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Characterization and Epitope Mapping of Human Monoclonal Antibodies to PDC-E2, the Immunodominant Autoantigen of Primary Biliary Cirrhosis

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Further to define the epitopes of PDC-E2, the major autoantigen in primary biliary cirrhosis (PBC), we have developed and characterized five human monoclonal antibodies. These antibodies were derived by fusing a regional hepatic lymph node from a patient with PBC with the mouse human heterohybrid cell line F3B6. Previous studies of epitope mapping of PDC-E2 have relied on whole sera and have suggested that the immunodominant epitope lies within the inner lipoyl domain of the molecule. However, selective absorption studies using whole sera and a series of overlapping recombinant peptides of PDC-E2 have suggested that the epitope may also include a large conformational component. Moreover, several laboratories have suggested that autoantibodies against the 2-oxo acids dehydrogenase autoantigens are cross-reactive. The five monoclonal antibodies generated included three IgG2a and two IgM antibodies and were studied for antigen specificity using recombinant PDC-E2, recombinant BCKD-E2, histone, dsDNA, IgG (Fc), collagen and a recombinant irrelevant liver specific control, the F alloantigen. The antibodies were also used to probe blots of human, bovine, mouse and rat mitochondria. Finally, fine

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specificity was studied by selective ELISA and absorption against overlapping expressing fragments of PDC-E2. All five monoclonals, but none of the other mitochondrial autoantigens were specific for PDC-E2. In fact, although affinity purified antibodies to PDC-E2 from patients with PBC cross-reacted with protein X, the human monoclonals did not, suggesting that protein X contains an epitope distinct from that found on PDC-E2. Additionally, all three IgG2 monoclonals recognized distinct epitopes within the inner lipoyl domain of PDC-E2.

Introduction

Although the etiology of PBC remains enigmatic [1], antimitochondrial autoantibodies (AMA) from patients with PBC react with components of the 2-oxo acids dehydrogenase pathway, i.e., the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2), the E2 subunit of the branched chain α -keto acid dehydrogenase complex (BCKD-E2), the E2 subunit of 2-oxo-glutarate acid dehydrogenase complex (OGDC-E2); protein X and the E1 α and E1 β subunits of pyruvate dehydrogenase complex [2-7]. Among these autoantigens, human PDC-E2 has been cloned, sequenced and expressed [8, 9]. Previous studies of epitope mapping have relied on whole sera and have suggested that the immunodominant epitope lies within the inner lipoyl domain of the molecule and may include a large conformational component [9, 10]. Additionally, several laboratories have proposed that AMA against the 2-oxo acid dehydrogenase autoantigens are cross-reactive [11-14]. These issues have also been studied with mouse monoclonal antibodies to PDC-E2; however, murine antibodies are not true antibodies and map to a different region of PDC-E2 than human patients with PBC [15]. Because of these problems, we have developed and report herein the characterization of five human monoclonal antibodies against PDC-E2. The availability of these reagents provides a valuable tool to address the fine specificity of antigenic epitopes in patients with PBC.

Methods and materials

Generation of hybridomas

A regional lymph node from a patient with end-stage and well-established PBC was removed during liver transplantation. The serum from this patient was known to contain antimitochondrial antibodies to PDC-E2, BCKD-E2, PDC-E1 α , protein X and OGDC by a combination of immunoblotting and ELISA assays [16-17]. In addition, a control normal lymph node was studied in parallel (see below). The resected lymph node was teased with forceps and scissors in Hanks' balanced salt solution (HBSS) without calcium and magnesium. It was then passed through a 22 gauge nylon mesh to prepare a single cell suspension at 5×10^6 cells/ml. A similar single cell suspension was prepared from normal human lymph node cells, removed during a diagnostic laparotomy. The fusion partner used was the mouse-human heterohybrid cell line F3B6.

F3B6, which is known to be 6-thioguanine- and ouabain-resistant, was produced by fusing NS-1 mouse myeloma cells with human peripheral blood lymphocytes.

Previously, hybridomas produced with this fusion partner have been shown to produce human Ig in the range of 1–50 µg/ml [18, 19]. The optimal ratio for fusions was established in pilot experiments using regional lymph nodes and peripheral blood from several other patients. The lymph node cells were washed in HBSS without calcium and with 2 mM magnesium (HBSS \pm). F3B6 was washed separately in HBSS \pm . Thence, 5×10^6 lymph node cells were mixed with 5×10^6 F3B6 cells and the total cells added to a Falcon 6-well plate which had been previously treated with 5 mg/ml peanut agglutinin at 37°C. After centrifuging at $500 \times g$ for 6 min at room temperature, the supernatant was aspirated off and 2 ml of prewarmed (37°C) 40% PEG fusion mixture (8 g PEG 4000, 2 ml DMSO to 20 ml HBSS \pm) added down the side of the wells. One min later, 4 ml of warm HBSS \pm with 5% DMSO (FDM) was added slowly, followed by 4 ml of FDM. The wells were then aspirated and an additional 2 ml of FDM added. The wells were then washed with HBSS \pm and the plates centrifuged at $400 \times g$ for 5 min. The supernatants were then removed and the monolayer washed by the addition of 2 ml of HBSS \pm followed by centrifugation at $400 \times g$ for 5 min. After a final aspiration, 3 ml of growth medium (GM-Iscoves' Modified Dulbecco's medium supplemented with 10% prescreened heat inactivated fetal bovine serum, 5×10^{-3} IU/ml bovine insulin, 5 µg/ml human transferrin, 5 ng/ml sodium selenite, 5 µg/ml human low density lipoprotein, 50 IU/ml penicillin and 50 µg/ml streptomycin) was added. The plates were incubated overnight at 37°C in a 7% CO₂ incubator. The fused cells were then diluted with GM containing 100 µM hypoxanthine (HA) and seeded into 96-well flat-bottom plates at a density of 1×10^5 cells/well; the cells were fed HAGM every third day. The hybrids were assayed for antibodies to PDC-E2 by ELISA, beginning on the 14th day. Positive wells were subcloned with two rounds of limiting dilution. Wells were screened microscopically daily and only wells containing one colony were regarded as monoclonal.

