Heterogeneity of Autoreactive T Cell Clones Specific for the E2 Component of the Pyruvate Dehydrogenase Complex in Primary Biliary Cirrhosis

By Judy Van de Water,* Aftab Ansari,‡ Thomas Prindiville,* Ross L. Coppel,‖ Nancy Ricalton,¶ Brian L. Kotzin,¶ Shengjiang Liu,§ Thomas E. Roche,§ Sheri M. Krams,* Santiago Munoz,** and M. Eric Gershwin*

*From the *Division of Rheumatology, Allergy and Clinical Immunology, School of Medicine, University of California Davis, Davis, California 95616; the ‡Department of Pathology, Emory University School of Medicine, Winship Cancer Center, Atlanta, Georgia 30322; the §Department of Biochemistry, Kansas State University, Manhattan, Kansas 66506; the ‖Department of Microbiology, Monash University, Clayton, Victoria 3168, Australia; the ¶Departments of Pediatrics and Medicine, National Jewish Center for Immunology and Respiratory Medicine, Denver, Colorado 80206; and the **Division of Gastroenterology, Department of Medicine, Jefferson Medical College, Philadelphia, Pennsylvania 19107*

Summary

The extraordinary specificity of bile duct destruction in primary biliary cirrhosis (PBC) and the presence of T cell infiltrates in the portal tracts have suggested that biliary epithelial cells are the targets of an autoimmune response. The immunodominant antimitochondrial humoral response in patients with PBC is directed against the E2 component of pyruvate dehydrogenase (PDC-E2). Hitherto, there have only been limited reports on the characterization and Vβ usage of PDC-E2–specific cloned T cell lines. In this study, we examined peripheral blood mononuclear cells (PBMC) for their reactivity to the entire PDC complex as well as to the E1- and E2-specific components. We also examined the phenotype, lymphokine profile, and Vβ usage of PDC-specific T cell clones isolated from cellular infiltrates from the livers of PBC patients. We report that PBMC from 16/19 patients with PBC, but not 12 control patients, respond to the PDC-E2 subunit. Interestingly, this response was directed to the inner and/or the outer lipoyl domains, despite the serologic observation that the autoantibody response is directed predominantly to the inner lipoyl domain. Additionally, lymphokine analysis of interleukin (IL) 2/IL-4/interferon γ production from individual liver-derived autoantigen-specific T cell clones suggests that both T helper cell Th1- and Th2-like clones are present in the liver. Moreover, there was considerable heterogeneity in the T cell receptor for antigen (TCR) Vβ usage of these antigen-specific autoreactive T cell clones. This is in contrast to murine studies in which animals are induced to develop autoimmunity by specific immunization and have an extremely limited T cell Vβ repertoire. Thus, our data suggest that in human organ–specific autoimmune diseases, such as PBC, the TCR Vβ repertoire is heterogenous.

Primary biliary cirrhosis (PBC)[1] is an idiopathic hepatic disorder characterized by the presence of autoantibodies to mitochondrial antigens (AMA), lymphoid infiltrates in the portal tracts of the liver, bile duct destruction, and proliferation of bile duct epithelial cells (1–3). The immunodominant response of AMA is directed at the E2 component of pyruvate dehydrogenase (PDC-E2) (4, 5). The reactive region of PDC-E2 recognized by autoantibodies has been mapped and includes specific recognition of the inner lipoyl domain and, to a 100-fold lesser extent by sera titration, the outer lipoyl domain (6, 7).

The extraordinary specificity of bile duct destruction in PBC, the presence of lymphoid infiltrates in the portal tracts, and the aberrant expression of class II antigens on biliary epithelium, suggest that biliary epithelial cells are the targets of an intense autoimmune response (8–10). Although it has been suggested that the infiltrating T cells are oligoclonal,

[1] *Abbreviations used in this paper:* AMA, autoantibodies to mitochondrial antigens; PBC, primary biliary cirrhosis; PDC-E1, -E2, E1 and E2 components of pyruvate dehydrogenase, respectively; PSC, primary sclerosing cholangitis.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/95/02/0723/11 $2.00
Volume 181 February 1995 723–733

there have been only limited reports on characterization of PDC-E2 specific-cloned T cell lines (11–15). In this study, we not only studied PBMC but also examined cells cultured from liver tissues from patients with PBC for reactivity to the entire PDC complex, and to the E1- and E2-specific subcomponents. We report the response of patients' peripheral blood T cells to different PDC components and, in addition, the phenotype, lymphokine profile, and Vβ usage of PDC-specific cloned T cell lines isolated from cellular infiltrates from the livers of PBC patients. Based on cytokine profiles, these PDC-E2-specific cloned T cell lines were noted to be composed of both Th1- and Th2-like cells. More importantly, unlike in animal models of autoimmunity, there was considerable heterogeneity of TCR Vβ usage in autoreactive T cell clones specific for PDC-E2.

Materials and Methods

Clinical Specimens. Blood was collected from 19 female subjects, ages 42–65 yr, with PBC (16); all were known to be positive for AMA by use of Hep-2 cells and immunofluorescence. All 19 subjects were, by histology, considered to have stage II through stage IV disease. In addition, 12 control subjects were studied. These included eight healthy volunteers (six female, two male) and four patients with primary sclerosing cholangitis (PSC) (three female, one male). The sera from all subjects were analyzed by immunoblotting with beef heart mitochondria and by ELISA with recombinant proteins (7). From this analysis, sera from 19/19 patients with PBC reacted with PDC-E2, 9/19 reacted with PDC-E1α, and 13/19 reacted with branched-chain ketoacid dehydrogenase. None of the control subjects showed any detectable reactivity. Finally, liver tissue was obtained at transplantation from two patients with PBC (one female and one male) and one male patient with PSC, ages 52–65 yr.

Antigen Preparation. The native antigens used for in vitro stimulation were prepared from beef heart mitochondria (17, 18); sera from PBC patients react equally well with both human and bovine mitochondrial antigens (4). First, a parbomb was used to disrupt mitochondria, followed by polyethylene glycol fractionation. After homogenization, the PDC fraction was centrifuged through 7.5, 10, and 15% sucrose gradients at 28,000 rpm in a SW27 rotor at 4°C for 4 h. The resulting pellet was then fractionated into the E1, E2, and E3 components by use of a Sephacryl s400 column (18). Fractions were collected, analyzed for purity by SDS-PAGE, and filtered through a 0.22-μ filter. In addition, the two lipoic acid–binding regions of human PDC-E2 were prepared from our previously defined recombinant human PDC-E2 (pHumPDC-E2-2A) (6, 7). As a control, another mitochondrial antigen, bovine malic dehydrogenase (Sigma Chemical Co., St. Louis, MO) was studied.

Briefly, all domains were derived by PCR using purified pHumPDC-E2-2A as a template with EcoRI cloning sites at both ends. The resulting constructs were cloned into the pGEX-2T system and treated with thrombin to cleave the rPDC from the glutathione-s-transferase moiety. Positive clones were selected by ELISA and immunoblotting and subsequently sequenced. The constructs used in this study were amino acid residues 1–98 (E2L1; containing the outer lipoyl domain), 120–233 (E2L2; containing the inner lipoyl domain), and 1–233 (E2L1 + L2). All fragments were then purified after thrombin treatment.

