Cryptic Antigenic Determinants on the Extracellular Pyruvate Dehydrogenase Complex/Mimeotope Found in Primary Biliary Cirrhosis

A PROBE BY AFFINITY MASS SPECTROMETRY*

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Affinity mass spectrometry (AMS) was used to evaluate the structural diversity of the E2 component of pyruvate dehydrogenase complex (PDC) in normal and diseased liver cells, including those from patients with the autoimmune disease primary biliary cirrhosis (PBC). Two different antibodies to PDC-E2, the immunodominant mitochondrial autoantigen in patients with PBC, were used. AMS was performed directly on frozen liver sections and purified bile duct epithelial cells. Mass spectrometric signals associated with the molecular recognition of PBC-specific antigenic determinants were enhanced by an in situ enzyme-linked signal amplification process. Samples from patients with PBC gave strong positive signals for the antigen(s) recognized by the monoclonal antibody C355.1. Conversely, tissues from normal and disease controls showed only a minimal signal. AMS was used to identify specific antigenic determinants within the E2 component of PDC for comparison with unknown antigenic determinants observed by affinity capture with C355.1 monoclonal antibody from PBC samples. PDC components bound to C355.1 were mapped and identified by mass before dissociation from the E2 component. A similar approach was used to identify unknown antigenic determinants associated with PBC. We believe AMS may be an important new approach with wide application to the identification of molecules associated with a number of disease states.

A multi-enzyme structure known as the pyruvate dehydrogenase complex (PDC), common to both prokaryotic and eukaryotic cells, has been characterized primarily to understand its essential role in the maintenance of carbohydrate utilization. Three different multimeric enzyme components are assembled, together with regulatory components, to generate the highly regulated function(s) of PDC.

Pyruvate dehydrogenase (E1) is a large (150 kDa) tetrameric protein (ααββ) at the periphery of the assembled PDC (1, 2). E1 is a highly regulated enzyme responsible for the oxidative decarboxylation of pyruvic acid and the reductive acetylation of lipoic acid within the dihydrolipoamide acetyl transferase complex (E2). Phosphorylation of E1 by E1-kinase (bound to E2) and dephosphorylation by E1-phosphatase appears to be important in the regulatory process (3, 4). E2 is a macromolecular assembly of 60 identical (52 kDa) but highly segmented (four “domain”) proteins. A homodimer (51 kDa) known as dihydrolipoamide dehydrogenase (E3) completes the PDC complex (5).

The ability to evaluate more closely the molecular basis of PDC function and its regulation improves continuously as subunit molecular recognition events are clarified and specific binding domains identified. E2 is thought by many to hold the key to understanding the PDC self-assembly process, its final quaternary structure, and its function (6). Several aspects of PDC-E2 structure remain elusive.

Monoclonal antibodies are becoming increasingly important in the process of defining subunit composition, alterations in subunit composition, and conformational epitopes associated with functional capacity (7, 8). Recently, due to a growing awareness of the role of PDC in a number of clinical disorders, there has been a concerted effort to identify and define new antibodies to PDC. It is especially important to identify antibodies capable of inhibiting PDC function and, hopefully, antibodies capable of identifying the dysfunctional PDC assembly. Thus, new approaches to the generation and characterization of useful antibodies have been explored. Most recently, these included the use of surface plasmon resonance to conduct competitive (i.e. indirect) epitope mapping studies with libraries of monoclonal antibodies (7, 8).

Structural information is needed beyond general knowledge of operational molecular specificity. In the case of complex multimeric protein antigens, it has not always been possible, even with monoclonal antibodies, to verify by indirect means the identity of individual target antigens or specific epitopes. This is particularly true in situations where antibodies must be used to investigate antigenic determinants that are limited in quantity. This issue is exacerbated in cases where the antigenicity of target proteins must be evaluated in situ to determine possible contributions of local environment on conformational...
epitopes and the differential presentation of otherwise occluded (i.e. cryptic) epitopes.

In this study, we have addressed an opportunity to compare more closely the biochemistry and structural biology of normal PDC with that of an abnormal, extracellular form of PDC (or molecular mimic). Specifically, the structural diversity of PDC-E2 was investigated in human liver collected from normal individuals and individuals with diseased livers, including those with an autoimmune disease known as primary biliary cirrhosis (PBC). PBC results from autoimmunemediated destruction of intrahepatic bile ducts with progressive inflammatory scarring (9). The association of PBC with high titer autoantibodies to mitochondrial antigens (AMA) has long been recognized and the possible significance discussed (9–11). It is of considerable interest that the major AMA activity is directed against PDC-E2.