Antigens

PDC-E2 were purified from bovine kidney as described [20]. Human PDC-E2 and bovine BCKD-E2 were cloned respectively into the expression plasmids PUR [9, 21] and pKK233-2 [22, 23]. *E. coli* transformed with an expression plasmid of human PDC-E2 [9] or bovine BCKD-E2 [22, 23] were grown overnight in L-broth with 25 µg/ml ampicillin at 37°C. The overnight cultures were then diluted 1:10, incubated at 37°C for 1 h and induced with 1 mM isopropyl thiogalactosidase (IPTG) for 3 h at 37°C. The cells were harvested and lysed by sonication. The lysate was resolved by SDS-PAGE stained with Coomassie blue, and the percentage of recombinant protein in the lysate was determined [24]. Typically, recombinant PDC-E2 and BCKD-E2 represent 10–15% of the total protein. Mouse histones were a gift of Drs Peter Yau and Morton Bradbury of the Department of Biological Chemistry at the University of California, Davis. They were prepared by 0.4 N H₂SO₄ extraction of nuclei from mouse EL-4 thymoma cells. DNA was removed by centrifugation and the solubilized histones dialysed extensively against water and lyophilized. Double stranded DNA was prepared by extracting calf thymus DNA (Sigma Chemical Co., St. Louis, MO, USA) with phenol/chloroform. The DNA were then precipitated in ethanol, resuspended at 0.5 mg/ml in PBS and stored

frozen in small aliquots. IgG (Fc) (Jackson ImmunoResearch, West Grove, PA, USA) was dissolved in carbonate buffer at 10 µg/ml. Collagen (Eureka Laboratories, Sacramento, CA, USA) was solubilized in acetic acid and then resuspended at 10 µg/ml in carbonate buffer pH 9.6. The recombinant liver specific F alloantigen was prepared as described [25]. The F antigen served as both an additional antigen control and as a recombinant control.

Screening of monoclonals

Supernatants were initially screened with native PDC-E2 as previously described [17]. Known positive and negative sera were used throughout as quality controls. Since there were no differences in using native or recombinant PDC-E2 in the screening procedure, subsequent ELISA were performed using recombinant PDC-E2. To determine the class and subclass specificities of the monoclonal antibodies, the monoclonal antibodies were probed with optimal dilutions of mouse monoclonal anti-human μ , γ , α , IgG1, IgG2, IgG3, IgG4, κ and λ chains (Caltag Laboratories, South San Francisco, CA, USA) with known positive and negative reagents. Goat anti-mouse heavy and light chain antibodies were also used throughout to confirm the absence of mouse antibodies in the monoclonal supernatants.

Specificity

Antigen specificity was first studied by ELISA using recombinant human PDC-E2 [9], recombinant bovine BCKD-E2 [22, 23], histone, dsDNA, IgG (Fc), collagen and a recombinant irrelevant liver specific control, the F antigen [25] at 10 µg/ml. After blocking, 100 µl of monoclonal supernatant was added and incubated at room temperature for 2 h and washed three times with PBS containing 0.05% Tween 20. The wells were then incubated with peroxidase conjugated anti-human Ig (Tago, Burlingame, CA, USA) at a predetermined optimal dilution of 1:3,000. After 1 h incubation at room temperature, the wells were washed three times with PBS Tween and the color developed by incubating with 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) [22]. The reaction was stopped by adding 100 µl of 5% SDS after an additional 10 min and the results were recorded at 495 nm.

Following ELISA, all reagents specific for PDC-E2 were further studied by immunoblotting against mammalian mitochondria. Mitochondrial preparations from human placenta, bovine heart, and mouse and rat liver were prepared as described [15]. The samples were boiled in SDS sample buffer before loading onto 10% SDS-polyacrylamide gel at 10 µg protein per lane. The resolved proteins were then transferred to nitrocellulose filters and probed with human monoclonals, PBC sera (1:1,000 dilution) and sera from healthy volunteers as described [10]. Immunoreactivity was detected using I^{125} anti-human Ig (Amersham, Arlington Heights, IL, USA) and autoradiography. Since protein X is abundant in bovine mitochondrial preparations, immunoreactivity against protein X was scored from the bovine heart mitochondrial blot [13].

In both the ELISA and the immunoblotting assays, human sera from patients with PBC with known reactivities to PDC-E2, BCKD-E2, OGDC-E2, PDC-E1 α and protein X were used throughout as controls.

Fine specificity against the PDC-E2 domain

A series of overlapping expressing clones of PDC-E2 were generated as described [9]. Firstly, an EcoRI fragment (nucleotide 745–2536) of the human clone encoding the entire mature PDC-E2, including 16 residues of the leader sequence, was ligated into the frame-matched plasmid pUR [21]. The fusion protein expressed from the plasmid was purified in the presence of a reducing agent and SDS. The reducing agent and SDS were removed by gel filtration chromatography. Fractions containing the recombinant proteins were pooled and concentrated. To localize the antigenic determinants on human PDC-E2, restriction fragments encoding most or all of each domain were generated and fusion proteins expressed. Briefly, individual aliquots of the large EcoRI partial fragment were digested with DraI and the resulting fragments subcloned into frame-matched derivatives of pUR. The second DraI fragment encoding the inner lipoyl domain was further digested with either AluI, RsaI or NcoI, and the resulting partial or fully digested fragments subcloned. These plasmids were used to transform *E. coli* JM101 cells and the fusion protein induced with 10 mM IPTG. As noted above, an irrelevant control, a cDNA fragment encoding the rat liver specific F alloantigen [25] was ligated into the same vector and utilized throughout.

These expressing subclones were studied by immunoblotting. The clones were induced with IPTG and lysates transferred onto nitrocellulose paper as described [9, 20]. The transferred protein was incubated with supernatants of the human monoclonals for 2 h at room temperature, washed three times with PBS Tween and incubated with I¹²⁵ anti-human Ig (Amersham) for 1 h at room temperature and thereafter washed with PBS Tween as before. The blot was then incubated overnight on X-ray film. Known positive and negative sera were used throughout these studies.

Finally, these overlapping expressing clones were also studied by selective absorption. The supernatants were absorbed with either the full length human PDC-E2 or with individual PDC-E2 subclones as described [9].