Culture and Proliferation of PBMC. PBMC were purified by density centrifugation, washed, and incubated in media (X-Vivo 15; Bio Whittaker, Walkersville, MD) containing 4% T-Stim (Collaborative Research, Inc., Bedford, MA) overnight at 37°C in 10% CO_2. Cellular proliferation of the PBMC was measured with the [^{3}H]thymidine assay (19). Briefly, 2×10^5 PMBC were washed and incubated with the following antigens: PDC at 10, 0.1, and 0.001 μg/ml; PDC-E2 at 50, 10, and 0.001 μg/ml; PDC-E1 at 10, 0.1, and 0.001 μg/ml; and, for purposes of control, BSA at 10 μg/ml. The antigens were diluted in X-Vivo 15 media containing 1% T-Stim and incubated with the isolated PBMC for 6 d at 37°C in 10% CO_2. On day 6, 1 μCi of [^{3}H]thymidine was added to each well, incubated for 18 h, harvested, and counted in a scintillation counter (Betaplate; Wallac, Inc., Gaithersburg, MD).

Liver-derived T Cell Clones. The liver was minced into 1-cm^3 pieces and digested with 1 mg/ml type I collagenase (Sigma Chemical Co.) in HBSS for 2 h at 37°C with agitation. The digested material was then washed three times in HBSS and the pellet purified by density centrifugation as above. The cells at the density gradient interface were collected and washed three times in PBS and cultured in 24-well plates in 4% T-Stim in X-Vivo 15 media at 37°C for 5–7 d. The cells were then cloned by limiting dilution to 0.3 cells/well. After ~2 wk, the clones were expanded into 24-well plates by use of irradiated JY feeder cells (20) at 5×10^5 cells/ml and 4% T-Stim. Positive T cell clones were identified by the proliferation assay described above except that two sources of APC were used for this study. One consisted of EBV-transformed cell lines derived from the PBMC of the PBC patients (21). The second source was an enriched population of adherent cells that were isolated from the PBMC from normal adult volunteers who shared the MHC class II type with the PBC patients being studied. The difference between EBV-transformed B cells versus MHC class II matched "professional" APC was quite marked. For example, clone 1, derived from patient 1 when cocultured with antigen-pulsed autologous irradiated EBV-transformed cells, gave a proliferative response of 7,526 ± 119 cpm (mean ± SEM) with an IL-2 production of 4.6 U/ml. In contrast, the same T cell clone, when cocultured with the antigen-pulsed enriched population of MHC class II identical adherent APC resulted in an increased proliferative response of 29,744 ± 166 cpm and an IL-2 concentration of 53.9 U/ml. The difference in the relative responses to the same antigen presented by autologous irradiated EBV-transformed cells compared with MHC class II identical adherent cells was not secondary to dose of antigen and/or differences in kinetics of the response (data not shown). Each cloned T cell line was assayed for antigen-specific proliferative responses by use of both autologous irradiated EBV-transformed cells lines as well as adherent cells as APC. However, the data presented here are from the studies performed with the adherent cell APC preparations.

Tissue typing of MHC class I and II alleles expressed by the EBV-transformed cell lines was defined by use of standard serological tissue typing techniques. MHC class II typing was further refined and confirmed by use of molecular typing techniques. These data allowed us to select for individuals who shared the identical MHC class II type for use as a source of APC in our assays.

Adherent APC were pulsed overnight with media alone (control) or antigen at optimum concentrations of 10 μg/ml PDC-E1, 25 μg/ml PDC-E2, 0.1 μg/ml E2L1, and 0.1 μg/ml E2L2. Malic dehydrogenase was also used at concentrations of 0.1, 1.0, and 10 μg/ml for purposes of antigen specificity. The antigen-pulsed APC were dispensed into 96-well microtiter plates in a volume of 0.1 ml containing 2×10^5 cells in media consisting of RPMI 1640

supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 2×10^{-5} M 2-mercaptoethanol, and 15% heat-inactivated pretested human AB plasma. The frequency of the adherent cell subsets was confirmed by FACS® analysis (Becton Dickinson & Co., Mountain View, CA) to be 5–9% $CD3^+$, 55–75% $CD14^+$, and 24–36% $CD20^+$ cells. To triplicate wells was added either 0.1 ml of media alone (control) or 0.1 ml of media containing 10^5 of the cloned T cell line being analyzed for proliferation. The cultures were incubated at 37°C in a 7% CO_2–humidified atmosphere. At 48 h, 0.1 ml of the supernatant from each well was aspirated and assayed for levels of IL-2 by use of the IL-2–dependent HT-2 cell line as described elsewhere (15). Recombinant human IL-2 (courtesy of Roche Laboratories, Nutley, NJ) was used to derive a standard curve. The cultures were then fed fresh media and allowed to incubate for an additional 3 d, then pulsed with 1 μCi of methyl-[^{3}H]thymidine (2 Ci/mmol; New England Nuclear, Boston, MA) in 0.02 ml of media. Cultures were harvested 16–18 h later and the mean uptake of [^{3}H]TdR was determined. Supernatants from clones derived from patient 1 were also assayed for IL-4 and IFN-γ by use of EIA kits (Genzyme Corp., Boston, MA) and a bioassay.

Cryopreserved aliquots of each cloned T cell line were thawed and cultured in media containing 10 U/ml of recombinant human IL-2 for 3–7 d. The expanded cell population was then allowed to rest for 24–48 h in media without IL-2 and layered over a Ficoll-Hypaque gradient. The cells at the interface were washed with media and then assayed for antigen-specific proliferation. Cloned T cell lines showing specificity for E1, E2, E2L1, E2L2, or mixed specificity were subsequently maintained by coculture with the appropriate antigen pulsed normal matched APC at a ratio of 1:2. For reproducibility studies, such maintained cells were washed and layered onto Ficoll-Hypaque gradients. The cells at the interface were collected, washed twice in media, and then assayed for antigen-specific proliferation and IL-2 production. In cases in which a lack of concordance was noted between data obtained with the IL-2 readout and [^{3}H]TdR uptake, the clones were studied in further detail by use of APC pulsed with a broad range of antigen concentrations (from 0.001 to 25 μg/ml for PDC-E2 and from 0.001 to 10 μg/ml for PDC-E1, E2L1, and E2L2) in efforts to rule out differences, if any, that could be secondary to antigen dose. In addition, each clone was also tested for MHC-restricted responses by coculture of the cloned T cell line with non-MHC compatible APC pulsed with the same antigen at the same dose used with the MHC-compatible APC.

A total of 254 T cell clones were obtained from liver tissues from PBC patient 1, 196 clones from PBC patient 2, and 156 clones from the control patient with PSC.

Phenotypic Analysis. Antigen-specific T cell clones from both patients were phenotyped for CD4, CD8, TCR-α/β and CD45RO by standard flow microfluorometric techniques by use of FACS-Star Plus® (Becton Dickinson & Co., Mountain View, CA) and fluorescent conjugated mAbs. The Vβ usage of the antigen-specific T cell clones obtained from the liver tissues of patients 1 and 2 was analyzed by PCR. 10^7 cells of each clone were collected, snap-frozen, and stored at −70°C. TCR Vβ expression was determined by PCR (22). Each PCR reaction contained specific oligonucleotide primers to expand a particular Vβ gene segment (170–220 bp) as well as an α chain constant gene segment (600 bp) (23). The primer used to identify Vβ8 expression does not distinguish between Vβ8.1 and Vβ8.2, and the primer used to identify Vβ6 expression does not distinguish between Vβ6.1 and Vβ6.3. Amplified products were separated on 2% agarose gels and identified by use of ethidium bromide.

