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Immunology

Combinatorial autoantibodies to dihydrolipoamide acetyltransferase, the major autoantigen of primary biliary cirrhosis

(combinatorial immunoglobulin antibodies/primary biliary cirrhosis/pyruvate dehydrogenase complex-E2/autoimmunity)

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ABSTRACT **mRNA from a regional lymph node of a patient with primary biliary cirrhosis (PBC) was used to construct a combinatorial immunoglobulin library in the λ phage vector system. Six human monoclonal IgG Fab clones (LC1–LC6) specific for the major autoantigen of PBC—dihydrolipoamide acetyltransferase, the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2)—were isolated, appearing at a frequency of 0.01% in the combinatorial immunoglobulin library. These Fab clones recognize human PDC-E2 with high affinity (K_a = 10^{-7}–10^{-9} M^{-1}). Using both immunoblotting and ELISA, LC1–LC6 showed little cross-reactivity to any of the other autoantigens commonly recognized by PBC sera or to other antigens commonly recognized by PBC sera or to other antigens such as histone, calf thymus DNA, and bovine serum albumin. The Fab monoclonal antibodies show a typical anti-mitochondrial staining pattern in HEp-2 cells but react strongly with the luminal aspect of biliary epithelial cells of patients with PBC. Our results demonstrate that a recombinant combinatorial immunoglobulin library can be used to isolate high-affinity Fabs against a specific autoantigen. Such reagents will facilitate the analysis of immunoglobulin gene structure, idiotype, and antigen–antibody interactions in autoimmune disease.**

The generation of human monoclonal antibodies is of great potential importance in the study, diagnosis, and therapeutic treatment of immunological diseases (1). Conventional methodologies of production of human monoclonal antibodies, such as hybridoma fusion technique and Epstein–Barr virus transformation of human B lymphocytes, are unreliable and often produce unstable cell lines (2–4). One alternative, the combination of rodent variable (V) regions and human constant regions to produce chimeric antibodies (5, 6), has had some success, although foreign framework regions may induce autoantibodies (7).

Recently, murine and human monoclonal antibodies have been successfully generated by PCR and repertoire cloning into phage vectors of various types (8–19). Recombinant antibodies to antigens such as keyhole limpet hemocyanin coupled to *p*-nitrophenyl phosphonamidate antigen 1 (8), the hemagglutinin of influenza virus (9), tetanus toxoid (10, 11, 17), 2-phenyloxazol-5-one (12–14), turkey egg white lysozyme (14), gp120 of human immunodeficiency virus (18), and hepatitis B core antigen (19) have been obtained by screening combinatorial immunoglobulin libraries. These antibodies were directed against exogenous antigens and it was not known whether such an approach would be useful for isolating anti-self specificities.

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We attempted to isolate such autoimmune recombinant Fabs, using a library derived from lymph node mRNA of a patient with primary biliary cirrhosis (PBC). PBC is a chronic autoimmune disease of the liver of unknown etiology and is characterized by the presence of high-titer anti-mitochondrial antibodies, inflammation of the septal and interlobular bile ducts, followed by necrosis, and ultimately cirrhosis (20, 21). Within the past few years, the mitochondrial autoantigens have been identified as components of the 2-oxo acid dehydrogenase enzymes including the E2 subunits of the pyruvate dehydrogenase complex (PDC), the branched-chain oxo acid dehydrogenase complex (BCKD), the 2-oxo acid glutarate dehydrogenase complex, and other protein components of PDC—protein X and PDC-E1α and -β subunits (22–33). Murine monoclonal antibodies to the mitochondrial autoantigen PDC-E2 have been isolated. Although these monoclonal antibodies were able to inhibit PDC-E2 enzymatic activity, they recognized a different region of the target autoantigen than did patient sera (34), suggesting that monoclonal antibodies of rodent origin differ in fine specificity from human autoantibodies.

In this study, we report the isolation and characterization of human combinatorial immunoglobulins specific to a human autoantigen, in this case dihydrolipoamide acetyltransferase, the E2 subunit of pyruvate dehydrogenase complex (PDC-E2). These monoclonal Fab clones share striking similarities with autoantibodies found in the sera of patients with PBC.

