Identification and Precursor Frequency Analysis of a Common T Cell Epitope Motif in Mitochondrial Autoantigens in Primary Biliary Cirrhosis

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Abstract

The immunodominant antimitochondrial antibody response in patients with primary biliary cirrhosis (PBC) is directed against the E2 component of the pyruvate dehydrogenase complex (PDC-E2). Based on our earlier observations regarding peripheral blood mononuclear cell (PBMC) T cell epitopes, we reasoned that a comparative analysis of the precursor frequencies of PDC-E2 163-176–specific T cells isolated from PBMC, regional hepatic lymph nodes, and from the liver of PBC patients would provide insight regarding the role of T cells in PBC. Results showed a disease-specific 100–150-fold increase in the precursor frequency of PDC-E2 163-176–specific T cells in the hilar lymph nodes and liver when compared with PBMC from PBC patients. Interestingly, autoreactive T cells and autoantibodies from PBC patients both recognize the same dominant epitope. In addition, we demonstrated cross-reactivity of PDC-E2 peptide 163-176–specific T cell clones with PDC-E2 peptide 36-49 and OGDC-E2 peptide 100-113 thereby identifying a common T cell epitope "motif" ExETDK. The peptide 163-176–specific T cell clones also reacted with purified native PDC-E2, suggesting that this epitope is not a cryptic determinant. These data provide evidence for a major role for PDC-E2 peptide 163-176 and/or peptides bearing a similar motif in the pathogenesis of PBC. (*J. Clin. Invest.* 1998. 102: 1831–1840.) Key words: autoreactive T cells • primary biliary cirrhosis • pyruvate dehydrogenase • epitopes • liver

Introduction

Primary biliary cirrhosis (PBC)[1] is an autoimmune chronic cholestatic liver disease characterized by the presence of antimitochondrial antibodies, inflammation, and destruction of interlobular bile ducts in the liver (1). The major mitochondrial antigens recognized by antimitochondrial antibodies have been identified as the E2 components of 2-oxo acid dehydrogenase complexes (2–10). The extraordinary specificity of bile duct destruction in PBC, the presence of lymphoid infiltrates, including B cells that produce anti–pyruvate dehydrogenase (PDC)–E2 antibodies in the portal tracts, the aberrant expression of MHC class II antigens, and cell adhesion molecules on biliary epithelium all suggest that biliary epithelial cells are the target of an intense autoimmune response. Much of the immune response in the disease have been shown to be directed against PDC-E2, but mounting evidence suggests that the molecule expressed on biliary epithelium may in fact be a molecular mimic, as it lacks all the immunological features of authentic PDC-E2 (11–13).

Initially, our studies were focused on defining the epitope of human PDC-E2 that was the target of the specific humoral response in PBC (14, 15). These studies were followed by attempts to define T cell epitopes for PDC-E2. Two different approaches were used. One involved the use of cloned and expressed recombinant truncated fragments of PDC-E2 (14), and the other involved use of a series of synthetic overlapping peptides covering the entire sequence of PDC-E2 (15). These two different studies were used based on the reasoning that in the former case antigen-presenting cells (APCs) will process and present appropriate peptides, whereas in the latter case, peptides were prepared that would not require processing, which would allow for the rapid identification of the relevant peptide. Results of the former study provided evidence for the presence of epitopes localized to both the PDC-E2 inner and outer lipoyl domain fragments (14). Use of synthetic peptides, on the other hand, led to the identification of an inner lipoyl domain–encoding peptide 163-176 (GDLLAEIETDKATI) restricted to HLA DRB4 0101, a haplotype that is present in 80% of the population (15).