Results

PBMC Response. Of 19 PBC patients studied, PBMC from 16 gave a proliferative response ranging from two- to sixfold above background to one or more mitochondrial antigens (Table 1, Fig. 1). A positive response was designated as at least two SD above the mean of the media control for each patient. Of these 16 patients, PBMC from 10 produced a two- to sixfold response to the PDC-E2 subunit, which was optimal at 50 μg/ml (Fig. 1). PBMC from 3 of these 16 patients also responded to PDC-E1 (Fig. 2 *A*), and 4 demonstrated reactivity to both the PDC-E1 and PDC-E2 compo-

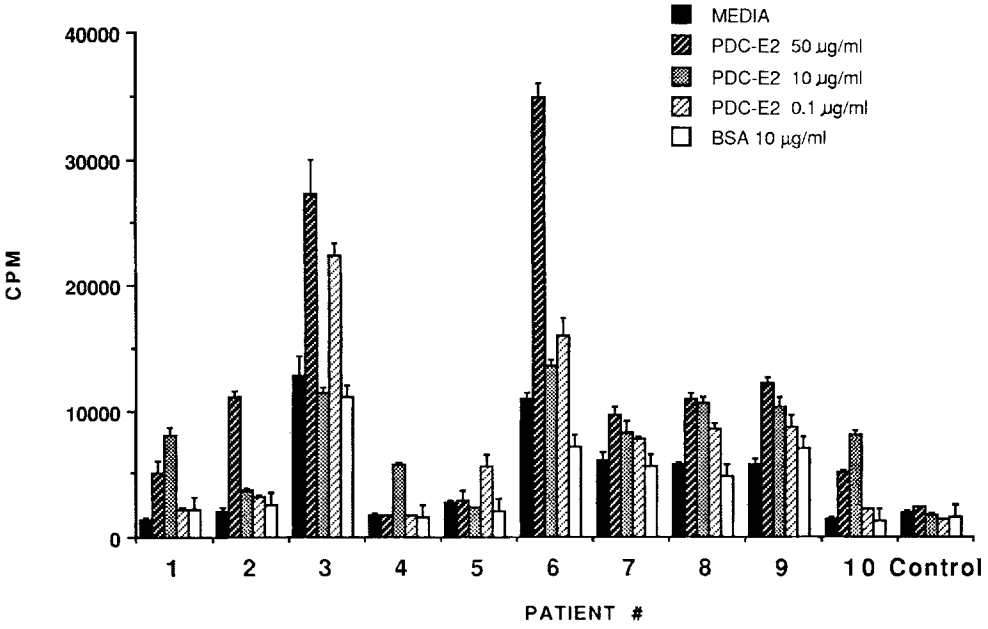

Figure 1. In vitro proliferative response (PBMC) from 10 patients with PBC and a representative control subject cultured with media, various concentrations of PDC-E2, and BSA. A positive response was at least two standard deviations above the mean of the media control. The data are expressed as mean cpm ± SEM.

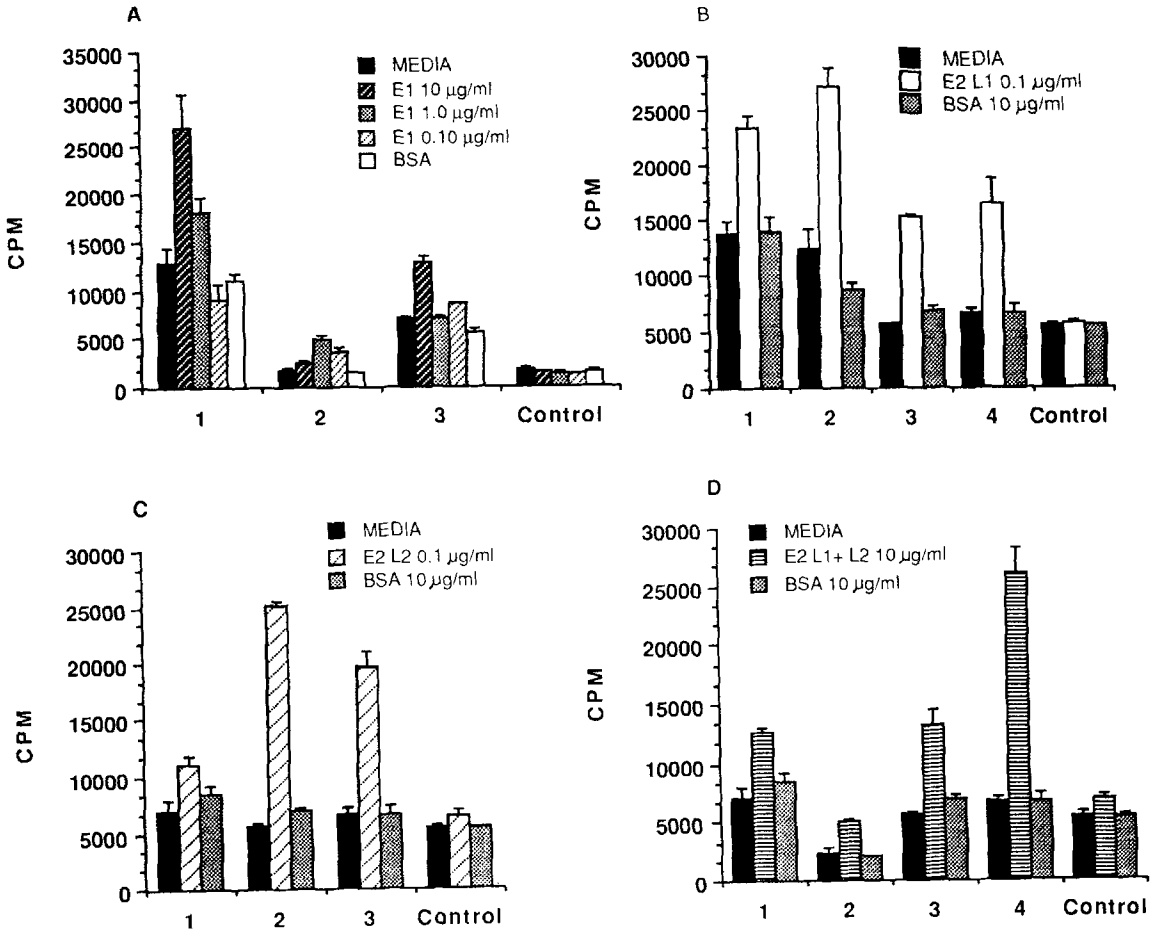

Figure 2. PBMC from patients with PBC were stimulated with either PDC-E1 (*A*), the PDC-E2 outer lipoyl domain (E2L1) (*B*), the inner lipoyl domain (E2L2) (*C*), or a combination of the two domains (E2L1+L2) (*D*). (*A*) Three representative patients and a negative control are shown. Maximum stimulation was seen most often with a PDC-E1 antigen concentration of 10 µg/ml. (*B*) Four representative patients who responded to a 0.1-µg/ml concentration of E2L1 are shown. Note the substantial response directed against the outer lipoyl domain of PDC-E2. (*C*) Three representative patients that responded to the inner lipoyl domain (E2L2) are illustrated. Note the particularly strong response of patient 2. (*D*) Four representative patients who responded to the E2L1+L2 domains at a concentration of 10 µg/ml are shown. Patient 4 produced a particularly strong response when both domains were tested. The numbers assigned to each of the patients depicted were for graphing purposes only and do not reflect the same patient in each graph. A positive response was designated as two SD above the mean of the media control and are expressed as mean cpm ± SEM. Representative negative control subjects were included in experiments and are shown in all graphs.