MATERIALS AND METHODS

RNA Isolation. A regional lymph node from a female patient with PBC was obtained at liver transplantation and frozen in liquid nitrogen. Total RNA was extracted as described (35) and stored at −70°C until used.

Construction of the Combinatorial Immunoglobulin Library. The library was constructed using the ImmunoZAP cloning kit (Stratagene) according to the manufacturer's instructions (10). Briefly, cDNA was synthesized from the PBC lymph node RNA with oligo(dT) priming. The γ (Fd part), κ, and λ immunoglobulin genes were amplified by PCR with a human immunoglobulin primer kit (Stratagene catalogue no. 2001). After restriction digestion and purification, heavy- and light- (H and L) chain DNA fragments were ligated into modified Lambda ZAP II vectors (ImmunoZAP H and ImmunoZAP L, respectively) and packaged *in vitro* (Gigapack II Gold). Each library was amplified and phage

Abbreviations: PBC, primary biliary cirrhosis; PDC-E2, E2 subunit of the pyruvate dehydrogenase complex; V, variable; H and L, heavy and light; BCKD, branched-chain oxo acid dehydrogenase complex; HRP, horseradish peroxidase.

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DNA was prepared as described (36). For construction of the combinatorial library, the H-chain library λ phage DNA yielded the left arm of the final construct, and the L-chain library λ phage DNA yielded the right arm. Both fragments were digested with *Eco*RI separately, ligated together, packaged, and plated.

Screening of Libraries for Positive Clones. Plaque-lift assays were performed to check for expression of V_H and V_L fragments from each H- and L-chain library. Briefly, *Escherichia coli* (XL-1 Blue) was infected with recombinant phages and plated. Nitrocellulose filters soaked in 10 mM isopropyl β-D-thiogalactopyranoside were overlaid onto the agar plates and incubated at room temperature overnight to induce expression of antibodies (8). For identification of V_H fragment expression, the filters were incubated with rabbit anti-decapeptide antibody (TagTeam kit, Stratagene), which binds to a decapeptide tag expressed in V_H fragments. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody was used as a secondary antibody. The expression of V_L fragments was performed similarly except that a mixture of HRP-conjugated goat anti-human κ and λ antibodies was used. The screening for PDC-E2-specific Fabs was performed similarly except that the filters were incubated with 1 μM recombinant fusion protein consisting of the inner lipoyl domain of human PDC-E2 (residues 91–227) fused to glutathione *S*-transferase (37, 38) at 4°C overnight and probed for 2 h at room temperature with a 1:500 dilution of rabbit anti-glutathione *S*-transferase antibody and then with HRP-conjugated goat anti-rabbit IgG antibody. Positive clones were identified and passaged to homogeneity.

Preparation of Fabs. Phagemids were excised from the positive clones with helper phage R408 (Stratagene) and used to transform XL-1 Blue cells as described (39, 40). Transformants were picked from each Fab clone and induced with 1 mM isopropyl β-D-thiogalactopyranoside to induce Fab production (8). After induction, the cells were pelleted and the supernatants were saved for subsequent experiments. An irrelevant clone was recovered, manipulated as described above, and used as a negative control. The Fabs were concentrated by ammonium sulfate precipitation.

Immunoblots. The presence of human Fab fragments in the *E. coli* supernatant of PDC-E2-positive Fab clones was verified by immunoblotting. The concentrated Fabs were resuspended in nonreducing SDS/PAGE sample buffer and electrophoresed by SDS/PAGE followed by immunoblotting as described (37, 38). Human Fab fragment was detected by ^{125}I-labeled goat anti-human IgG antibody (Amersham) and autoradiography. The same immunoblot technique was applied to determine the binding specificity of recombinant Fab clones to SDS/PAGE separated bovine, rat, and mouse mitochondrial preparations.