T cell clones prepared from the PBMC of PBC patients with specificity for peptide 163-176 also appeared to cross-react with a peptide 36-49 (GDLIAEVETDKATV) encoded by the outer lipoyl domain of PDC-E2. The finding of this cross-reactivity is consistent with results obtained using truncated recombinant inner/outer lipoyl domains of PDC-E2 in which both these domains contain the sequences with sufficient homology to induce proliferation leading to the identification of cross-reactive epitopes. These initial findings identified ExDK as a motif that is required to bind HLA DRB4 0101 or to be

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1. *Abbreviations used in this paper:* a.a., amino acid; APC, antigen-presenting cells; E2L1, inner lipoyl domain of PDC-E2; MBP, myelin basic protein; MS, multiple sclerosis; OGDC, oxo-glutarate acid dehydrogenase; OVA, ovalbumin; PBC, primary biliary cirrhosis; PDC, pyruvate dehydrogenase; PSC, primary sclerosing cholangitis; RLN, regional lymph node; SI, stimulation index.

recognized by T cells in PBC. This led us to carry out further studies on the specificity and cross-reactivity of a large number of T cell clones with specificity for peptide 163-176 restricted to HLA DRB4 0101. In addition, it was reasoned that the reactivity of each of these T cell clones also needs to be examined using autologous APC pulsed with the native purified molecules. This would ensure that such reactivity was not due to our accidental identification of an as yet undefined and cryptic epitope but was due to the natural processing and presentation of the appropriate peptides by autologous APC. Finally, it was reasoned that the definition of the precursor frequency of T cells specific for the defined autoantigenic peptide in both PBMC and those derived from the liver or regional lymph nodes of PBC patients may provide important information as to the relevance of such T cells in the pathogenesis of this disease.

Methods

Clinical specimens. Blood was collected from 13 female subjects, ages 38–67 yr, with PBC. PBMCs from each of the 13 patients were subjected to HLA class II molecular typing and were all found to express HLA DRB4 0101. Each of these patients were classified histologically as having stage II through stage IV disease (stage II, three patients; stage III, four patients; stage IV, six patients). In addition, PBMCs from six control subjects who were HLA DRB4 0101 were studied in parallel. These six controls included three healthy female volunteers and three female patients with primary sclerosing cholangitis (PSC). Finally, explanted liver tissue was obtained at transplantation from six female patients with PBC; of these six transplanted PBC patients, PBMC was available on six out of six and regional lymph node (RLN) on four out of six patients. Explanted liver was also studied in three patients with PSC. All transplanted patients were HLA DRB4 0101.

Antigen preparation. Native PDC-E2 used for in vitro stimulation was purified from beef heart mitochondria (16, 17). The two lipoic acid–binding regions of human PDC-E2 were prepared from recombinant human PDC-E2 (pHumPDC-E2-2A) (4, 18). In brief, all domains were derived by PCR using purified pHumPDC-E2-2A as a template with Eco RI cloning sites at both ends. The resulting constructs were cloned into the pGEX-2T system and treated with thrombin to cleave the rPDC fragments 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 (a.a.) 1–98 (E2L1; containing the outer lipoyl domain), residues 120–233 (E2L2; containing the inner lipoyl domain), and residues 1–233 (E2L1+L2). All fragments were then purified after thrombin treatment. In addition, 42 different peptides each composed of 14–20 amino acid residues corresponding to the amino acid sequence of human PDC-E2 were synthesized by F-moc chemistry (15). The peptides were designed according to analysis of amphipathic regions of the antigenic molecules and the presence of consensus motifs (19). Synthetic peptides (Sps) were indicated by the amino acid number from the NH_2 terminus of PDC-E2 and used as a cocktail mixture for the studies presented herein. Each mixture was referred to individually as group 1–9. Group 1 contained Sps 15-34, 23-42, 38-56, 69-88, 80-99, and 87-106; group 2, Sps 107-126, 137-156, 144-163, 163-176, 168-187, and 182-201; group 3, Sps 198-217, 208-227, 216-230, 235-254, and 255-274; group 4, Sps 261-280, 269-288, 289-306, and 302-321; group 5, Sps 310-329, 320-339, 332-351, and 339-358; group 6, Sps 341-360, 353-372, 355-374, and 363-382; group 7, Sps 373-392, 392-411, 396-413, and 406-425; group 8, Sps 442-461, 449-466, 468-487, and 483-500; and group 9, Sps 495-514, 503-522, 521-540, 536-553, and 543-560. For the cross-reactivity experiments, the a.a. sequence of PDC-E2 and another immunodominant autoantigen OGDC-E2 were screened for sequences with shared homology to the PDC-E2 peptide 163-176. Two such peptides were identified and include human PDC-E2 peptide 36-49 (GDLIAEVETDKATV) and the human OGDC-E2 residues 100-113 (DEVVCEIETDKTSV). These peptides were synthesized and used to determine the potential cross-reactivity and/or specificity of the 163-176 peptide–specific T cell response in a T cell proliferation assay. The recombinant fusion protein fragments of rat OGDC-E2 was prepared from cDNA clones expressed in *Escherichia coli* as described previously.