Table 1. *Proliferative Response of PBMC from Patients with PBC to PDC Antigens*

		Antigen					
Group	Positive/total*	PDC	PDC-E1	PDC-E2	E2L1 (aa1-98)	E2L2 (aa120-233)	E2L1 + L2 (aa1-233)
PBC	16/19	1/19	7/19§	10/19	6/11‖	4/11	4/11
	(84%)	(5%)	(47%)	(52%)	(54%)	(36%)	(36%)
Control‡	0/12	0/12	0/12	0/12	0/12	0/12	0/12
	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)

* Positive to one or more mitochondrial antigens.
‡ Controls include eight healthy and four PSC samples.
§ Three of the seven reactive patients reacted only to PDC-E1.
‖ 2 of 11 patients responded to both the outer and inner lipoyl domains.

nent (Table 1). Interestingly, PBMC from only 1 of 19 patients responded to the entire PDC complex (Table 1).

PBMC from 11 PBC patients and 12 control subjects were also tested for reactivity to the outer (E2L1) and inner (E2L2) lipoyl domains. Of these, 6/11 or 54% reacted to the outer domain region (Fig. 2 *B*) compared with 4/11 (36%) to the inner lipoyl domain (E2L2) (Fig. 2 *C*). PBMCs from 2 of 11 patients responded to both the E2L1 and E2L2 regions. PBMCs from 1 of 11 patients did not show a detectable response to any of the antigens tested. Surprisingly, when the construct containing both domains (E2L1 + L2, amino acids 1–233) was studied, only 4 of these same 11 patients showed a proliferative response (Fig. 2 *D*). Moreover, at comparable concentrations, one of these latter four patients responded only to E2L1 + L2 and full-length PDC-E2 antigens, and not to the individual E2L1 and E2L2 domains. Only 3 of 11 patients responded to the full-length PDC-E2 antigen and one or both of the E2L1 or E2L2 domains. The eight healthy and four PSC control subjects tested showed no significant response to any of the antigens used here.

Liver-derived Cloned T Cells. Of the 254 clones generated from PBC patient 1, 28 showed specificity for one or more of the mitochondrial antigens (Table 2). Of these 28 clones, 6/28 demonstrated specificity for PDC-E2, 7/28 showed specificity for PDC-E1, 8/28 for the E2L1 domain, and 11/28 for the E2L2 domain of PDC-E2 (Table 2). Data from representative clones are illustrated in Fig. 3, *A–D*. Only four clones from patient 1 showed specificity for more than one antigen; this includes two clones that showed specificity for both PDC-E1 and E2L2 and two clones positive for both PDC-E2 and E2L1. Similarly, 23 antigen-specific clones were found out of the 196 clones isolated from the liver of PBC patient 2 (Table 2). Of these 23 clones, 8 (35%) showed specificity for PDC-E2 and only 2/23 were positive for the E2L1 region. The majority of the clones, 19/23 or 83%, responded to the second lipoic acid domain, E2L2. Data from representative clones are illustrated in Fig. 4, *A–C*. Unlike patient 1, there were no PDC-E1–positive T cell clones derived from this liver. Interestingly, only one clone, 2-123, responded to PDC-E2, E2L1, and E2L2. This patient had four clones that responded to both PDC-E2 and E2L2. This is in contrast to clones from patient 1, in which none of the dual-positive clones responded to both of those antigens simultaneously. Overall, these responses were quite reproducible with respect to both proliferation and IL-2 production when data from three different assays were compared (Table 3). When tested with similar concentrations of the same antigen-pulsed MHC-incompatible APC, the cpm values were at background levels (data not shown). In addition, none of the clones showed proliferative responses or IL-2 production upon coculture with malic dehydrogenase–pulsed MHC-matched APC. Finally, none of the 156 PSC control liver clones showed any detectable response to the antigens tested.

Supernatant fluids derived from antigen-stimulated cultures of the T cell clones from both patients were assayed for IL-2 production. Clones from patient 1 were also tested for IL-4 and IFN-γ production. As seen in Table 4, only 4 of the 28 cloned T cells from patient 1 appeared to secrete IL-4. The rest of the cloned T cell lines from this patient secreted either IL-2, IFN-γ, or both. Based on the concept that IL-2/IFN-γ secretion is a property of Th1-like clones and IL-4 a property attributed to Th2-like clones, these data suggest a dominant Th1 profile for these T cell clones. Clones from patient 2 showed strong IL-2 production, especially when stimulated with PDC-E2 (Table 5).

Phenotypic analysis of the 28 liver-derived T cell clones from patient 1 revealed that 27/28 were $CD4^+$, with 1 CD4/CD8 dual-positive clone (Table 4). Out of 23 clones from patient 2, 21 were $CD4^+$ and 2 were $CD4/CD8^+$ (Table 5). 9 of 28 clones from patient 1 were $CD45RO^+$, while 3 were weakly $CD45RO^+$. For patient 2, 2 out of 23 clones were $CD45RO^+$. These data suggest the presence of at least 32% antigen-specific memory T cells in patient 1.

The TCR phenotype of all but one of the clones from patient 1 were $\alpha\beta$. Analysis of Vβ usage was performed, and the antigen-specific T cell clones showed a remarkably heterogeneous repertoire (Table 4). For example, among the 30 E2L2-reactive clones with identifiable Vβ expression from both patients, at least 14 different Vβs were involved in the response. However, there appeared to be a slight increase with respect to Vβ 3, 6.1, 13.1, and 13.2. Vβ8 and Vβ3 utilization was associated with all of the mitochondrial antigens tested.

The TCR phenotype of the clones from patient 2 were all $\alpha\beta$ (Table 5). Analysis of Vβ usage revealed that 3 of 8 clones reactive with PDC-E2 expressed Vβ8.1/8.2, 4 of 19

Table 2. *Autoantigen-specific T Cell Clones Isolated from the Liver of Patients with PBC*

Patient	Positive/total clones	PDC-E2	PDC-E1	PDC-E1 only	E2L1 (aa 1-98)	E2L2 (aa 120-233)	E2L1 + L2 (aa 1-233)
PBC 1	28/254	6/28 (21%)	7/28 (25%)	5/28 (18%)	8/28 (29%)	11/28 (39%)	0/28 (0%)
PBC 2	23/196	8/23 (35%)	0/23 (0%)	0/23 (0%)	2/23 (9%)	19/23 (83%)	1/23 (4%)
Control (PSC)	0/156	0/156 (0%)	0/156 (0%)	0/156 (0%)	0/156 (0%)	0/156 (0%)	0/156 (0%)