Briefly, overnight cultures of the PDC-E2 subclones were diluted 1:10 with L-broth and 25 μ g/ml ampicillin, then induced with 1 mM IPTG for 6 h; cells were then harvested and lysed by sonication. The human monoclonals were incubated with 10 mg/ml sonicated extract at 4°C overnight and centrifuged at 10,000 \times *g* for 10 min to pellet the *E. coli* lysate. Monoclonals were also absorbed with the control plasmid as a control. The absorbed supernatants were then probed against the full length PDC-E2 by ELISA as described above.

PDC inhibition assay

The effects of human monoclonals on PDC enzyme activity were studied by monitoring substrate-dependent NADH production [26]. Briefly, a predetermined optimal concentration of monoclonal was first incubated with purified PDC enzyme

(Sigma Chemical Co., St. Louis, MO, USA) for 10 min at room temperature in 0.1 ml of 0.3% BSA-PBS and then with 0.9 ml of reaction mixture. The reaction mixture contained 50 μM potassium phosphate, pH 8.0, 2 μM sodium pyruvate, 2.5 μM NAD^+ , 0.2 μM thiamine pyrophosphate, 0.13 μM coenzyme A, 1 μM MgCl_2 and 0.3 μM dithiothreitol. The change in absorbance at 30°C was monitored at 340 nm and compared with control enzyme complex without antibody.

Results

Generation and characterization of human monoclonals against PDC-E2

Following fusion, hybridoma growth was observed in 25 of 500 culture wells; five of these wells were stable and produced antibody against PDC-E2. The antibodies include three IgG and two IgM monoclonals. Further characterization revealed that the three IgGs all belong to the IgG2 subclass, with two of them having λ -light chain and one κ -light chain. Both of the two IgM monoclonals were κ -light chain. All five monoclonals were reactive with anti-human Ig but not to either goat anti-mouse heavy or light chains, indicating the absence of any murine antibodies. Finally, the fusion of B cells from lymph nodes of a non-PBC patient did not yield any PDC-E2 specific human monoclonals.

Antigen specificity of human monoclonals

All five monoclonals reacted strongly and specifically to PDC-E2 but not to histone, dsDNA, human IgG (Fc) collagen, the liver specific F antigen [25] or recombinant BCKD-E2 using ELISA (Figure 1). The reactivity of these monoclonals was also tested by immunoblotting against beef heart mitochondria. The three IgG monoclonals were reactive only to PDC-E2, the 74 kDa mitochondrial antigen, but not to the other mitochondrial autoantigens, namely BCKD-E2, OGDC-E2, PDC-E1 α or protein X (Figure 2). The two IgM monoclonals did not blot.

Reactivity of human monoclonals against various mammalian species

The monoclonal antibody reagents were compared by analysing reactivity against human, bovine, rat and mouse mitochondrial extracts (Table 1). PBC sera and the three IgG monoclonals reacted to the PDC-E2 from all species. However, reactivity to protein X was only observed in PBC sera and was not noted in any of the three human IgG monoclonals (Figure 2).

Inhibition of PDC enzyme activity by human monoclonals

PBC sera and the human monoclonals were able to inhibit PDC enzyme activity to a similar degree. For example, polysera from patients with PBC were able to inhibit enzyme activity to about 64%. Inhibition of PDC-E2 activity by the human monoclonals ranged from 74–79% (Table 1). Neither an irrelevant monoclonal control nor sera from normal volunteers demonstrated inhibition of enzyme activity.

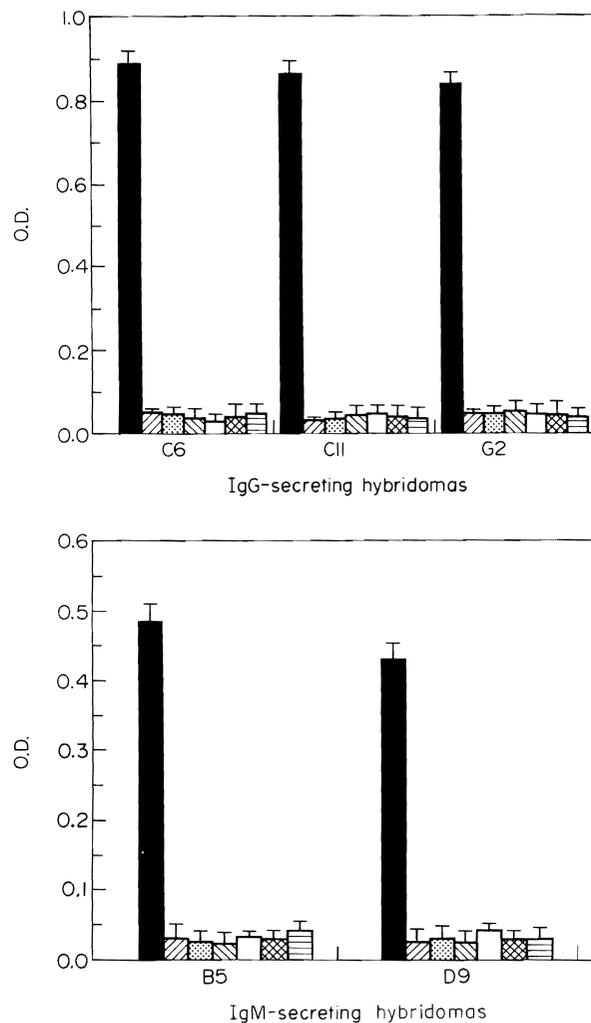


Figure 1. Antigen specificity of IgM and IgG secreting human monoclonals. Supernatants from human hybridoma were assayed for their reactivity against a panel of antigens by ELISA. The results were scored by measuring the optical densities of the individual wells. Note the high optical density (mean \pm SEM) observed with PDC-E2 as antigen but not the other antigens, $P < 0.01$ Student's *t*-test. ■ PDC-E2; ▨ histone; ▩ ds DNA; ▧ IgG (Fc); □ collagen; ▣ F protein; ▤ rBCKD-E2

Immunoreactivity against the structural domains of PDC-E2

Using expression clones of full length PDC-E2 (amino acid residues 1–560) and subclones containing the outer lipoyl domain (amino acid residues 1–96), the inner lipoyl domain (amino acid residues 91–227), the E1/E3 binding site (amino acid residues 229–401) and the catalytic domain (amino acid residues 396–506) of human PDC-E2 [9], we addressed the issue of binding specificities by selective absorption. Immunoblotting results show that the incubation of the monoclonals with *E. coli* expression clones of full length PDC-E2 and the inner lipoyl domain removed