We have previously reported the development and characterization of several different murine monoclonal antibodies specific for PDC-E2 (10). These antibodies were found to recognize four different regions of PDC-E2 when evaluated by enzyme-linked immunosorbent assay with overlapping recombinant fragments (10). Each of the monoclonal antibodies showed typical mitochondrial immunofluorescence on biliary epithelium and on hepatocytes from patients with PBC, primary sclerosing cholangitis, or hepatocarcinoma (12). However, one of the eight antibodies (C355.1) showed greater intensity and specificity for the apical region of biliary epithelial cells only from patients with PBC. Similar results have also been obtained with affinity-purified anti-PDC-E2 and combinatorial (13–15). Attempts to characterize this antigen have failed for a variety of reasons, including the small number and low viability of biliary epithelial cells in diseased livers. There is a clear need for both enabling new technologies and the implementation of alternative strategies to identify structural variations in PDC-E2.

We have previously outlined new strategies for the enhanced detection and structural characterization of biopolymers by laser desorption/ionization time-of-flight mass spectrometry (e.g. Ref. 16). We demonstrated that laser desorption probe elements and the necessary energy absorbing molecules can be much more defined and independent of the empirical process imposed on them previously. Indeed, inert probe element “platforms” have been chemically and physically transformed (i.e. redesigned) at the molecular level to become active participants in both the process of analyte docking and the subsequent laser-induced desorption/ionization process (16). Because of the unique involvement of the probe element surfaces in both the analyte adsorption and desorption process, surface-enhanced laser desorption/ionization (SELDI) has been referred to simply as affinity mass spectrometry (AMS). Affinity mass spectrometry may be considered simply a solid phase mass spectrometric approach to molecular detection, the evaluation of molecular recognition, and a wide variety of in situ chemical and enzymatic reactions necessary for structural analyses.

We present here our use of SELDI in the AMS mode to detect and help identify epitopes that distinguish normal PDC-E2 from what is apparently an abnormal form of PDC-E2 or a mimicope (17). We demonstrate the ability to detect two important epitopes with very low levels of purified PDC-E2. One of these epitopes, or a highly reactive mimicope, has also been detected at very low levels directly within target tissues and cells. The apparent structural diversity of “PDC-E2” observed in livers from patients with PBC is discussed in the context of three competing hypotheses, including the existence of conformation-dependent PDC-E2 epitopes, altered presentation (i.e. cryptic) of PDC-E2 epitopes after translocation to an extracellular location, and molecular mimicry. The SELDI technology provides a very high level of sensitivity and an opportunity to directly evaluate tissue sections, cells, or crude cell lysates. We believe the strategy and capabilities demonstrated here will find wide application in the study of other biomolecules present in trace quantities, especially those associated with disease states.

MATERIALS AND METHODS

Clinical Specimens—Liver specimens were obtained from 9 patients with PBC, 3 patients with primary sclerosing cholangitis, 1 patient with α-trypsin deficiency, and 2 patients with alcoholic cirrhosis. The diagnosis of all patients was based on established disease criteria and confirmed by histologic review by an independent observer (18). The specimens were divided into two sets. The first gel was prepared as described (19). We used the region of liver most likely to contain small intrahepatic ducts and ductules, which are the primary target of disease, rather than the larger interhepatic ducts. Approximately 30 g of liver was minced and incubated with 1 mg/ml type 1A collagenase with agitation for 4 h at 37 °C. The resulting mixture was washed five times with PBS before the cells were suspended in 30 ml of PBS, layered onto 15 ml of Lymphoprep for density gradient separation, and centrifuged at 2400 rpm for 25 min. The cells at the differential interphase were harvested and washed three times with PBS. The washed cells were then incubated with 5 mg/ml HEA123 (epithelial cell-specific monoclonal antibody) for 30 min at 37 °C. After washing three times with PBS, 1 ml of 1% biotinylated streptavidin (Dynal) in 7 ml of PBS was added to the cells and incubated at 37 °C for 30 min, followed by magnetic separation (20).

Monoclonal Antibodies—We utilized a mouse monoclonal antibody, termed C355.1, that is specific to PDC-E2 but produces a distinctive staining pattern of biliary epithelial cells of patients with PBC as well as a non-disease-specific PDC-E2 mAb, C150, as a control. As an additional control, we used an irrelevant mAb (SAG-1) throughout (10, 12). The characteristics of these mAbs have been described in detail previously (10).