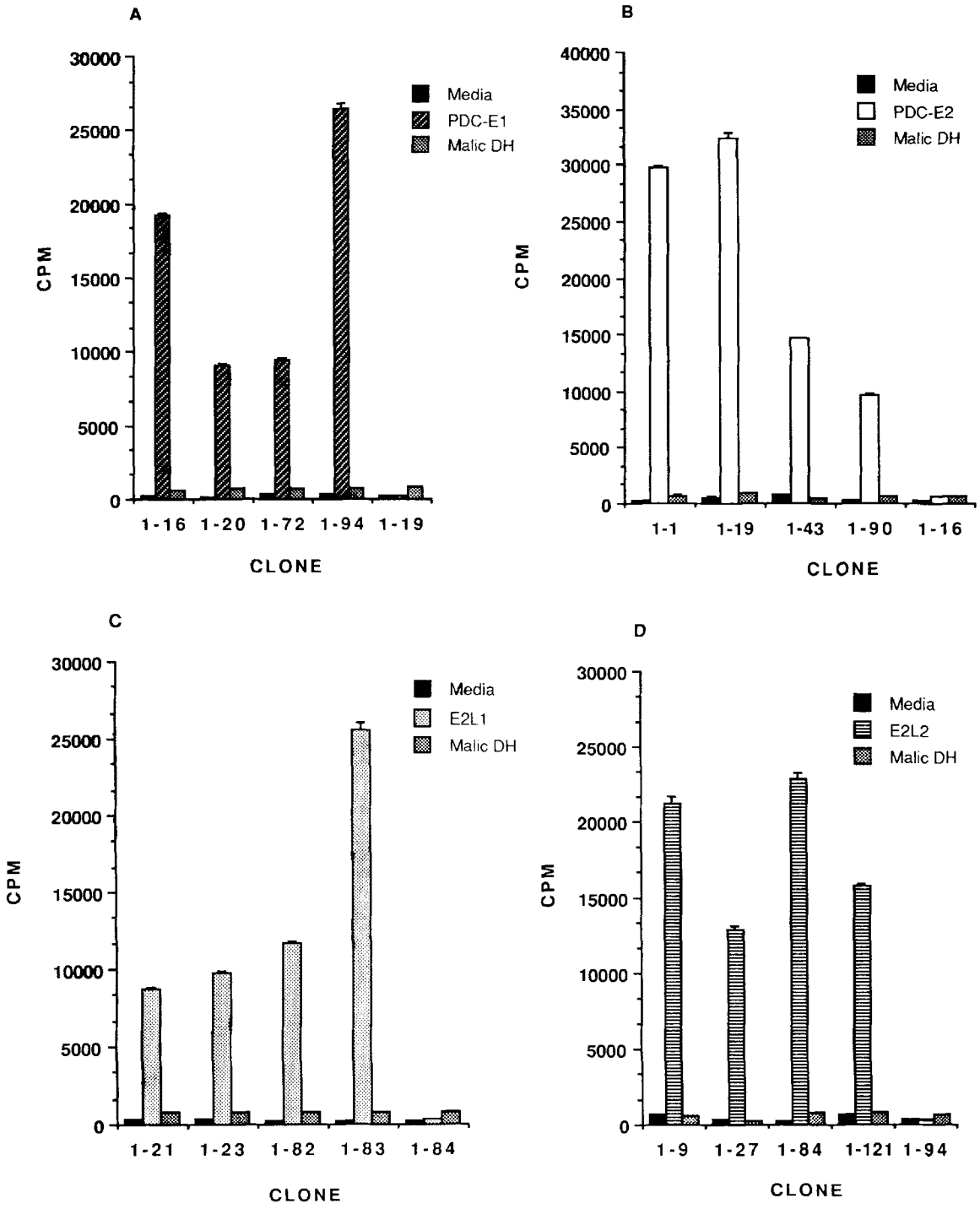

Figure 3. T cell clones derived from the liver of patient 1 were stimulated with various mitochondrial antigens and [^{3}H]thymidine uptake was measured. Four representative positive clones and one negative clone for PDC-E1 (*A*), PDC-E2 (*B*), E2L1 (*C*), and E2L2 (*D*) are illustrated. The data are presented as mean cpm ± SEM.

clones reactive with E2L2 expressed Vβ6.1 and 3 of 19 expressed Vβ3. Overall, the expression of these gene segments appeared to be increased compared with that expected in normal blood (for Vβ8 and Vβ6.1, 17% of the clones vs. 3–7% in blood; for Vβ3, 22% vs. 1–8% in blood).

Discussion

PBC is best classified as belonging to a group of spontaneous autoimmune diseases in which specific organ systems are targeted for destruction. Included in this group are myasthenia gravis, multiple sclerosis, autoimmune thyroiditis, and type 1 diabetes (23–26). As in primary PBC, there has

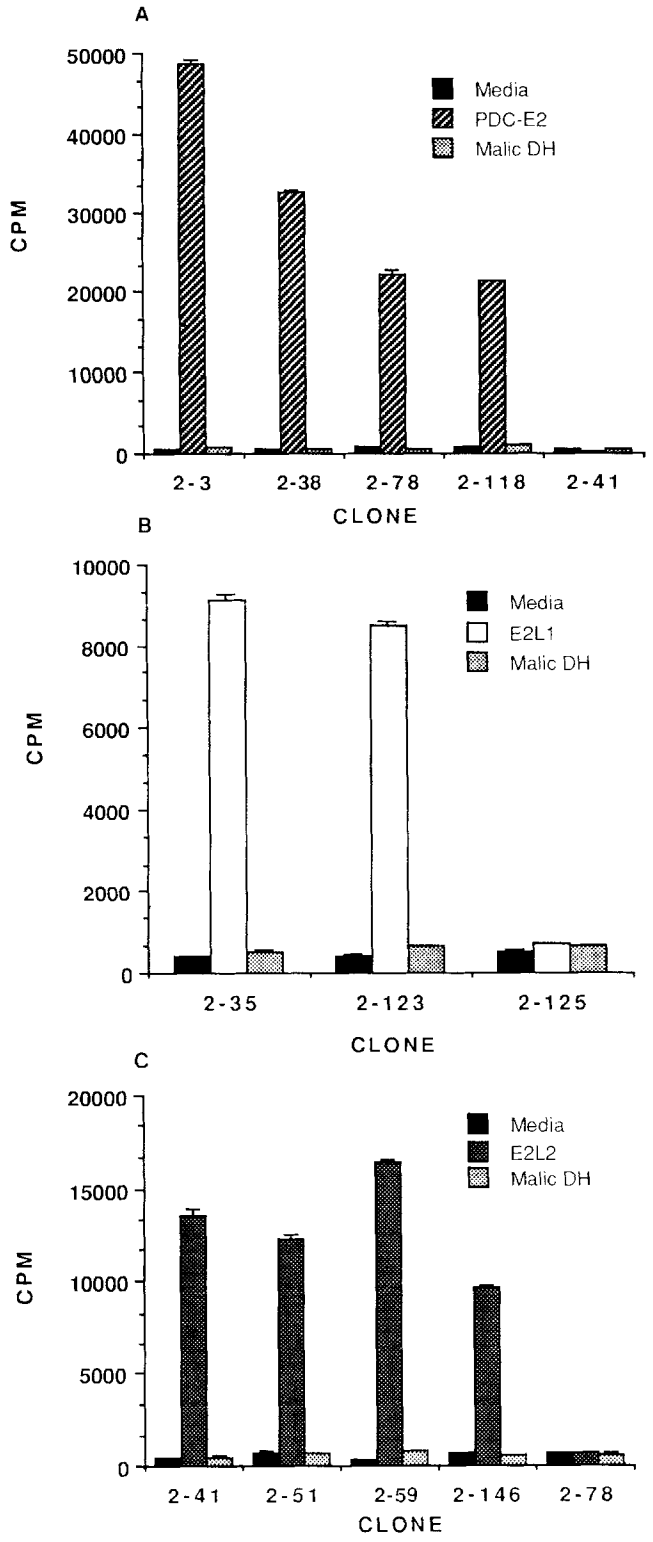

Figure 4. T cell clones derived from the liver of patient 2 were stimulated with various mitochondrial antigens and [^{3}H]thymidine uptake was measured. Representative positive clones and a negative clone for PDC-E2 (*A*), E2L1 (*B*), and E2L2 (*C*) are illustrated. As noted in the text, there were no liver-derived T cell clones from this patient that reacted to PDC-E1.

been difficulty in obtaining and analyzing samples from the target organ. By necessity, many investigators have chosen to work with peripheral blood T cells to define specificity and T cell receptor usage, even though the relevance of these cells to the organ-infiltrating T cells is uncertain (27, 28). Our study is therefore significant in that it defines the phenotype and specificity of T cells derived from damaged livers. In addition, the specificity of these cells matches that of a major antibody response that can be detected early in the disease process.