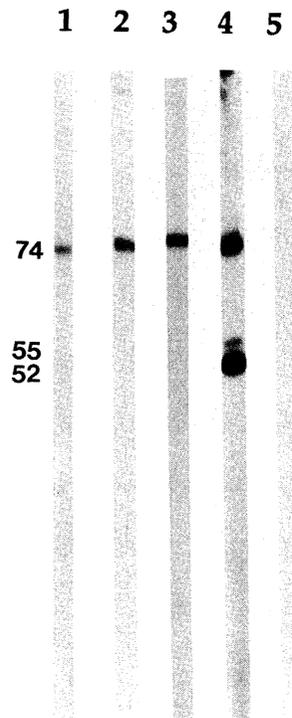


Figure 2. Beef heart mitochondria were separated by SDS-PAGE, blotted and probed with antisera. Lanes 1, 2 and 3 correspond to the IgG monoclonals C6, C11 and G2 respectively. Lane 4 has been probed with a known positive PBC serum control. Lane 5 has been probed with a normal human serum control. Note that the human monoclonals in lanes 1, 2 and 3 react only to PDC-E2 and not to either protein X or BCKD-E2. In contrast, sera from the patient with PBC react to the 74 kDa PDC-E2, the 55 kDa protein X and the 52 kDa BCKD-E2. This pattern of unique reactivity to only the 74 kDa antigen with the human monoclonals was also found even with more concentrated preparations of antibody (data not shown).

Table 1. Immunological profiles of human IgG monoclonal antibodies*

| Antibodies | Isotypes | L chain | Immunoblotting† | | | | % Inhibition activity‡ | Epitope§ |
|------------|----------|---------|-----------------|--------|-------|-----|------------------------|----------|
| | | | Human | Bovine | Mouse | Rat | | |
| C6 | IgG2 | λ | + | + | + | + | 74 | 128–227 |
| C11 | IgG2 | λ | + | + | + | + | 77 | 136–227 |
| G2 | IgG2 | κ | + | + | + | + | 79 | 91–227 |
| Sera | IgG2 | λ, κ | + | + | + | + | 64 | 91–227 |

*PDC-E2 specific human monoclonal antibodies were generated by fusing B cells from the lymph node of PBC patient with the mouse–human heterohybrid cell line F3B6.

†Immunoblotting was performed against purified mitochondria from human placenta, bovine heart, rat liver and mouse liver.

‡The ability of monoclonal antibodies to inhibit PDC enzyme activity *in vitro*. Normal sera and irrelevant monoclonal antibody control do not inhibit.

§Epitopes are represented as amino acid residues of PDC-E2. All epitopes were localized to the inner lipoyl domain.

||Sera from the same PBC patient from whom the monoclonal antibodies were derived.

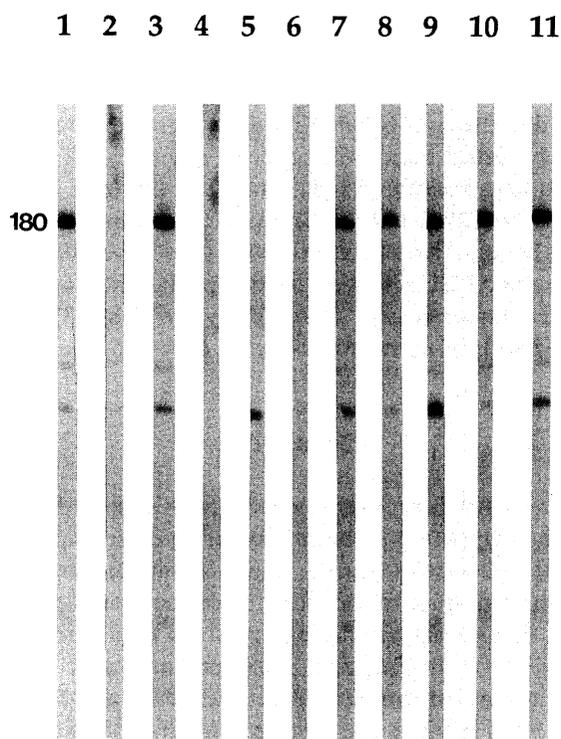


Figure 3. Lysates of *E. coli* containing the full length human PDC-E2 were separated by SDS-PAGE, blotted and probed with various preparations of monoclonal C11. The 180 kDa band is the recombinant fusion protein. The monoclonal antibodies used were initially absorbed with *E. coli* crude lysates containing expression clones of various domains of PDC-E2. The monoclonal C11 was absorbed with the following: an irrelevant control plasmid (lane 1); the full length PDC-E2 (lane 2); amino acids 1-96 (lane 3); amino acid residues 91-227 (lane 4); amino acid residues 128-227 (lane 5); amino acid residues 136-227 (lane 6); amino acid residues 146-227 (lane 7); amino acid residues 160-227 (lane 8); amino acid residues 181-227 (lane 9); amino acid residues 229-401 (lane 10); amino acid residues 396-560 (lane 11). Note the removal of reactivity by the full-length PDC-E2, amino acid residues 91-227 (lane 4), 128-227 (lane 5) and 136-227 (lane 6). In contrast, control plasmid (lane 1) and clones containing amino acid residues 1-96 (lane 3), 146-227 (lane 7), 160-227 (lane 8), 181-227 (lane 9), 229-401 (lane 10) and 396-560 (lane 11) do not absorb reactivity. A similar pattern was seen with the other IgG monoclonal reagents.

immunoreactivity against the full length protein (Figure 3, lane 2 and 4). All three IgG human monoclonals reacted to the full length PDC-E2 and the inner lipoyl domain, but not to the outer lipoyl, the E1/E3 binding site or the catalytic domain of PDC-E2 (Table 2).