Surface-enhanced Laser Desorption/Ionization (SELDI) Time-of-flight (TOF) Mass Spectrometry—The generic use of this methodology has been previously published in detail (16, 21–23). Energy absorbing molecules such as sinapinic acid or c-syanic-4-OH-cinnamic acid were prepared in 60% acetonitrile/0.5% trifluoroacetic acid. An aliquot of 1–2 μl was deposited on the sample affinity captured on the probe (SEAC) device and allowed to air dry. The resulting sample preparations were analyzed with either a modified Hewlett Packard 1700XP laser desorption/ionization time-of-flight or a Molecular Analytical System SELDI mass spectrometer. The instruments were supplied with three-stage ion optic assembly with a variable high voltage source (up to 30 kV potential) and a 1.5–2.0-m flight path length. Laser pulses were generated from either a nitrogen laser or a neodymium:yttrium-aluminum-garnet (Nd:YAG) laser. The nitrogen laser (model VSL 377 ND from Laser Science, Inc., Boston, MA) generates up to 300 mJ/pulse maximum output with a 3-ns pulse width at a variable repetition rate of 0.2–5 Hz. The spot size is an ellipse with a long axis width of 180 mm and a short axis width of 80 mm. The Nd:YAG laser (Lumonics, Livonia, MI or Continuum, Santa Clara, CA) generates several (up to 90) mJ/pulse with an output as a 3-ns pulse (at a 0.03–3-mm2 spot size) width at a repetition rate of 10–20 Hz. The spot size is an ellipse with a long axis width of about 100 mm and a short axis width of about 50 mm. The laser irradiance was adjusted real-time, while monitoring the process on an oscilloscope or computer, in order to achieve optimum ion signal (significant signal versus maximum resolution). Ion signals were detected on the Hewlett Packard 1700XP using a secondary ion generator with an array of microchannel plates; one N-type microchannel plate followed by a high output technology (HOT) microchannel plate manufactured by Galileo Electro-Optics Corp. (Sturbridge, MA). The microchannel plates are held at a constant ~1000 V and the secondary ion generator was set for post acceleration with a potential of ~5 kV. The SELDI instrument from MAS uses a discrete dynode electron multiplier (Hamamatsu Corp, Bridgewater, NJ) with microchannel plates, and the signals were amplified using a fast pre-amplifier prior to being recorded by a transient recorder capable of fast signal averaging. The data was captured with a 400–500 megasample/s digital oscilloscope with a 350 MHz bandwidth and analyzed on PC-based software. Spectra presented typically represent the average of 50–250 shots unless otherwise stated.

Affinity Mass Spectrometry to Demonstrate Specificity of Monoclonal Antibodies—The antigen specificity of the monoclonal antibodies C355.1 and C150 and the sensitivity of the AMS technique with PDC
was performed as follows. C150 or C355.1 monoclonal antibodies were immobilized on Protein G-sepharose beads according to the manufacturer's instructions (Pharmacia Biotech Inc.). The pure PDC antigen was prepared as described previously (18). An aliquot of PDC was incubated with a slurry of beads bound to either C355.1 or C150 at 4°C for 30–60 min. The beads were washed with 0.1% Tween 20 in phosphate-buffered saline, pH 7, then with 50 mM ammonium citrate, and, finally, with water. An aliquot of 0.5 μl of the beads was added to the mass spectrometer probe element, an aliquot of 1 μl of sinapinic acid was added, and the mixture was allowed to air-dry. The samples were analyzed with a mass spectrometer, and averages from 250 laser shots were obtained from multiple spots on each sample. The sample of PDC affinity captured on monocolonal antibodies immobilized on Protein G-sepharose beads were further washed with 3x urea in phosphate-buffered saline and 50 mM ammonium citrate, and, finally, with water. An aliquot of 0.5 μl of the beads was added to the mass spectrometer probe element, an aliquot of 1 μl of sinapinic acid was added, and the mixture was allowed to air-dry. The samples were analyzed with a mass spectrometer, and averages from 250 laser shots were obtained from multiple spots on each sample.