Specificity Determination. Antigen specificity was determined by ELISA as described (41). Microtiter plates were coated overnight at 4°C with recombinant human PDC-E2 containing the inner lipoyl domain (37), porcine PDC (Sigma), recombinant BCKD-E2 (42, 43), histone (Worthington), calf thymus double-stranded DNA (Sigma), and bovine serum albumin (Sigma) at 10 μg/ml in bicarbonate buffer (pH 9.5). After washing and blocking, 100 μl of *E. coli* supernatants from each Fab clone was added to the wells and incubated for 1 h at 37°C. Binding was identified by HRP-conjugated goat anti-human IgG $(Fab')_2$ (Tago). Color was developed by adding 100 μl of 2,2′-azinobis(3-ethylbenzthiazoline 6-sulfonic acid) in 0.1 M citrate buffer as substrate and optical density at 405 nm was measured. The Fabs were also studied by immunofluorescence using HEp-2 cell slides (Antinuclear Antibody Kit, Antibodies, Inc.) as described (28). Immunofluorescence of liver biopsies was performed as described (45).

Competitive Inhibition ELISA. Affinity binding constant clones were measured as described (46). Briefly, microtiter plates were coated with 100 μl of recombinant PDC-E2 inner lipoyl domain fusion protein (10 μg/ml) and incubated overnight at 4°C. The *E. coli* supernatants of PDC-E2-specific Fab clones were mixed with the same volume of serial dilutions of PDC-E2 inner lipoyl domain fusion protein at final concentrations of 10^{-5}–10^{-12} M and incubated overnight at 4°C to allow equilibration. Microtiter plates were washed and blocked and mixtures of bacterial supernatants from PDC-E2-specific Fab clones and serial dilutions of PDC-E2 fusion protein prepared as described above were added to the microplates and incubated at 37°C for 1 h. HRP-conjugated goat anti-human $(Fab')_2$ and 2,2′-azinobis(3-ethylbenzthiazoline 6-sulfonic acid) were used as secondary antibody and substrate, respectively.

RESULTS

Titering of Immunoglobulin Library. H- and L-chain libraries contained 7.8×10^4 and 1.1×10^5 clones, respectively. Over 85% of the libraries expressed H or L chains, confirmed by screening with anti-decapeptide antibodies or a mixture of anti-human κ and λ immunoglobulin antibodies. The two libraries were combined by ligation and, after packaging, the combinatorial immunoglobulin library was titered. The H/L combinatorial library contained 3.4×10^6 independent clones, with the number of clones expressing H-chain only or L-chain only being 5.3×10^4 and 1.4×10^5 clones, respectively, ≈6% of the total number of clones. We estimate that >70% of the clones in this combinatorial immunoglobulin library are expressing clones that coexpress H and L chains.

Identification of PDC-E2-Specific Clones. Screening with PDC-E2 identified six positive clones (LC1–LC6) from ≈60,000 phage plaques at a frequency of 0.01%. These positive clones were recovered from the plates and passaged to homogeneity. The presence of Fab fragments in the supernatant of lysed phage particles was confirmed by ELISA (data not shown), and immunoblotting of phage proteins separated under nonreducing conditions with ^{125}I-goat anti-human IgG antiserum. All Fab preparations clearly indicated the presence of a 48-kDa band, the expected size for an immunoglobulin molecule produced in this system (data not shown). LC1 also showed a signal at 25 kDa, implying some degradation of Fab fragments in this preparation.

Specific Reactivity of the Fabs. The antigen specificity of LC1–LC6 was examined by a variety of procedures. In a direct binding ELISA, a number of protein preparations were coated on microtiter plates and incubated with *E. coli* supernatants containing Fab. All clones reacted with porcine PDC and recombinant human PDC-E2, but not with bovine BCKD-E2, another commonly recognized autoantigen in PBC, or with bovine serum albumin histone, or calf thymus double-stranded DNA (Fig. 1). There was no binding detected when supernatant from a clone with the Lambda ZAP II vector without insert was used. Serum from a patient with PBC reacted strongly with the PDC, PDC-E2, and BCKD preparations (data not shown). The supernatant from an irrelevant control clone, which contained a Fab insert having the same λ L-chain isotype as PDC-E2-specific clones, did not show any reactivity to PDC, PDC-E2, or any of the other antigen preparations (data not shown). Thus, specific binding activity to PDC-E2 derived from antigen binding sequences in the Fab clones and not from *E. coli* proteins or mere expression of human IgG Fab sequences. To test further the specificity of the Fab fragments for other mitochondrial autoantigens, we performed immunoblotting of mitochondrial preparations from human placenta, bovine heart, rat liver, and mouse liver. The combinatorial Fabs reacted with a single band of 72 kDa in all lanes that migrated at the same