Since the specific immunoreactivity of the IgG human monoclonals against the inner lipoyl domain resembles the polyclonal sera reactivity of PBC patients, the fine reactivity of human monoclonals was further examined with an overlapping series of expression clones of the inner lipoyl domain of PDC-E2. Lysates of a series of overlapping expression clones of the PDC-E2 inner lipoyl domain were incubated with human monoclonals and were then probed with an expression clone of full length PDC-E2. Although the three PDC-E2 specific IgG2 monoclonals were derived from the same patient, fine mapping of their specificities revealed differences. For example, the reactivity of monoclonal G2 was absorbed within

amino acid residues 91–227 and partially within amino acid residues 128–227. In contrast, expression clones containing amino acid residues 91–227, 128–227 and 136–227 were all able to absorb immunoreactivity of monoclonal C11 to a similar degree, suggesting that the epitope of C11 maps within amino acid residues 136–227 of PDC-E2. On the other hand, the immunoreactivity of monoclonal C6 was absorbed by amino acid residues 91–227 and 128–227 and partially even within amino acid residues 160–227 (Table 2). Plasmid control and the other clones bearing amino acid residues beyond that range showed negligible reactivity (Figures 3 and 4). When the same absorbed PBC sera were used to probe a full length expressing clone of human PDC-E2, similar results were observed (Table 2). Finally, it was not possible to map the two IgM monoclonals by ELISA (see Discussion).

Discussion

Several autoantigens have now been cloned and sequenced and their immunodominant epitopes mapped by both human polysera and murine monoclonals [15, 27–30]. However, murine monoclonal antibodies to autoantigens have often been found to map to a different antigenic epitope than human autoantibodies [15, 28]. In contrast, attempts to produce human monoclonal antibodies have often been hindered by the failure to develop immortalized human B cells. Most procedures require the transformation of peripheral B cells by Epstein-Barr virus prior to fusion [31, 32]. Herein we report the generation of three IgG and two IgM PDC-E2 specific human monoclonals using the mouse human heterohybrid F3B6.

Although the predominant Ig of AMA from sera of PBC patients are IgM and IgG3 [33], the IgG subclass specificities seen in the serum samples from the patients studied herein was predominantly IgG2 (data not shown). Thus, the predominant IgG2 AMA from the patient was identical to the IgG2 subclass of the human monoclonals. This suggests that the antibodies that resulted from the fusion reflected either the *in vivo* regional lymph node of this patient or, alternatively, that starter fusion occurs only with IgG2-producing B cells. However, the mouse-human heterohybrid line F3B6 has been previously shown to generate IgG3 and IgM monoclonals from rheumatoid synovial cells [34]. Thus, the observation that we developed only IgG2 monoclonals is unlikely owing to the limitations of the starter fusion. We should also note that our success is likely due to the large number of available B cells in lymph nodes in contrast to use of peripheral blood lymphocytes. The use of F3B6 also minimizes possible artefacts that might be induced by B cells that are activated or transformed by Epstein-Barr virus.

Earlier studies have shown that the autoepitope of PDC-E2 is localized to the lipoyl domain of PDC-E2 [3, 9, 11, 35]. Van de Water *et al.* [3] have previously identified that a 20 amino acid peptide within the lipoyl domain was able to absorb the antibody reactivity from PBC sera against rat PDC-E2. Surh *et al.* [9] have suggested that a minimum stretch of 92 amino acids (residues 136–227) within the inner lipoyl domain of human PDC-E2 is essential for antibody recognition and imply that a specific conformation is required. Similarly, several studies have also demonstrated that PBC sera react with the PDC-E2 component of *E. coli* [14, 35]. Using genetically engineered variants of the *E. coli* PDC-E2, Fussey *et al.* [14] reported that sera

Table 2. Reactivity of PBC sera and human monoclonal antibodies against expression clones of human PDC-E2*

| Amino acid residue | Human monoclonal antibodies | | | | | | | |
|--------------------|-----------------------------|-------------|---------------|-------------|---------------|-------------|---------------|-------------|
| | PBC Sera | | C6 | | C11 | | G2 | |
| | Mean | Range | Mean | Range | Mean | Range | Mean | Range |
| Control | 0.686 ± 0.025 | 0.663-0.711 | 0.179 ± 0.006 | 0.174-0.183 | 0.567 ± 0.018 | 0.541-0.585 | 0.278 ± 0.0 | 0.278-0.278 |
| 1-560 | 0.106 ± 0.006 | 0.101-0.111 | 0.035 ± 0.001 | 0.035-0.036 | 0.054 ± 0.006 | 0.049-0.060 | 0.059 ± 0.004 | 0.057-0.062 |
| 1-96 | 0.545 ± 0.025 | 0.512-0.573 | 0.203 ± 0.018 | 0.190-0.215 | 0.552 ± 0.028 | 0.523-0.588 | 0.339 ± 0.11 | 0.332-0.347 |
| 91-227 | 0.159 ± 0.010 | 0.147-0.170 | 0.042 ± 0.008 | 0.036-0.048 | 0.092 ± 0.008 | 0.084-0.099 | 0.062 ± 0.002 | 0.061-0.064 |
| 128-227 | 0.408 ± 0.019 | 0.390-0.435 | 0.033 ± 0.002 | 0.032-0.035 | 0.082 ± 0.009 | 0.074-0.090 | 0.219 ± 0.002 | 0.217-0.220 |
| 136-227 | 0.425 ± 0.020 | 0.406-0.449 | 0.127 ± 0.001 | 0.126-0.128 | 0.111 ± 0.015 | 0.097-0.128 | 0.307 ± 0.0 | 0.307-0.307 |
| 146-227 | 0.509 ± 0.023 | 0.481-0.534 | 0.126 ± 0.014 | 0.116-0.136 | 0.542 ± 0.005 | 0.536-0.546 | 0.281 ± 0.018 | 0.268-0.293 |
| 160-227 | 0.489 ± 0.020 | 0.461-0.506 | 0.111 ± 0.006 | 0.107-0.113 | 0.542 ± 0.034 | 0.504-0.570 | 0.302 ± 0.004 | 0.299-0.304 |
| 181-227 | 0.589 ± 0.051 | 0.529-0.652 | 0.193 ± 0.028 | 0.173-0.213 | 0.560 ± 0.023 | 0.536-0.579 | 0.274 ± 0.001 | 0.273-0.274 |
| 229-401 | 0.495 ± 0.017 | 0.471-0.507 | 0.186 ± 0.016 | 0.175-0.197 | 0.553 ± 0.022 | 0.533-0.572 | 0.324 ± 0.013 | 0.314-0.333 |
| 396-506 | 0.593 ± 0.034 | 0.549-0.627 | 0.189 ± 0.007 | 0.184-0.194 | 0.544 ± 0.014 | 0.527-0.555 | 0.326 ± 0.0 | 0.326-0.326 |

* Absorption studies show that expression clones of either full length PDC-E2 (residue 1-560) or subclones containing amino acid residues 91-227, 128-227 and 136-227 can reduce immunoreactivity of the PBC sera and the human monoclonals to various degrees.