Detection of PDC-E2 by Affinity Mass Spectrometry with Enzyme-linked Signal Amplification—Various amounts (from 0.5–50 fmol) of PDC-E2, prepared as described (24, 25), were immobilized on a 96-well plate by incubating with shaking overnight at 37°C in the presence of phosphate-buffered saline, pH 7. Each well was probed with C150 or C355.1 monoclonal antibodies diluted in phosphate-buffered saline, pH 7, by incubating with shaking at 25°C for 1–2 h. In another series of wells, 10 fmol of PDC-E2 was immobilized and probed with serial dilutions (100–18,000-fold) of C150 or C355.1 mAbs under the same conditions. The unbound antibodies were washed away with 0.1% Tween 20 in phosphate-buffered saline, pH 7. Each well was then incubated with goat anti-mouse IgG antibody conjugated to bovine intestinal alkaline phosphatase (Calbiochem, 1 mg/ml, diluted 5000-fold in phosphate-buffered saline, pH 7). The wells were washed with 0.1% Tween 20 in phosphate-buffered saline, pH 7, then with 50 mM ammonium citrate, and finally with water. A synthetic phosphopeptide, Gly-Leu-phosphoSer-Pro-Ala-Arg (680 Da, prepared in TWH laboratory), in 50 mM ammonium carbonate, 0.05 mM MgCl2, pH 9.5, was added to monitor the activity of bound alkaline phosphatase. The amount of dephosphorylated peptide (600 Da) produced indicates the amount of antigen-specific monoclonal antibodies bound to the immobilized enzyme sample. Nitrogen laser desorption/ionization time-of-flight mass spectrometry was used to detect both the substrate and product peptide with α-cyano-4-OH-cinnamic acid as the energy absorbing molecule.

Detection of Marker Protein(s) by Affinity Mass Spectrometry with Enzyme-linked Signal Amplification—This was performed on both frozen liver sections and isolated bile duct epithelial cells. Frozen liver sections (8 μm thick) on glass coverslips were cut up into approximately four 4-mm pieces. They were used either directly without treatment or fixed with 95% methanol, 5% acetic acid to facilitate handling. The purified bile duct epithelial (BDE) cells and the liver tissue were washed with phosphate-buffered saline, pH 7. An aliquot of 5 μl of C150 or C355.1 monoclonal antibodies was added and allowed to incubate at 25°C in a moist chamber for 30–60 min. For the controls, the cells or tissue were incubated with phosphate-buffered saline only. The cells or tissue were washed with 3x urea in phosphate-buffered saline, pH 7, then with 0.1% Tween 20 in phosphate-buffered saline, pH 7. After washing with phosphate-buffered saline, an aliquot of 5 μl of goat anti-mouse IgG antibodies conjugated to bovine intestinal alkaline phosphatase (Calbiochem, 1 mg/ml, diluted 5000-fold in phosphate-buffered saline, pH 7) was added and allowed to incubate at 25°C in a moist chamber for 30–60 min. The cells or tissue were washed with 3x urea in phosphate-buffered saline, then with 0.1% Tween 20 in phosphate-buffered saline, with 50 mM ammonium citrate, and finally with 50 mM ammonium bicarbonate, pH 7.8. An aliquot of 5 μl of synthetic phosphopeptide (Gly-Leu-phosphoSer-Pro-Ala-Arg) in 50 mM ammonium carbonate, 0.05 mM MgCl2, pH 9.5, was added and allowed to incubate at 25°C in a moist chamber for various periods of time. For the liver sections on glass coverslips, an aliquot of 2 μl of α-cyano-4-OH-cinnamic acid was added and allowed to air-dry. For the BDE cells, after brief centrifugation to remove the cells, an aliquot of 0.5 μl of the supernatant was mixed with an aliquot of 1 μl of α-cyano-4-OH-cinnamic acid on the mass spectrometer probe and allowed to air-dry. The samples were analyzed with a mass spectrometer, and averages from 250 laser shots were obtained from multiple spots on each sample.

Affinity Mass Spectrometry to Demonstrate the Detection and Identification of Previously Unknown Molecular Determinants of PBC—C150 and C355.1 mAbs were immobilized separately on Affi-Prep 10 polymer beads according to the manufacturer's instructions (Bio-Rad). The PBC liver homogenate was prepared by homogenization in PBS containing 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, pH 7.4. An aliquot of PBC liver homogenate was incubated with a slurry of either C355.1- or C150-bound beads at 4°C for 18 h. The beads were washed with 0.1% Tween 20 in PBS, pH 7, then with 50 mM ammonium citrate, and, finally, with water. An aliquot of 0.5 μl of the beads was added to the mass spectrometer probe element, followed by an aliquot of 1 μl of sinapinic acid, and the mixture was allowed to air-dry. The samples were analyzed with a mass spectrometer, and averages from 250 laser shots were obtained from multiple spots on each sample.