PDC-E2 contains two lipoic acid–binding regions (E2L1 and E2L2), which share a 63% sequence homology (7). In previous studies by our laboratory, patient sera were tested for antibody activity against these two lipoyl domains, and 80–90% of patients show activity to both domains. However, antibody titers to the inner domain are frequently 100-fold greater. Previous data suggested that the B cell epitopes for these two domains had partial cross-reactivity based on selective absorption data (29). In the current study, 54% of patients showed a peripheral T cell response to the outer lipoic acid–binding region, while only 36% responded to the inner lipoyl domain. Thus, the peripheral T cell response appears to be directed against two entirely separate regions of the PDC-E2 molecule. Surprisingly, only 4 of the 10 patients that responded to the E2L1 and E2L2 domains also reacted to the full length PDC-E2 molecule. This may indicate that an epitope that overlaps the E2L1 and E2L2 proteins is present within the full length PDC-E2 molecule but is not present in either of the smaller lipoyl domains. It is less likely that the data are secondary to antigenic differences between species (30), but it is possible that the processed forms of the entire PDC-E2 molecule may yield several different peptide fragments, some of which may have a higher affinity for the class II molecule and thereby not allow efficient presentation of the autoantigenic peptide. Indeed, biliary epithelial cells may also have a different composition of proteases than monocytes or EBV cells. Finally, this phenomenon could also be explained by a failure to efficiently process the large PDC complex.

The peripheral T cell PDC-E1 response was also examined in this study. Autoantibody reactivity to PDC-E1, in both this and previous studies (31), is found in about half of patients with PBC. In contrast, nearly all patients have activity for PDC-E2. Interestingly, if one compares the T cell response in the PBMC and T cell clones of patient 1 to PDC-E1 and PDC-E2 (52%), they are nearly equal. This is in marked contrast to the antibody profile. It is recognized that T cells recognize epitopes distinct from B cells, as highlighted by hapten-carrier systems. Thus, epitopes of PDC-E1 may induce T cell activation, which may secrete cytokines that induce B cell–specific responses to other moieties of the PDC. Another possibility is that the PDC-E2–specific T cells home preferentially to the liver and are less abundant in the periphery.

The difference in T cell specificity between patients 1 and 2 was also remarkable. For example, 25% of the cloned T cell lines from patient 1 showed specificity for PDC-E1, while none of the T cell clones from patient 2 showed similar specificity. In addition, 83% of the cloned T cell lines from

Table 3. *Reproducibility of Antigen-specific Proliferation and Lymphokine Responses by Representative T Cell Clones from PBC Patients 1 and 2*

Clone	Antigen specificity	cpm (1)*	IL-2(1) U/ml	cpm (2)	IL-2 (2)	cpm (3)	IL-2 (3)
1-11	E2L2	7,647 ± 76	21.0	5,988 ± 24	7.7	8,667 ± 104	8.7
1-72	PDC-E1	9,422 ± 137	15.6	7,642 ± 101	9.6	9,824 ± 134	8.9
1-90	PDC-E2	9,647 ± 142	39.9	8,456 ± 68	18.6	8,125 ± 71	26.3
1-105	E2L2	6,006 ± 108	5.9	9,924 ± 100	4.8	9,376 ± 76	5.7
1-122	E2L1	8,145 ± 145	28.7	4,738 ± 85	6.9	5,145 ± 48	6.9
2-34	E2L2	8,014 ± 137	18.2	4,393 ± 22	12.6	9,014 ± 147	14.6
2-35	E2L1	5,055 ± 70	22.9	9,122 ± 139	14.5	6,926 ± 48	9.2
2-177	PDC-E2	18,374 ± 100	11.2	22,667 ± 30	15.4	8,456 ± 100	5.9

* Values are expressed as mean ± SEM.

Table 4. *Response and Phenotype of Antigen-specific T Cell Clones from PBC Patient 1*

Clone	Antigen specificity	S.I.	Lymphokine	CD	TCR	Vβ	CD45RO
1	PDC-E2	38	IL-2*, IFN	CD4	αβ	1	Positive
3	E2L1	19	IL-2, IFN	CD4	αβ	5.1	Weak
9	E2L2	29	IL-2	CD4	αβ	13.1	Negative
11	E2L2	16	IL-2	CD4	?	NEG‡	Negative
16	PDC-E1	80	IL-2, IFN	CD4	αβ	8.1, 20§	Positive
19	PDC-E2, E2L1	60, 34‖	IL-2	CD4	αβ	2	Weak
20	PDC-E1, E2L2	15, 57	IL-2	CD4	αβ	2	Weak
21	E2L1	12	IL-2, IFN	CD4	αβ	7	Positive
23	E2L1	27	IL-2, IL-4	CD4	?	3	Negative
27	E2L2	17	IL-2, IFN	CD4	?	1	Negative
43	PDC-E2	19	IL-2, IFN	CD4	αβ	4	Negative
47	E2L2	18	IL-2, IFN	CD4	αβ	13.1	Negative
72	PDC-E1	29	IL-2	CD4	αβ	NEG‡	Negative
75	PDC-E2	2	IL-2, IFN	CD4	αβ	2	Negative
82	E2L1	59	IL-2	CD4/CD8	αβ	NEG‡	Positive
83	E2L1	95	IL-2	CD4	αβ	5.1	Positive
84	E2L2	23	IL-2, IFN	CD4	αβ	5.1, 13.2	Positive
90	PDC-E2	14	IL-2, IFN	CD4	αβ	14	Negative
94	PDC-E1	77	IL-2, IL-4	CD4	αβ	3	Positive
105	E2L2	21	IL-2, IFN	CD4	αβ	6.1, 13.2	Negative
121	E2L2	23	IL-2	CD4	αβ	3, 10	Positive
122	PDC-E2, E2L1	11	IL-2	CD4	αβ	13.1, 13.2	Positive
123	E2L2	27	IL-2	CD4	αβ	7	Negative
152	PDC-E1	15	IL-2	CD4	αβ	2	Negative
161	PDC-E1	22	IL-2	CD4	αβ	NEG‡	Negative
170	E2L2	17	IL-4	CD4	αβ	8.1	Negative
219	E2L1	28	IL-4	CD4	αβ	8.1	Negative
230	PDC-E1, E2L2	16, 38	IL-2	CD4	αβ	ND	Negative

* The range of IL-2 U/ml values was 1.9–53.9.
‡ The Vβ expressed was not identified with the panel of Vβ primers used in this analysis.
§ Although designated Vβ8.1, the primer used does not distinguish between 8.1 and 8.2. Similarly, the primer used to identify Vβ6 members will amplify Vβ6.1, 6.2, and 6.3 gene segments.
‖ Where more than one value is given, they correspond to the order of the antigens.
S.I., stimulation index.