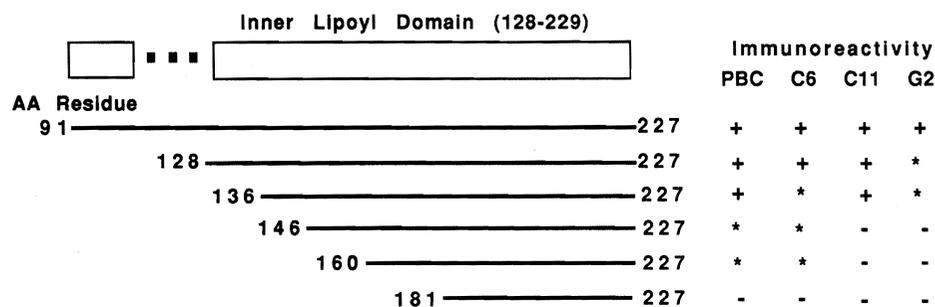


Figure 4. A schematic representation of the reactivity of PBC sera and C6, C11 and G2 monoclonal antibodies against expression cDNA clones of human PDC-E2. Note the specific reactivity against the inner lipoyl domain and within amino acid residues 91–227, 128–227, 136–227 of the inner lipoyl domain. Other expression clones do not react. (+) Positive reactivity; (–) negative reactivity; (*) partial reactivity. Data compared with the control value of the full length PDC-E2 (Table 2) and for each monoclonal respectively.

of PBC patients recognized the lipoyl domain of *E. coli* PDC-E2 as the immunodominant region. Moreover, substitution of lipoic acid by octanoic acid mimics the unique peptide-cofactor conformation, suggesting that both the lipoyl moiety and the peptide backbone are the major integral part of the B-cell epitope. In contrast, removal of lipoic acid by site directed mutagenesis of the lysine residue within a 413 base pair cDNA clone encoding the inner lipoyl domain of the human PDC-E2 did not affect antibody recognition by PBC sera [36]. Thus, the role of lipoic acid as an epitope is still unclear. Finally, a recent study took advantage of the ability of PBC sera to inhibit the *in vitro* catalytic function of the PDC enzyme and reported the presence of a population of non-blotting, but enzyme inhibiting autoantibodies in PBC. These were interpreted as indirect evidence for reactivity to a conformational determinant [37].

Epitope mapping with the IgM monoclonals was not possible owing to their inability to blot and their multiple reactivity to various expression clones of the PDC-E2 structural domains by ELISA. We believe that the main difficulties of studying IgM lie in the oligomeric structure and the stickiness of IgM which results in a heteroclitic response. Nevertheless, the selective absorption of anti-PDC-E2 reactivities by a number of overlapping subclones of PDC-E2 and the fact that a minimum stretch of 92 amino acid residues is required for the three IgG human monoclonals (Table 2, Figure 4) strongly indicates the presence of conformational epitope(s).

The presence of conformational epitopes in PDC-E2 can be discussed with respect to other autoantigens. For example, the histidy t-RNA transferase of myositis-associated anti-Jo-1 autoimmune response [30] contains both conformational and multiple linear epitopes. Multiple epitopes, including linear and discontinuous epitopes, have also been mapped in p70 [38, 39] in systemic lupus erythematosus (SLE). The U SnRNP associated B/B polypeptide of SM autoantibodies in SLE [40] has six epitopes, some of which are conformational. In some autoantigens, such as Sm B/B, a repeated proline rich motif PPGMRPP is the dominant epitope recognized by both human and murine antibodies [41]. In the case of the La antigen of Sjögren's syndrome, three distinct epitopes, LaA, LaC and LaL2/3, have been

identified. The LaA contains the first 107 amino acid residues from the N-terminal, the LaC contains amino acid residues 111–242, which is comprised of a RNA consensus sequence and helical core, and LaL2/3 constitutes amino acid residues 346–408, which contains a linear epitope [29, 42]. Evidently, therefore, both linear and conformational epitopes are common among autoantigens.

The PDC-E2-specific human IgG2 monoclonal antibodies reported herein inhibit PDC enzyme activity *in vitro*. This is similar to PBC polysera [43–46]. The ability of autoantibodies to inhibit enzyme function has also been demonstrated in other autoimmune diseases. For example, such data include the inhibition of t-RNA aminoacylation by autoantibodies to t-RNA synthetases in polymyositis sera [47] and inhibition of thyroid peroxidase activity by thyroid microsomal antibody [48]. Moreover, antibodies to the proliferating cell nuclear antigen in SLE have been reported to inhibit DNA replication *in vitro* [49] and RNA polymerase I specific antibodies from scleroderma sera inhibit the synthesis of 28S and 18S RNA [50]. The inhibition of enzyme activity by autoantibodies suggests that these autoantibodies are most likely to be directed against the functional sites of the enzyme. However, the functional significance of enzyme inhibition by autoantibodies and their possible role in disease development are unclear. Finally, it is believed that antigens recognized by IgG2 are primarily carbohydrate in nature. In contrast, PDC-E2 is devoid of carbohydrate moieties. Clearly, more work on the genetic origin of clones reported herein and on similarly derived hybridomas from other patients is needed for continued investigation of the fundamental questions regarding autoantibody formation and pathogenic significance.