RESULTS

Strategy for Detection of PBC Antigenic Determinants by Affinity Mass Spectrometry with Enzyme-linked Signal Amplification—Fig. 1 shows the design and architecture of the laser desorption SEAC probe of the biomolecular reagents necessary for the detection of PBC antigenic determinants. The presence of specific marker analyte, in this case either PDC-E2 or the PDC-E2-like PBC antigen with specific monoclonal antibodies. The primary signal is amplified by interaction of the secondary antibody with a coupled enzyme (alkaline phosphatase). The enzyme converts added substrate (phosphopeptide, 680 Da) to product (dephosphorylated peptide, 600 Da) directly in situ (i.e. on the probe surface). The mass spectrum (inset) shows the detection of both the substrate and product, which are separated by 80 Da (one phosphate group). The ratio of product to substrate gives an indication of the amount of specific marker analyte being detected.

Biomolecule Detection and Quantitation in SELDI TOF Mass Spectrometry—The mass spectra were analyzed by IBM-compatible computers using the softwares HP MALDITOF (Hewlett Packard) and GRAMS/386 (Galactic Industries Corp.). The software evaluates the noise of the base line relative to the peaks and automatically set peak detection factors such as data smoothing (noise filter or data banching), minimum area and height reject thresholds, minimum detectable slope, fused/shoulder peaks detection, and grouped peaks detection. Peak area was estimated by Gaussian fit. Normal statistical protocols such as standard deviation and linear regression were used to analyze the data.
use of these same SEAC probes to compare the recognition affinity and specificity of C355.1 mAbs (PBC-specific) and C150 mAbs (control) for the surface-immobilized PDC-E2. The utility of AMS for quantitation of surface-immobilized PDC-E2 with C355.1 and C150 mAbs was determined to be significant over the background signal at 0.5 fmol of surface-immobilized antigen. The product peak increased in intensity, with a corresponding decrease of substrate peak intensity when increasing amounts of surface-immobilized antigens were probed with the mAbs. Since the efficiency of immobilization of femtomole quantities of PDC-E2 on a 96-well plate is difficult to estimate, this represents only a semiquantitative estimation of the detection sensitivity and response. Serial dilutions of C150 or C355.1 monoclonal antibodies were also used to detect 10 fmol of immobilized PDC-E2. A significant difference in efficacy was detected for the two mAbs. An 18,000-fold dilution of C150 could still generate a strong product peptide signal, whereas a 600-fold dilution of C355.1 reduced the signal to just over background (Fig. 2B).

Detection of PDC-E2-like Antigens Directly within Frozen Liver Sections by AMS with Enzyme-linked Signal Amplification—To determine whether a macromolecule specific to PBC could be detected in frozen liver sections using C355.1 and the control mAb C150, specimens from 9 patients with PBC and 6 control livers (1 normal and 5 disease controls) were analyzed by SELDI in the AMS mode with SEAC probes using the enzyme-linked amplification system. Our results indicate that an intense C355.1 specific product signal is present at 600 Da (mass/charge) in 9/9 of the PBC samples, but was found in much lower intensities in the 5 disease controls or the normal control liver (Fig. 3A). There were variations in avidity among the PBC patients as expected. However, the specificity and intensity of the signal is consistent with immunohistochemical data obtained using confocal microscopy (12). In contrast, the control mAb C150 produced a strong product peak in the normal control specimen, moderate peaks in the disease controls, and very little signal for the PBC sections (Fig. 3B).

Direct Detection of PDC-E2-like Antigens in Isolated BDE Cells by AMS with Enzyme-linked Signal Amplification—The detection of PDC-E2 or PDC-E2-like molecular determinants associated with PBC was also performed using purified BDE cells isolated from either PBC livers or normal (non-PBC livers). Purified BDE cells were analyzed with both the PBC-specific mAb C355.1 and the control mAb C150. A distinct difference in product signal generation was revealed in the PBC epithelial cells when probed with the C355.1 mAb (PBC-positive) versus the C150 mAb (negative control) (Fig. 4A). The solid line profiles illustrate the positive enzyme activity (strong product peaks) detected in the PBC BDE cells when probed with C355.1. The dashed line profiles represent the reduced enzyme activity detected in PBC BDE cells when probed with C355.1. Similarly, a distinct difference in product signal generation was revealed in the non-PBC epithelial cells when probed with the C355.1 mAb (little or no signal)