Table 5. *Response and Phenotype of Antigen-specific Liver T Cell Clones from PBC Patient 2*

Clone	Antigen specificity	S.I.	Lymphokine	CD	TCR	Vβ	CD45RO
3	PDC-E2	78	IL-2*	CD4	αβ	8.1‡	Negative
12	E2L2	2	IL-2	CD4	αβ	14	Negative
25	E2L2	6	IL-2	CD4	αβ	15	Negative
34	E2L2	12	IL-2	CD4	αβ	13.1,13.2	Negative
35	E2L1	9	IL-2	CD4	αβ	3	Negative
38	PDC-E2	73	IL-2	CD4	αβ	8.1	Negative
41	E2L2	27	IL-2	CD4	αβ	3	Negative
43	E2L2	10	IL-2	CD4	αβ	3	Negative
51	E2L2	16	IL-2	CD4	αβ	13.1	Negative
59	E2L2	57	IL-2	CD4	αβ	12	Negative
63	E2L2	13	IL-2	CD4	αβ	2	Negative
78	PDC-E2	27	IL-2	CD4	αβ	3,8.1,11	Negative
85	PDC-E2, E2L2	57, 9§	IL-2	CD4	αβ	15	Negative
91	E2L2	20	IL-2	CD4/CD8	αβ	6.1	Negative
108	E2L2	14	IL-2	CD4/CD8	αβ	ND	Negative
118	PDC-E2, E2L2	26, 9	IL-2	CD4	αβ	6.1	Negative
123	PDC-E2, E2L1, E2L2	43, 20, 22	IL-2	?	αβ	20	Negative
125	E2L2	23	IL-2	CD4	αβ	13.2	Positive
139	E2L2	1.4	IL-2	?	αβ	8.1	Negative
146	E2L2	19	IL-2	CD4	αβ	5.1	Positive
156	E2L2	3	IL-2	CD4	αβ	6.1	Negative
177	PDC-E2, E2L2	28	IL-2	CD4	αβ	6.1	Negative
179	PDC-E2, E2L2	24, 9	IL-2	CD4	αβ	3	Negative

* The range of IL-2 U/ml was 5.4-53.4.
‡ Although designated Vβ8.1, the primer used does not distinguish between 8.1 and 8.2. Similarly, the primer used to identify Vβ6 members will amplify Vβ6.1, 6.2 and 6.3 gene segments.
§ Where more than one value is given, they correspond to the order of the antigens.

patient 2 responded to E2L2, in contrast to only 39% for patient 1. These differences could be attributed to chance during cloning or could be due to a difference in the stage of the liver at the time of transplant. A final possibility could be MHC differences and their ability to recognize and present the various antigens. These data again raise the issue of whether there is preferential homing of the E2L2-reactive T cells to the liver and whether the epitope recognized by these E2L2-specific T cells may cross-react with a non-PDC antigen, as has been suggested by previous histologic studies (32). Also of great interest were the number of clones that responded to more than one antigen. The combination of antigens was also somewhat unexpected. For example, patient 1 had two clones, one of which had a single Vβ, that responded to both PDC-E1 and E2L2. Perhaps there is an epitope that is common to both antigens, or, less likely given the single Vβ usage, they were not homogenous clones. Patient 1 also had two dual PDC-E2-E2L1–reactive clones. Again, there was single Vβ usage in both of these pairs. Although it is not as surprising to find shared reactivity to these two antigens, it is interesting that there is seldom reactivity to all three antigens derived from PDC-E2 by a single clone (1 out of 51 clones tested). These studies suggest that there is in fact more than one T cell epitope on the PDC-E2 antigen and that some of these may have shared epitopes, while others are unique to the two lipoyl regions. Moreover, there is a gap of 22 amino acids between E2L1 and E2L2 that is unique to the entire PDC-E2 complex which may also contain an additional epitope. Further studies with synthetic peptides may clarify this issue.

The T cell receptor Vβ repertoire utilized by PDC-reactive clones infiltrating the liver was remarkable in its heterogeneity. This heterogeneity was especially apparent within subsets that responded to particular domains. For example, in both patients, E2L2-reactive T cell clones utilized multiple different Vβs, and only a few examples of repetitive Vβ usage were noted. This contrasts greatly with studies of peripheral blood–cloned T cell lines in multiple sclerosis (23), but is consistent with other studies (27, 33). It also is distinct from autoimmune T cell receptor repertoires defined

in mice immunized with autoantigens (34). A few Vβs were also noted to be possibly overutilized, but the relatively small number of clones used to estimate total repertoire limits any definite conclusions. Since the patients studied were advanced in their disease process, additional epitopes may have elicited responses related to the tissue damage analogous to determinant spreading. Further observations involving patients at different stages of disease will help to clarify these interesting issues.

The authors appreciate the excellent technical help of Helen Smith and the secretarial support of Theresa Andreozzi.

This work was supported by National Institutes of Health grants DK-39588 and AR-37070 and Liver Tissue Procurement and Distribution System grant 1-DK-6-2274.

Address correspondence to Dr. M. Eric Gershwin, Division of Rheumatology, Allergy and Clinical Immunology, TB-192, School of Medicine, University of California Davis, Davis, CA 95616.

Received for publication 25 February 1994 and in revised form 11 October 1994.