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References

1. Kaplan, M. M. 1987. Primary biliary cirrhosis. *Adv. Intern. Med.* **32**: 359–377
2. Gershwin, M. E. and I. R. Mackay. 1991. Primary biliary cirrhosis: paradigm or paradox for autoimmunity. *Gastroenterol.* **100**: 822–833
3. Van de Water, J., C. D. Surh, P. S. 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. *Sem. Liv. Dis.* **9**: 132–137
4. Mackay, I. R. and M. E. Gershwin. 1990. Primary biliary cirrhosis: considerations on pathogenesis based on identification of the M2 autoantigens. *Springer Semin. Immunopathol.* **12**: 101–119
5. Bassendine, M. F., S. P. Fussey, D. J. Mutimer, O. F. James, and S. J. Yeaman. 1989. Identification and characterization of four M2 mitochondrial autoantigens in primary biliary cirrhosis. *Sem. Liv. Dis.* **9**: 124–131
6. James, O. F., S. J. Yeaman, and M. F. Bassendine. 1989. Molecular aspects of the M2 autoantigens in primary biliary cirrhosis: what a difference a year makes. *Hepatology* **10**: 247–251
7. Mackay, I. R. and M. E. Gershwin. 1989. Molecular basis of mitochondrial autoreactivity in primary biliary cirrhosis. *Immunol. Today* **10**: 315–319

8. Coppel, R. L., L. J. McNeilage, C. D. Surh, J. Van de Water, T. W. Spithill, S. Whittingham, and M. E. Gershwin. 1988. Primary structure of the human M2 mitochondrial autoantigen of primary biliary cirrhosis: dihydrolipoamide acetyltransferase. *Proc. Natl. Acad. Sci. USA* **85**: 7317–7321
9. Surh, C. D., R. Coppel, and M. E. Gershwin. 1990. Structural requirement for autoreactivity on human pyruvate dehydrogenase-E2, the major autoantigen of primary biliary cirrhosis. Implication for a conformational autoepitope. *J. Immunol.* **144**: 3367–3374
10. Van de Water, J., M. E. Gershwin, P. Leung, A. Ansari, and R. L. Coppel. 1988. The autoepitope of the 74-kD mitochondrial autoantigen of primary biliary cirrhosis corresponds to the functional site of dihydrolipoamide acetyltransferase. *J. Exp. Med.* **167**: 1791–1799
11. Flannery, G. R., A. K. Burroughs, P. Butler, J. Chelliah, J. Hamilton-Miller, W. Brumfitt, and H. Baum. 1988. Antimitochondrial antibodies in primary biliary cirrhosis recognize both specific peptides and shared epitopes of the M2 family of antigens. *Hepatology* **10**: 370–374
12. Baum, H. 1988. Nature of the mitochondrial antigens of primary biliary cirrhosis and their possible relationships to the etiology of the disease. *Semin. Liv. Dis.* **9**: 117–123
13. Surh, C. D., T. E. Roche, D. J. Danner, A. Ansari, R. L. Coppel, T. Prindiville, E. R. Dickson, and M. E. Gershwin. 1988. Antimitochondrial autoantibodies in primary biliary cirrhosis recognize cross-reactive epitope(s) on protein X and dihydrolipoamide acetyltransferase of pyruvate dehydrogenase complex. *Hepatology* **10**: 127–133
14. Fussey, S. P. M., S. T. Ali, J. R. Guest, O. F. W. James, M. F. Bassendine, and S. J. Yeaman. 1990. Reactivity of primary biliary cirrhosis with *Escherichia coli* dihydrolipoamide acetyltransferase (E2p): Characterization of the main immunogenic regions. *Proc. Natl. Acad. Sci. USA* **87**: 3987–3991
15. Surh, C. D., A. Ahmed-Ansari, and M. E. Gershwin. 1990. Comparative epitope mapping of murine monoclonal and human autoantibodies to human PDH-E2, the major mitochondrial autoantigen of primary biliary cirrhosis. *J. Immunol.* **144**: 2647–2652
16. Coppel, R. L., M. E. Gershwin, and A. D. Sturgess. 1989. Cloned autoantigens in the study and diagnosis of autoimmune diseases. *Mol. Biol. Med.* **6**: 27–34
17. 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 recombinant mitochondrial proteins in patients with primary biliary cirrhosis. *N. Engl. J. Med.* **320**: 1377–1380
18. Pollack, M., A. A. Raubitschek, and J. W. Larrick. 1987. Human monoclonal antibodies that recognize conserved epitopes in the core-lipid A region of lipopolysaccharides. *J. Clin. Invest.* **79**: 1421–1438
19. Larrick, J. W., O. Gray, T. Deinhart, J. Wang, J. Flatgaard, and K. T. Chong. 1988. Generation of a protective human monoclonal for the treatment of gram-negative sepsis. *Prog. Clin. Biol. Res.* **272**: 383–393
20. Fujikura, S., P. Davis, T. Prindiville, P. Leung, R. I. Fox, and M. E. Gershwin. 1990. Sjögren's syndrome and primary biliary cirrhosis: Presence of autoantibodies to purified mitochondrial 2-oxo-acid dehydrogenase. *J. Rheumatol.* **17**: 1453–1457
21. Ruther, U. and B. Muller-Hill. 1983. Easy identification of cDNA clones. *EMBO J.* **2**: 1791–1794
22. Griffin, T. A., R. Max Wynn, and D. T. Chuang. 1990. Expression and assembly of mature apotransacylase (E₂b) of bovine branched-chain α -keto acid dehydrogenase complex in *Escherichia coli*. *J. Biol. Chem.* **265**: 12104–12110
23. Griffin, T. A. and D. T. Chang. 1990. Genetic reconstruction and characterization of the recombinant transacylase (E₂b) component of bovine branched-chain α -keto acid dehydrogenase complex. *J. Biol. Chem.* **265**: 13174–13180
24. Salemmuddin, A., H. Ahmad, and A. Husain. 1980. A simple rapid and sensitive procedure for the assay of endoproteases using Coomassie brilliant blue G-250. *Anal. Biochem.* **105**: 202–206
25. Gershwin, M. E., R. L. Coppel, E. Bearer, M. G. Peterson, A. Sturgess, I. R. Mackay. 1987. Molecular cloning of the liver-specific rat F antigen. *J. Immunol.* **139**: 3828–3833