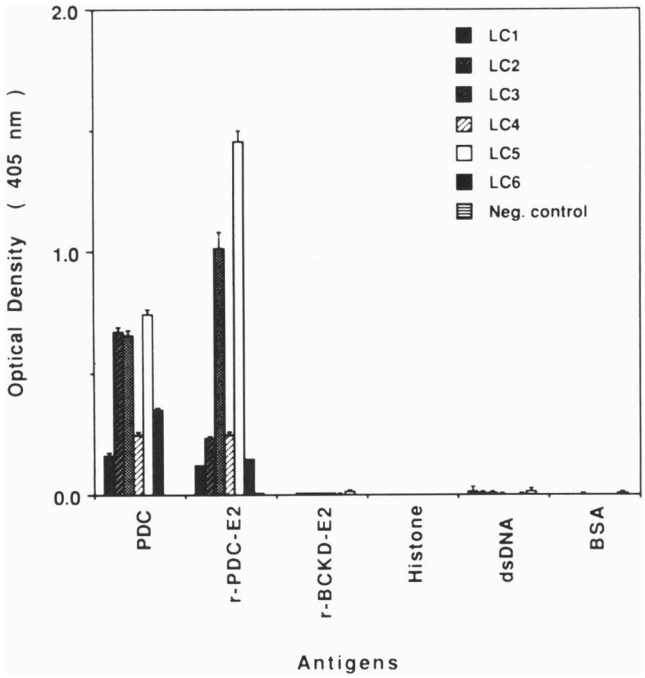

FIG. 1. PDC-E2-specific binding activity of recombinant human Fab clones. Wells were coated with different antigens and the supernatant from each Fab clone was examined for specific binding activity. The supernatant from the clone with the same Lambda ZAP II vector without the Fab insert was used as a negative control. Optical density measurements are mean ± SD (n = 3).

mobility as the 72-kDa protein recognized by PBC patient sera—i.e., PDC-E2 (data not shown). This experiment demonstrated that the Fabs reacted with an epitope that was conserved in several different species, precisely as found with patient sera. The PBC patient sera reacted with a number of other bands on the filter that corresponded to the other PBC autoantigens—BCKD-E2, 2-oxo acid glutarate dehydrogenase complex subunit E2, protein X, PDC-E1α, and PDC-E1β (data not shown). When the six Fabs were reacted with immunoblotted mitochondrial preparations, there was weak reactivity to protein X, detectable after prolonged exposure of the immunoblots, but no detectable reactivity to the other proteins, demonstrating marked specificity for PDC-E2.

Immunofluorescent Reactivity of the Fabs. Indirect immunofluorescence staining using the Fabs was performed on a number of substrates. HEp-2 cells were reacted with either PBC serum, a control serum, or a Fab, and the pattern of staining was visualized by a fluoresceinated secondary antibody (Fig. 2). The PBC serum and the Fab gave indistinguishable patterns of reactivity—i.e., typical anti-mitochondrial fluorescence. All Fabs gave identical patterns of fluorescence (data not shown). One of the Fabs (LC4) was reacted with biliary epithelial cells in liver sections obtained from a patient with PBC and a patient with progressive sclerosing cholangitis (PSC). There was intense reactivity detected with biliary epithelial cells in the PBC liver, especially in the luminal region of the cells (Fig. 3). Hepatocytes in this section showed normal mitochondrial staining. In contrast, reactivity with PSC biliary cells was less intense and had a mitochondrial pattern, similar to that seen in surrounding hepatocytes or in hepatocytes of PBC liver (data not shown). When the PBC liver was examined using a murine monoclonal antibody directed to PDC-E2, reactivity of biliary epithelial cells was found to be identical to that seen in normal liver—i.e., fluorescence was less intense and without luminal concentration (data not shown).