References

1. Kaplan, M.M. 1987. Primary biliary cirrhosis. *N. Engl. J. Med.* 316:521–528.
2. Ahrens, E.H., M.A. Payne, and H.G. Kunkel. 1950. Primary biliary cirrhosis. *Medicine.* 29:299–304.
3. Walker, J.G., D. Doniach, I.M. Roitt, and S. Sherlock. 1965. Serological tests in the diagnosis of primary biliary cirrhosis. *Lancet.* 1:827–830.
4. 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.
5. Fussey, S.P.M., J.R. Guest, O.F.W. James, M.F. Bassendine, and S.J. Yeaman. 1988. Identification and analysis of the major M2 autoantigens in primary biliary cirrhosis. *Proc. Natl. Acad. Sci. USA.* 85:8654–8658.
6. Coppel, R.L., L.J. McNeilage, C.D. Surh, J. Van de Water, T.W. Spithill, S. Thittingham, 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.
7. 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.
8. Nakanuma, Y., and N. Kono. 1991. Expression of HLA-DR antigens on interlobular bile ducts in primary biliary cirrhosis and other hepatobiliary diseases: an immunohistochemical study. *Hum. Pathol.* 22:431–436.
9. Ballardini, G., F.B. Bianelli, D. Doniach, R. Mirakiam, E. Pisi, and G.F. Botazzo. 1984. Aberrant expression of HLA-DR antigens on bile duct epithelium in primary biliary cirrhosis, relevance to pathologenesis. *Lancet.* 2:1009–1013.
10. Krams, S.M., J. Van de Water, C. Esquivel, J. Roberts, A. Ansari, and M.E. Gershwin. 1990. Analysis of hepatic T lymphocyte and immunoglobulin deposits in patients with primary biliary cirrhosis. *Hepatology.* 12:306–313.
11. Lohr, H., B. Fleischer, G. Gerken, S.J. Yeaman, K.-H. Meyer zum Buschenfeld, and M. Manns. 1993. Autoreactive liver-infiltrating T cells in primary biliary cirrhosis recognize inner mitochondrial epitopes and the pyruvate dehydrogenase complex. *J. Hepatol.* 18:322–327.
12. Moebius, U., M. Manns, G. Hess, G. Kober, K.-H. Meyer zum Buschenfeld, and S.C. Meuer. 1990. T cell receptor gene rearrangements of T lymphocytes infiltrating the liver in chronic active hepatitis B and primary biliary cirrhosis (PBC): oligoclonality of PBC-derived T cell clones. *Eur. J. Immunol.* 20: 889–896.
13. Si, L., T.L. Whiteside, R.R. Schade, T.E. Starzl, and D.H. van Thiel. 1984. T-lymphocyte subsets in liver tissues of patients with primary biliary cirrhosis (PBC), patients with primary sclerosing cholangitis (PSC) and normal controls. *J. Clin. Immunol.* 4:262–272.
14. Meuer, S.C., U. Moebius, M.M. Manns, H.P. Dienes, G. Ramadori, G. Hess, T. Hercent, and K.-H. Meyer zum Buschenfeld. 1988. Clonal analysis of human T lymphocytes infiltrating the liver in chronic active hepatitis B and primary biliary cirrhosis. *Eur. J. Immunol.* 18:1447–1452.
15. Van de Water, J., A.A. Ansari, C.D. Surh, R. Coppel, T. Roche, H. Bonkovsky, M. Kaplan, and M.E. Gershwin. 1991. Evidence for the targeting by 2-oxo-dehydrogenase enzymes in the T cell response of primary biliary cirrhosis. *J. Immunol.* 146:89–94.
16. MacSween, R.N.M., and E. Sumithran. 1989. Histopathology of primary biliary cirrhosis. *Semin. Liver Dis.* 1:282–285.
17. Rahmatullah, M., S. Gopalakrishnan, P.C. Andrews, C.L. Chang, G.A. Radke, and T.E. Roche. 1989. Subunit associations in the mammalian pyruvate dehydrogenase complex. Structure and role of protein X and the pyruvate dehydrogenase component binding domain of the dihydrolipoyl transacetylase component. *J. Biol. Chem.* 264:2221–2228.
18. Roche, T.E., M. Rahmatullah, S. Liu, G.A. Radke, C.L. Chang, and S.L. Powers-Greenwood. 1989. Lipoyl-containing components of the pyruvate dehydrogenase complex: roles in modulating and anchoring the PDH kinase and the PDH phosphatase. *Ann. NY Acad. Sci.* 573:168–173.
19. Yssel, H., D. Blanchard, A. Boylston, J.E. DeVries, and H.

Spits. 1986. T cell clones which share T cell receptor epitopes differ in phenotype, function and specificity. *Eur. J. Immunol.* 16:1187–1193.
20. Yssel, H., T. Nakamoto, P. Schneider, V. Freitas, C. Collins, D. Webb, N. Mensi, C. Soderberg, and G. Peltz. 1990. Analysis of T lymphocytes cloned from the synovial fluid and blood of a patient with lyme arthritis. *Int. Immunol.* 2:1081–1091.
21. Sly, W.S., G.S. Sekhon, R. Kennet, W.F. Bodner, and J. Bodmen. 1976. Permanent lymphoid lines from genetically marked lymphocytes: success with lymphocytes recovered from frozen storage. *Tissue Antigens.* 7:165–172.
22. Choi, Y.W., B. Kotzin, L. Herron, J. Callahan, P. Marrack, and J. Kappler. 1989. Interaction of Staphylococcus aureus toxin "superantigens" with human T cells. *Proc. Natl. Acad. Sci. USA.* 86(22):8941–8945.
23. Kotzin, B.L., S. Karuturi, Y.K. Chou, J. Lafferty, J.M. Forrester, M. Better, G.E. Nedwin, H. Offner, and A.A. Vandenbark. 1991. Preferential T-cell receptor β-chain variable gene use in myelin basic protein-reactive T cell clones from patients with multiple sclerosis. *Proc. Acad. Sci. USA.* 88:9161–9165.
24. Dayan, C.M., M. Londei, A.E. Corcoran, B. Grubeck-Loebenstein, R.F. James, B. Rapoport, and M. Feldmann. 1991. Autoantigen recognition by thyroid-infiltrating T cells in Graves disease. *Proc. Natl. Acad. Sci. USA.* 88:7415–7419.
25. Sommer, N., G.C. Harcourt, N. Willcox, D. Beeson, and J. Newsom-Davis. 1991. Acetylcholine receptor-reactive T lymphocytes from healthy subjects and myasthenia gravis patients. *Neurology.* 41:1270–1276.
26. Anderson, J.T., J.G. Cornelius, A.J. Jarpe, W.E. Winter, and A.B. Peck. 1993. Insulin-dependent diabetes in the NOD mouse model. II. Beta cell destruction in autoimmune diabetes is a TH2 and not a TH1 mediated event. *Autoimmunity.* 15:113–122.
27. Meinl, E., F. Weber, K. Drexler, C. Movelle, M. Ott, G. Saruhan-Direskeneli, N. Goebels, B. Erte, G. Jechart, G. Giegerich, et al. 1993. Myelin basic protein-specific T lymphocyte repertoire in multiple sclerosis. Complexity of the response and dominance of nested epitopes due to recruitment of multiple T cell clones. *J. Clin. Invest.* 92:2633–2643.
28. Chou, Y.K., P. Henderikx, M. Vainiene, R. Whitham, D. Bourdette, C.H. Chou, G. Hashim, H. Offner, and A.A. Vandenback. 1991. Specificity of human T cell clones reactive to immunodominant epitope of myelin basic proteins. *J. Neurosci. Res.* 28:280–290.
29. 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. *J. Immunol.* 144:3367–3374.
30. Palmer, M., M.F. Bassendine, O.F.W. James, and S.J. Yeaman. 1993. Human pyruvate dehydrogenase complex as an autoantigen in primary biliary cirrhosis. *Clin. Sci.* 85:289–293.
31. Gershwin, M.E., M. Rowley, P.A. Davis, P. Leung, R. Coppel, and I.R. Mackay. 1992. Molecular biology of the 2-oxo-acid dehydrogenase complexes and anti-mitochondrial antibodies. *Prog. Liv. Dis.* 60:47–61.
32. Van de Water, J., J. Turchany, P.S.C. Leung, J. Lake, S. Munoz, C.D. Surh, R. Coppel, A. Ansari, Y. Nakanuma, and M.E. Gershwin. 1993. Molecular mimicry in primary biliary cirrhosis. Evidence for biliary epithelial expression of a molecule cross-reactive with pyruvate dehydrogenase complex-E2. *J. Clin. Invest.* 91:2653–2664.
33. Wilson, K.B., A.J. Quayle, S. Suleyman, J. Kjeldsen-Kragh, O. Forre, J.B. Natvig, and J.D. Capara. 1993. Heterogeneity of the TCR repertoire in synovial fluid T lymphocytes responding to BCG in a patient with early rheumatoid arthritis. *Scand. J. Immunol.* 38:102–112.
34. Acha-Orbea, H., D.J. Mitchell, L. Timmerman, D.C. Wraith, G.S. Tausch, M.K. Waldor, S.S. Zamvil, H.O. McDevitt, and L. Steinman. Limited heterogeneity of T cell receptors from lymphocytes mediating autoimmune encephalomyelitis allows specific immune intervention. *Cell.* 54:263–273.