26. Sudin, U. 1990. Antibody binding and inhibition of pyruvate dehydrogenase (PDH) in sera from patients with primary biliary cirrhosis. *Clin. Exp. Immunol.* **81**: 238–243
27. Leung, P. S. C. and M. E. Gershwin. 1990. The molecular structure of autoantigens. *Curr. Opin. Immunol.* **2**: 567–575
28. Chambers, J. C., D. Kenan, B. J. Martin, and J. D. Kenne. 1988. Genomic structure and amino acid sequence domains of the human La autoantigen. *J. Biol. Chem.* **203**: 18043–18051
29. Sturgess, A. D., M. G. Peterson, J. McNeilage, S. Whittingham, and R. L. Coppel. 1988. Characteristics and epitope mapping of a cloned human autoantigen La. *J. Immunol.* **140**: 3212–3218
30. Ramsden, D. A., J. Chen, F. W. Miller, F. Misener, R. M. Bernstein, K. A. Simnovitch, and F. W. L. Tsui. 1989. Epitope mapping of cloned human autoantigen, histidyl tRNA synthetase. *J. Immunol.* **143**: 2267–2272
31. Casali, P. and A. L. Notkins. 1989. Probing the human B-cell repertoire with EBV: polyreactive antibodies and CD5⁺ B lymphocytes. *Annu. Rev. Immunol.* **7**: 513–535
32. Kunicki, T., K. Furihata, R. Kekomaki, J. P. Scott, and D. J. Nugent. 1990. A human monoclonal antibody specific for human platelet glycoprotein IIb (integrin alpha IIb) heavy chain. *Human Antibody Hybridomas* **1**: 83–95
33. Surh, C. P., A. E. Copper, R. L. Coppel, P. Leung, A. Ahmed, R. Dickson, and M. E. Gershwin. 1988. The predominance of IgG3 and IgM isotype antimitochondrial antibodies against recombinant fused mitochondrial polypeptide in patients with primary biliary cirrhosis. *Hepatology* **8**: 290–295
34. Robbins, D. L., T. P. Kenny, M. J. Coloma, J. V. Gavidondo-Cowley, R. W. Soto-Gil, P. P. Chen, and J. W. Larrick. 1990. Serologic and molecular characterization of a human monoclonal rheumatoid factor derived from rheumatoid synovial cells. *Arthritis Rheum.* **33**: 1188–1195
35. Fusey, S. P. M., M. F. Bassendine, O. F. M. James, and S. J. Yeaman. 1989. Characterization of the reactivity of autoantibodies in primary biliary cirrhosis. *FEBS Lett.* **246**: 49–53
36. Leung, P. S., T. Iwayama, R. L. Coppel, and M. E. Gershwin. 1990. Site-directed mutagenesis of lysine within the immunodominant autoepitope of PDC-E2. *Hepatology* **12**: 1321–1328
37. Rowley, M. J., L. J. McNeilage, J. M. Armstrong, and I. R. Mackay. 1991. Inhibitory antibody to a conformational epitope of the pyruvate dehydrogenase complex, the major autoantigen of primary biliary cirrhosis. *Clin. Immunol. Immunopathol.* **60**: 356–370
38. Reeves, W. H., A. Pierani, C. H. Chou, T. Ng, C. Nicastrì, R. G. Roeder, Z. M. Sthoeger. 1991. Epitopes of the p70 and p80 (Ku) lupus autoantigens. *J. Immunol.* **146**: 2678–2686
39. Cram, D. S., N. Fisicaro, R. L. Coppel, S. Whittingham, and L. C. Harrison. 1990. Mapping of multiple B-cell epitopes on the 70 kilodalton autoantigen of the U1 ribonucleoprotein complex. *J. Immunol.* **145**: 630–635
40. Rokeach, L. A., M. Jannatipour, and S. O. Hoch. 1990. Heterologous expression and epitope mapping of a human small nuclear ribonucleoprotein associated Sm-B/B autoantigen. *J. Immunol.* **144**: 1015–1022
41. Williams, D. G., N. G. Sharpe, G. Wallace, and D. S. Latchman. 1990. A repeated proline-rich sequence in the sm B/B and N is a dominant epitope recognized by human and murine autoantibodies. *J. Autoimmunity* **3**: 715–725
42. McNeilage, L. J., E. M. Macmillan, and S. F. Whittingham. 1990. Mapping of epitopes on the La(SS-B) autoantigen of primary Sjögren's syndrome: identification of a cross-reactive epitope. *J. Immunol.* **145**: 3829–3835
43. Van de Water, J., D. Fregeau, P. Davis, A. Ansari, D. Danner, P. Leung, R. Coppel, and M. E. Gershwin. 1988. Autoantibodies of primary biliary cirrhosis recognize dihydro-lipoamide acetyltransferase and inhibit enzyme function. *J. Immunol.* **141**: 2321–2324
44. 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 E1 α , a non-lipoic acid containing mitochondrial enzyme. *J. Immunol.* **144**: 1671–1676

45. Fregeau, D. R., P. A. Davis, D. J. Danner, A. Ansari, R. L. Coppel, E. R. Dickson, and M. E. Gershwin. 1989. Antimitochondrial antibodies of primary biliary cirrhosis recognize dihydrolipoamide acetyltransferase and inhibit enzyme function of the branched chain α -keto acid dehydrogenase complex. *J. Immunol.* **142**: 3815–3820
46. Uibo, R., I. R. Mackay, M. Rowley, P. Humphries, J. M. Armstrong, and J. McNeilage. 1990. Inhibition of enzyme function by human autoantibodies to an autoantigen pyruvate dehydrogenase E2: different epitope for spontaneous human and induced rabbit autoantibodies. *Clin. Exp. Immunol.* **80**: 19–24
47. Mathews, M. B. and R. M. Bernstein. 1983. Myositis, autoantibody inhibits histidyl-t-RNA synthetase: a model for autoimmunity. *Nature* **304**: 177–179
48. Okamoto, Y., N. Hamada, H. Saito, M. Ohno, J. Noh, K. Ito, and H. Morii. 1989. Thyroid peroxidase activity inhibiting immunoglobulins in patients with autoimmune thyroid disease. *J. Clin. Endocrinol. Metab.* **68**: 730–734
49. Tan, C. K., K. Sullivan, X. Y. Li, E. M. Tan, K. M. Downey, and A. G. So. 1987. Antibody to proliferating cell nuclear antigen neutralizes the activity of auxiliary protein for DNA polymerase delta. *Nucleic Acids Res.* **15**: 9299–9308
50. Reimer, G., K. M. Rose, U. Scheer, and E. M. Tan. 1987. Autoantibody to RNA polymerase I in scleroderma. *J. Clin. Invest.* **79**: 65–72