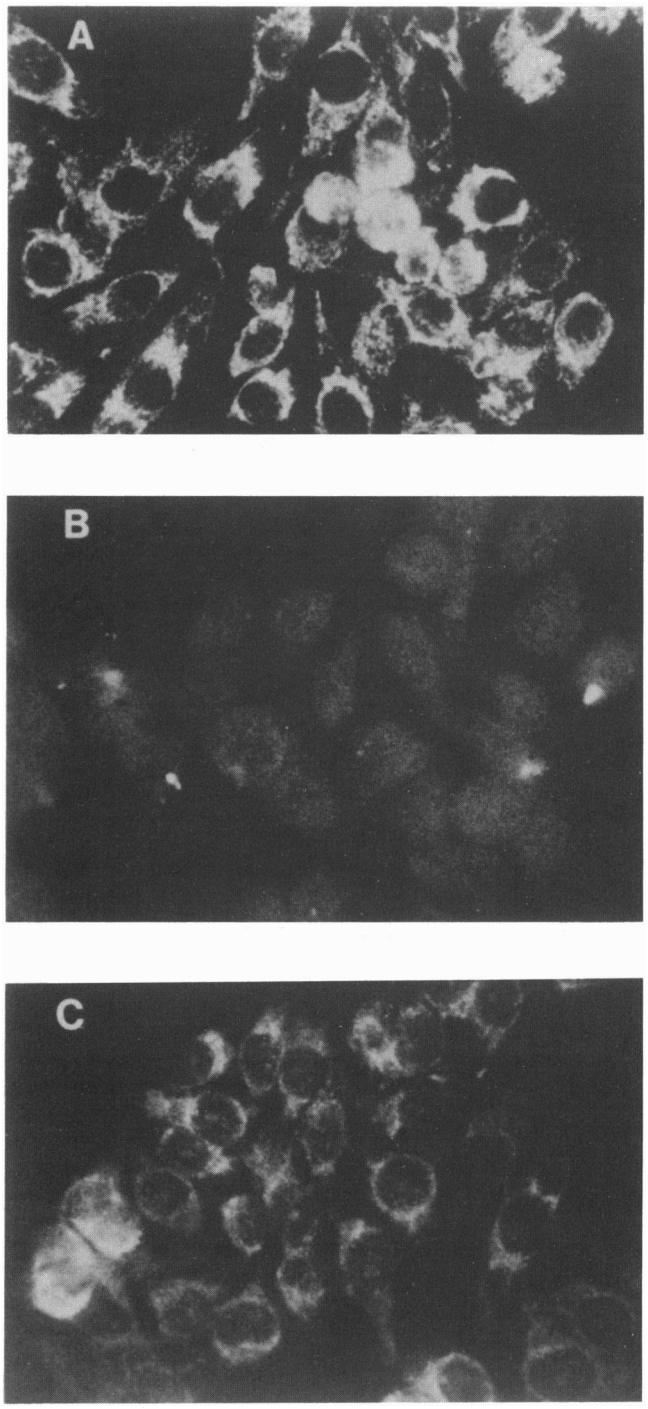

FIG. 2. Immunofluorescence of HEp-2 cells. Note the typical anti-mitochondrial pattern of reactivity by the Fabs. HEp-2 cell slides were incubated with a 1:100 dilution of PBC sera (*A*) or normal sera (*B*) or ammonium sulfate-precipitated LC5 (*C*). Thereafter, the slides were washed with phosphate-buffered saline and incubated with goat anti-human IgG antibody conjugated with fluorescein isothiocyanate.