**Fig. 2.** Detection sensitivity of surface immobilized antigen by AMS with enzyme-linked signal amplification. A, 0.5–50 fmol of PDC-E2 were immobilized on a 96-well plate. Each well was probed with C150 or C355.1 monoclonal antibodies. The amount of dephosphorylated peptide (600 Da, Product) produced indicates the amount of marker-specific monoclonal antibodies bound to the immobilized PDC-E2 sample. The x-axis delineates the peptide mass/charge values, and the y-axis represents the intensity of the peptide ion signal detected. Blank wells as well as an irrelevant monoclonal antibody (SAG-1) were used as controls and showed a small amount of background product peak formation. A significant increment over the background signal was detected for 0.5 fmol of surface immobilized antigen when probed with C355.1 mAb. This product peak increased in intensity with a corresponding decrease of substrate peak intensity when increasing amounts of surface immobilized PDC-E2 were probed with the mAbs. B, serial dilutions of the mAbs C150 or C355.1 were used to detect 10 fmol of PDC-E2 immobilized on a 96-well plate.
versus the stronger signal detected with C150 mAb (positive control) (Fig. 4B).

Evaluation of PDC and the PDC-E2 Component by AMS—AMS was used to characterize and distinguish the PDC-E2 antigenic determinants recognized by the mAbs C355.1 and C150 within PDC. Isolated PDC was bound to C150 or C355.1 mAbs that were tethered to the SEAC probe surface through immobilized Protein G. The PDC components bound to the immobilized mAb were mapped and identified by mass before their dissociation from the known antigenic determinant, the E2 component. The specificity of the C355.1 and C150 mAbs for PDC is illustrated in Fig. 5. When the PDC was captured on either of the two different monoclonal antibodies, C355.1 (Fig. 5A) or C150 (Fig. 5B), after exposure to 3 M urea in phosphate-buffered saline, only the E2 and X components of the original PDC remain tightly bound to the antibodies. These results further confirm the E2 component of PDC as the antigen specifically recognized by both C150 and C355.1 monoclonal antibodies. When the PDC was captured on the C150 or C355.1 SEAC probe surfaces, after exposure to 3 M urea to remove all but the bound E2 component, a trypsin digest of the C150- or C355.1-bound PDC-E2 component revealed C355.1 epitope containing fragments of 4.7, 5.4, 7.5, and 10 kDa (Fig. 6). When the SEAC probe was constructed with C150 and the trypsin digest performed, the bound fragments were identified as 1.6, 4.7, and 5.4 kDa.

SEAC Probes with Immobilized C355.1 for the Detection and Identification of Previously Unknown Molecular Determinants of PBC—Because the identity of the molecule recognized by C355.1 in livers from PBC patients is unknown, SEAC probe surfaces, with the C355.1 mAb as the tethered affinity capture device, were used in an attempt to detect and identify previously unknown molecular determinants of PBC. Several separate proteins or protein fragments (8.7, 13, 21, and 24 kDa) were identified in liver homogenates from PBC patients that were not recognized by the C150 mAb (Fig. 7). Additionally, when the homogenate was preadsorbed with the immobilized C150 beads prior to incubation with the C355.1 SEAC probe, the 8.7- and 13-kDa peaks became even more evident (data not shown).
DISCUSSION

PBC is an autoimmune cholangitis characterized by the destruction of intra-hepatic bile ducts/bile duct epithelial cells and the presence of AMA. Although AMA were first identified nearly 30 years ago, it was not until the cloning of the mitochondrial autoantigens that the targets were identified as components of the 2-oxodehydrogenase pathway with the E2 component of the pyruvate dehydrogenase complex (PDC-E2) as the immunodominant autoantigen. We have previously demonstrated that patients with PBC, but not controls, have either PDC-E2 or a cross-reactive molecule at the apical surface of biliary epithelium but the identity of this important molecular “marker” remains unclear. This molecule is detected in Stage I and II PBC, before the appearance of BB1/B7 or major histocompatibility class II, and therefore appears to be the earliest known marker of PBC (12, 26). This previous work has provided us with a monoclonal antibody that is highly specific for the bile duct epithelial cells of patients with PBC, yet the exact nature of this molecule and its epitope is still unknown.

