of primary biliary cirrhosis. *Semin. Liver Dis.* 9:132-137.
3. Van de Water, J., M. E. Gershwin, P. Leung, and R. L. Coppel. 1988. The autoepitope of the 74-kD mitochondrial autoantigen of primary biliary cirrhosis corresponds to the functional site of dehydrolipoamide acetyl transferase. *J. Exp. Med.* 167:1791-1799.
4. Fussey, S. P., J. R. Guest, O. F. James, M. F. Bassendine, and S. J. Yeaman. 1988. Identification and analysis of the major M2 autoantigens in primary biliary cirrhosis. *Proc. Natl. Acad. Sci. USA.* 85:8654-8658.
5. Surh, C. D., T. E. Roche, D. J. Danner, A. Ansari, R. L. Coppel, T. Prindville, E. R. Dickson, and M. E. Gershwin. 1989. Antimitochondrial antibodies in primary biliary cirrhosis recognize cross-reactive epitope(s) on protein X and dihydrolipoamide acetyl transferase of pyruvate dehydrogenase complex. *Hepatology* 10:127-133.
6. Fregeau, D. R., T. E. Roche, P. A. Davis, R. Coppel, and M. E. Gershwin. 1990. Primary biliary cirrhosis: inhibition of pyruvate dehydrogenase complex activity by autoantibodies specific for EN alpha, a non-lipoic acid containing mitochondrial enzyme. *J. Immunol.* 144:1671-1676.
7. Van de Water, J., A. A. Ansari, C. D. Surh, R. Coppel, T. Roche, H. Bonkovsky, M. Kaplan, and M. E. Gershwin. 1991. Evidence for the targeting by 2-oxo-dehydrogenase enzymes in the T cell response or primary biliary cirrhosis. *J. Immunol.* 146:89-94.
8. Joplin, R., J. G. Lindsay, S. G. Hubscher, G. D. Johnson, J. C. Shaw, A. J. Strain, and J. M. Neuberger. 1991. Distribution of dihydrolipoamide acetyltransferase (E2) in the liver and portal lymph nodes of patients with primary biliary cirrhosis: an immunohistochemical study. *Hepatology* 14:442-447.
9. Joplin, R., J. G. Lindsay, G. D. Johnson, A. Strain, and J. Neuberger. 1992. Membrane dihydrolipoamide acetyltransferase (E2) on human biliary epithelial cells in primary biliary cirrhosis. *Lancet.* 339:93-94.
10. Surh, C. D., A. Ahmed-Ansari, and M. E. Gershwin. 1990. Comparative epitope mapping of murine monoclonal and human autoantibodies to human PDC-E2, the major mitochondrial autoantigen of primary biliary cirrhosis. *J. Immunol.* 144:2647-2652.
11. Cha, S., P. S. C. Leung, M. E. Gershwin, M. P. Fletcher, A. A. Ansari, and R. L. Coppel. 1993. Use of a combinatorial immunoglobulin library to generate antibodies to pyruvate dehydrogenase complex-E2 (PDC-E2), the major autoantigen of primary biliary cirrhosis (PBC). *Proc. Natl. Acad. Sci. USA.* 90:2527-2531.
12. Mutimer, D. J., S. P. Fussey, S. J. Yeaman, P. J. Kelly, O. F. James, and M. F. Bassendine. 1989. Frequency of IgG and IgM autoantibodies to four specific M2 mitochondrial autoantigens in primary biliary cirrhosis. *Hepatology* 10:403-407.
13. Van de Water, J., A. Cooper, C. D. Surh, R. Coppel, D. Danner, A. Ansari, R. Dickson, and M. E. Gershwin. 1989. Detection of autoantibodies to the recombinant 74 Kd and 52 Kd mitochondrial autoantigens of primary biliary cirrhosis. *N. Engl. J. Med.* 320:1377-1380.
14. Hershko, A. 1991. The ubiquitin pathway of protein degradation and proteolysis of ubiquitin-protein conjugates. *Biochem. Soc. Trans.* 19:726-729.
15. Pape, G. R., U. Spengler, R. M. Hoffmann, and M.-C. Jung. 1991. Pathogenesis of primary biliary cirrhosis: new aspects of the role of T lymphocytes. In *Autoimmune Liver Diseases*. E. L. Krawitt and R. H. Wiesner, editors. Raven Press, Ltd., New York. pp. 124-130.
16. Lin, R.-H., M. J. Mamula, J. A. Hardin, and C. A. Janeway. 1991. Induction of autoreactive B cells allows priming of autoreactive T cells. *J. Exp. Med.* 173:1433-1439.
17. Brown, W. R., and T. M. Kloppel, T. M. 1989. The role of the liver in translocation of IgA into the gastrointestinal tract. *Immunol. Invest.* 18:269-285.
18. Delacroix, D. L., G. N. Malburny, and J. P. Vaerman. 1985. Hepatobiliary transport of plasma IgA in the mouse: contribution to clearance of intravascular IgA. *Eur. J. Immunol.* 15:893-899.
19. Hansen, B. U., S. Lindgren, S. Eriksson, V. Henricsson, A. Larsson, R. Manthorpe, and G. Warfringe. 1988. Clinical and immunological features of Sjögren's syndrome in patients with primary biliary cirrhosis with emphasis on focal sialadenitis. *Acta Med. Scand.* 224:611-619.
20. Domar, U., A. Danielsson, K. Hirano, and T. Stigbrand. 1988. Alkaline phosphatase isoenzymes in non-malignant intestinal and hepatic diseases. *Scand. J. Gastroenterol.* 23:793-800.
21. Culp, K. S., C. R. Fleming, J. Duffy, W. Baldus, and E. R. Dickson. 1982. Autoimmune associations in primary biliary cirrhosis. *Mayo Clin. Proc.* 57:365-370.