Affinity Determination of Fab Fragments. We attempted to determine whether the six Fabs were independent cloning events utilizing distinct V_H and V_L genes. To do this, we measured affinity constants of binding for the Fabs to the inner lipoyl domain of recombinant PDC-E2. Affinity constants for the six Fabs varied over 2 orders of magnitude and were in the range of 10^{-7} to 10^{-9} M^{-1}, demonstrating that the Fabs were indeed independent clones (Fig. 4).

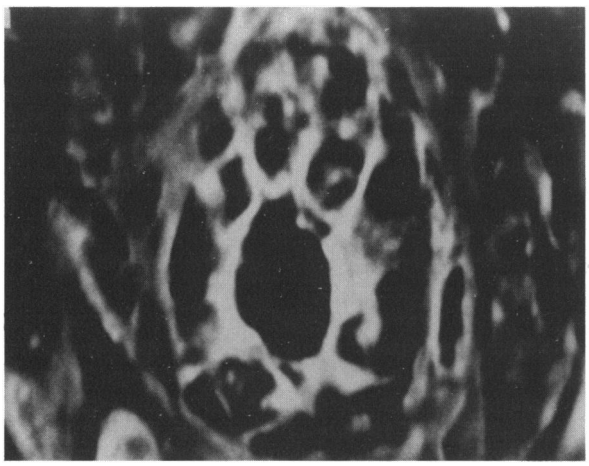

FIG. 3. Immunofluorescent staining of liver sections from a patient with PBC stained with Fab LC4. Note the high intensity staining of the luminal side of the biliary duct epithelium in the PBC patient.

DISCUSSION

The analysis of antibody–antigen reactions in autoimmune disease has been complicated by several factors. Autoantigens are often present in low concentrations, are difficult to purify, or may be poorly characterized. Patient sera may contain autoantibodies with multiple specificities. The generation of monoclonal antibodies of human origin against autoantigens will be of significant value in studying these reactions at both the biochemical and molecular level. Here we have successfully applied the combinatorial immunoglobulin library to isolate Fab fragments against PDC-E2, the major autoantigen in PBC (44). Previously, λ combinatorial immunoglobulin libraries have been constructed from immunoglobulin mRNA obtained from the spleen of immunized mice (8, 9) and peripheral blood of immunized healthy human volunteers (10, 11), but not from a lymphoid organ of a human with autoimmune disease.

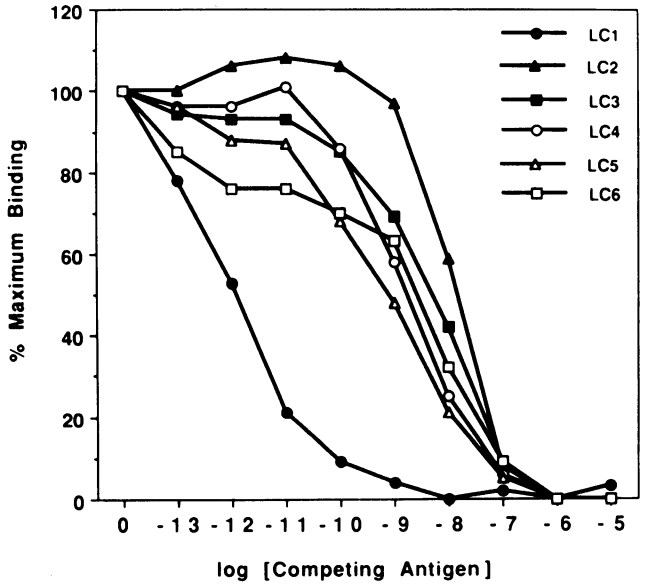

FIG. 4. Affinity determination by competitive inhibition ELISA. Affinity constants were determined at 50% of maximum binding. Affinity constants of LC1–LC6 were in the range of 10^{-7} to 10^{-9} M^{-1}. The high affinity shown by LC1 may be partly due to degradation of the LC1 preparation used in this experiment.