A variety of methods have been used to study monoclonal antibody-antigen interactions and the characterization of epitopes. Two of the most common approaches are competitive binding analysis using synthetic peptides and fine specificity studies with panels of evolutionary variant or recombinant proteins (27). Although these methods are well established, they do have significant limitations. Among these are the fact that discontinuous or conformationally defined epitopes may not be detectable using peptide probes (28). Therefore, a direct approach for epitope mapping has been more recently introduced based on several factors. Among these are the following: 1) mAbs exhibit resistance toward proteolytic enzymes; 2) in immune complexes, antigenic determinants can be protected from proteolytic degradation; and 3) proteolysis does not lead to dissociation of immune complexes (29–31). Previously, limited proteolytic cleavage of immune complexes has been used for epitope characterization by means of polyacrylamide gel electrophoresis (32) and high performance liquid chromatography (33). However, these methods may not enable unambiguous epitope identification due to unresolved peptides.

The high molecular specificity provided by mass spectrometric peptide mapping has been used successfully in various protein structural studies, such as the characterization of cDNA-derived sequences, identification of posttranslational modifications, and the differentiation of isoenzyme structures (34–36). In addition, this methodology has recently been used with great success in the analysis of antigen/antibody complexes such as the characterization of the epitope recognized by secondary antibody coupled to alkaline phosphatase. The amount of dephosphorylated peptide (600 Da, Product) produced indicates the amount of marker-specific monoclonal antibodies bound to the BDE cells. The x-axis delineates the protein mass/charge values, and the y-axis represents the intensity of the protein ion signal detected. The +1Na peak represents the peptide ion with a sodium ion adduct (+23 Da); the +2Na peak represents the peptide ion with two sodium ion adducts (+46 Da). In A, the upper profile (solid line) illustrates the positive enzyme activity detected in three PBC BDE cell specimens when probed with C355.1, where the dashed line profiles represent the reduced enzyme activity detected in PBC BDE cells when probed with the control mAb, C150. Note the strong product peak seen in the PBC BDE cell specimens when probed with C355.1 compared to C150. The enzyme activity is so strong that almost all the substrate was used up in the C355.1 probed samples and only the sodium adducts, peak +1Na and peak +2Na, remained. In B, the solid line profile illustrates the absence of enzyme activity detected in two non-PBC liver cells when probed with the mAb C150. The amount of product generated in the non-PBC liver cells when probed with C150 was significantly higher than that detected for the non-PBC liver cells probed with C355.
a mAb to the complement component C3a and mapping of the gastrin-releasing peptide/anti-bombesin monoclonal antibody complex (34, 37). Epitope extraction and direct identification of a single immune complex by mass spectrometry has been shown to be a sensitive and rapid method of high molecular specificity in the analysis of protein antigens.

In this study, we demonstrated the sensitivity and specificity of AMS in conjunction with highly specific mAb reagents using enzyme-linked signal amplification. Previous studies using these mAbs and confocal microscopy have shown that an increase in apical staining of the bile duct epithelial cells is seen only with C355.1 in patients with PBC (12). These observations are further substantiated by the current study. When frozen liver sections from patients with PBC and controls were probed with C355.1 and examined by AMS, strong enzyme activity was only found in the PBC specimens (9/9). In contrast, when the control mAb (C150) was used to analyze the same samples, there was little if any product peak in the PBC samples and reduced product in the disease controls compared to the normal, healthy liver. A similar analysis of purified BDE cells was performed to focus on the disease target tissue and to compare these results with the intact liver tissue sections. When pure BDE cells from patients with PBC and controls were analyzed by AMS with enzyme-linked signal amplification using C355.1 and C150, a strong C355.1 product peak was seen with PBC but not control BDE. These findings were almost identical to the crude frozen liver sections discussed above and indicate that the macromolecule recognized by C355.1 is also specific to the BDE cells of patients but not controls. Moreover, similar to the liver sections, when the PBC and disease-control BDE were tested with C150, the control liver BDE had a significantly higher product peak than the PBC BDE cells.