Our combinatorial immunoglobulin library was made from a mesenteric lymph node of a patient with PBC and was screened with a fusion protein expressing the inner lipoyl domain of PDC-E2. The frequency of anti-PDC-E2-specific Fab clones in our combinatorial library is 0.01%. This accords well with frequencies found in λ combinatorial immunoglobulin libraries from peripheral blood mRNA of humans immunized with tetanus toxoid [0.08% (10) and 0.02% (11)] and higher than seen in murine spleen-derived libraries [0.01% (8) and 0.008% (9)]. There is too little experience with these libraries to know how accurately plaque frequency reflects clonal frequency of B cells. However, it seems likely that anti-PDC-E2 immunoglobulin mRNA in patients with PBC is not a rare specificity but rather of the same approximate abundance as immunoglobulin mRNA for a moderately antigenic extrinsic antigen. These Fab clones have high affinity constants for their target antigen, ranging from 10^{-7} to 10^{-9} M^{-1}, similar to the affinities of Fab clones of combinatorial immunoglobulin libraries generated to exogenous antigens (11, 17, 18). LC1 is particularly interesting because it seemed to have extremely high affinity (K_a = 10^{-11}–10^{-12} M^{-1}), although it had the weakest signal among the clones in the reactivity to PDC-E2 in ELISA.

Several significant conclusions may be drawn from analysis of the six anti-PDC-E2 Fabs. The first concerns cross-reactivity among PBC autoantigens. Three of the most commonly recognized autoantigens are E2 subunits of 2-oxo acid dehydrogenases and all share similar active sites composed of lipoic acid bound to lysine. Data from our laboratory have demonstrated that the major autoepitope of PDC-E2 encompasses this region (24, 38) and our preliminary data would suggest that this region of BCKD is also an autoepitope. Absorption experiments suggest that each acetyltransferase is recognized by a distinct non-cross-reactive population of antibodies (30, 32). However, such experiments may give erroneous results as high-affinity antibodies may not elute from their target antigen and may therefore be unavailable for rebinding to a second antigen. The Fabs described here show high-affinity binding to PDC-E2 without any detectable binding to other acetyltransferases. Interestingly, there is minimal binding to protein X, an autoantigen that was believed to be recognized by virtue of cross-reactivity with PDC-E2 (31). This is similar to the binding profile of five human monoclonal antibodies derived by fusion of a regional lymph node from a PBC patient with a mouse–human heterohybrid cell line. All monoclonal antibodies were specific for PDC-E2 and showed minimal or no detectable binding to protein X (41).

Immunohistochemical staining of HEp-2 cells with the LC4 Fab fragment showed a typical mitochondrial staining pattern similar to that seen with PBC sera. Significantly, this Fab gave strong luminal staining of biliary epithelium in PBC livers, similar to the pattern seen with PBC sera (45) but distinct from the pattern seen with murine monoclonal antibodies to PDC-E2. Such a difference suggests that there is an immunologically distinct molecule found in PBC biliary epithelium that differs from normal PDC-E2. Whether this is a distinct isoform of PDC-E2 or a processed fragment with one but not all epitopes present, or indeed a cross-reactive molecule specific to the diseased cells, is currently unknown. Discovery of the identity of this molecule may provide a major insight into the etiology of the autoimmune process in this disease.

Thus, these Fabs have similar specificities to those found in human PBC sera and in human monoclonal antibodies (41) and should allow identification of the V_H and V_L gene usage of these autoantibodies. Comparison of V gene sequences from human monoclonal antibodies and combinatorial autoantibodies with corresponding germ-line V genes will allow determination of the role of somatic mutation in production of these high-affinity Fabs. As the sensitivity of screening and

efficiency of library construction improve, it may be possible to screen libraries derived from mRNA of healthy controls for PDC-E2 reactivity to extend such V gene sequence analyses.

We believe that, in the future, the combinatorial immunoglobulin library technique will provide a valuable alternative tool in the study of immunoglobulin repertoire and, perhaps in the future, development of monoclonal antibodies as blocking, diagnostic, or therapeutic reagents in medicine (47, 48).

Note Added in Proof. We note the recent report by Portolano *et al.* (49) of the isolation of three combinatorial Fab fragments directed to the human thyroid peroxidase, an autoantigen of Hashimoto thyroiditis.

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