The epitope mapping studies performed herein suggest that while both mAbs indeed recognize PDC-E2, there is a distinct difference in their antigenic determinants. This further substantiates earlier immunohistochemistry studies with C355.1 and C150 (10). Moreover, the moderate reduction of C150-probed elements in the disease controls and the dramatic reduction in the PBC liver suggest that normal mitochondrial production of PDC-E2 in liver is compromised by disease. These observations have been further substantiated by in situ hybridization studies where little signal for PDC-E2 is seen in the bile duct of liver from patients with PBC. The strong product peak in both the crude frozen liver sections and the BDE cells from

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Fig. 5. Demonstration of PDC-E2 specificity of mAbs C355.1 and C150. The mAbs C150 or C355.1 were immobilized on Protein G-agarose beads and incubated with PDC. The complexes were analyzed with a mass spectrometer. A, the composite mass spectrum of PDC proteins specifically captured by C355.1 mAb. B, the composite mass spectrum of PDC proteins specifically captured by C150 mAb. In both A and B, the top profile is a control mass spectrum of the monoclonal antibodies on Protein G-agarose beads alone. The major peak (48,670 Da) represents the triply charged ions of the antibodies ([IgG+3H]3+), which were desorbed by the laser from the Protein G-agarose beads. The middle profile shows the mass spectrum of proteins in the PDC captured by the monoclonal antibodies. The major peaks are the E2 (59,970 Da), E1α (39,970 Da), E1β (35,930 Da), and X (48,200 Da). Thus the whole PDC was captured by the mAb-SEAC probes as a single unit even after detergent and water washes. The individual components of the complex were revealed by the disruption with laser during the mass spectrometric analyses. When the samples of PDC affinity captured on mAb-SEAC probes were further washed with 3 M urea in phosphate-buffered saline, the lower profile shows that only E2 and X components remained tightly bound to both antibodies, confirming that the E2 component is the antigen specifically recognized by both C150 and C355.1 monoclonal antibodies.

K. Harada, J. Van de Water, P. S. C. Leung, R. L. Coppel, Y. Nakanuma, and M. E. Gershwin, submitted for publication.
patients with PBC using C355.1, as well as its absence in controls, suggests that C355.1 recognizes a molecule in the liver of these patients other than PDC-E2 or PDC-E2 in its native/immunodominant form.

When the liver homogenates from patients with PBC were analyzed with probe-bound C355.1 as an affinity capture device, several prominent protein fragments were observed (8.7, 13, 21, and 24 kDa) that were not recognized by C150. Moreover, when the homogenate were preincubated with immobilized C150 prior to probing with C355.1, the peaks at 8.7 and 13 kDa became more pronounced. Preliminary data obtained with BDE cells isolated from a patient with PBC showed similar results. The limited digestion of liver homogenate with collagenase during the preparation of BDE cells generated some protein fragments specifically captured by the immobilized antibodies that were substantially different and bigger in size than those generated from exhaustive digestion of PDC E2 with trypsin. However, some peptides in the PBC liver digest, particularly the 1670- and 4853-Da ones, did correspond in mass to some of the major PDC-E2 digest fragments captured by C150.

Thus, it appears that there are unique antigens recognized by C355.1 in the liver homogenate of patients with PBC. This could also be the result of different epitopes generated from the breakdown of PDC-E2. However, whatever the origin of these unique fragments, their identity would be instrumental in the determination of the differences between PBC and other liver disease bile ducts.

The eventual sequence identification could lead to more information regarding the possible role of these macromolecules in molecular mimicry or as cryptic determinants. Cryptic peptides of autoantigens may become expressed at sites of inflammation (38). This can lead to the engagement of T cells potentially directed against cryptic self-determinants that escaped tolerance induction in the thymus leading to induction and/or perpetuation of autoimmune reactivity. This self-directed T cell repertoire can also be activated silently to induce memory and participate unexpectedly in responses to foreign antigens and may be responsible for molecular mimicry (39). It is possible that the determinants recognized by C355.1 are in fact cryptic determinants of PDC-E2 that are also recognized by patients with PBC. In fact, immunohistochemical staining of PBC liver with human combinatorial antibodies generated from the lymph node of a patient with PBC has led to the discovery of one antibody, SP4, that stains in a PBC-specific apical pattern similar to C355.1. It has been suggested by studies using CD4+ T cell responses to two different strains of mycobacteria that T cell cross-reactive epitopes can exist in proteins with apparently not more than random levels of sequence homology (40). Thus, the potential of cross-reactive epitopes for unsuspected cross-sensitization may play a role in the maintenance of T cell memory, in the pathogenesis of autoimmune disease, and possibly in a wider range of host immune responses to infectious pathogens (40).

Also of much interest are the considerable advantages of this
technology. First, the use of AMS in the SELDI mode allows a level of sensitivity in the femtomole range. Second, without the need to isolate large numbers of cells, such analysis can even be performed on crude tissue sections. Third, using probes docked on crude tissues. Fourth, using probes docked in situ allows autoimmunity to be addressed and macromolecules identified using existing tools with extraordinary and versatile applications.

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