Preparation of cells. PBMCs were separated from heparinized blood by gradient centrifugation on Ficoll-Isopaque. Liver lymphocytes or RLN lymphocytes were prepared as described earlier (14). Mononuclear cells were cryopreserved at −196°C in FCS with 10% DMSO until use. Mononuclear cells were thawed at 37°C, washed three times, and after the last wash, cells were counted and checked for viability by using trypan blue dye exclusion (viability > 80%). 1×10^6 mononuclear cells from liver, RLN, or peripheral blood were seeded in media (X-Vivo 15; Bio Whittaker, Walkersville, MD) containing 4% T-Stim (Collaborative Research, Inc., Bedford, MA) with 1×10^6 irradiated (8,000 rad) autologous EBV-transformed B cells in individual wells of a 24-well plate for 7 d before analysis by a T cell proliferation assay or by limiting dilution analysis.

T cell proliferation assay. A total of 5×10^4 irradiated (8,000 rad) autologous EBV-transformed B cells from the appropriate PBC or control patient were dispensed into individual wells of a series of nine 96-well round-bottom plates. One plate (96 wells) each was pulsed with 10 μg/mL of a single mixture of peptides (group 1–9), and the nine plates were incubated overnight at 37°C 7% humidified CO_2 atmosphere. After overnight incubation, appropriate autologous mononuclear cells were added to each well (5×10^3/well) of each of the nine 96-well microculture plates. Media consisted of X-Vivo 15 containing 4% T-Stim (Becton Dickinson, Franklin Lakes, NJ). On day 10, the wells were restimulated with similarly prepared homologous peptide mixture–pulsed 5×10^4 irradiated (8,000 rad) autologous EBV-transformed B cells with 4% T-Stim. On day 20, the cells in each well were split into five aliquots, and two wells were cultured with 5×10^4 irradiated (8,000 rad) autologous EBV-transformed B cells pulsed with the same concentration of peptide mixture; two control wells received unpulsed autologous EBV-transformed B cells. Cells from the last well were reserved to isolate peptide-specific T cell lines. Cells were cultured for 72 h and then pulsed with 1.0 μCi of [^{3}H]thymidine per well during the last 12 h of culture and subsequently harvested and counted in a scintillation counter (Betaplate; Wallac, Inc., Gaithersburg, MD). Wells were scored positive if the mean cpm (mean cpm values of cells with antigen and mean cpm values of cells without antigen) was > 1,000 cpm and the stimulation index (SI) was at least threefold. SI equals mean value of [^{3}H]thymidine incorporation (cpm) in the wells containing peptide or other antigen/mean value of [^{3}H]thymidine incorporation (cpm) in the wells containing no antigen.

Generation of peptide mixture group 2 specific T cell lines. Since the peptides in group 2 were found to consistently induce T cell proliferation, attempts were made to identify which of the peptide(s) within this mixture was inducing proliferation. Lymphoid cells (5×10^3/well) cultured as described above were expanded in number by culture with group 2 mixture of peptides (each at 10 μg/mL) pulsed autologous EBV-transformed B cells in X-Vivo 15 medium containing 20 U/ml of recombinant human IL-2 (courtesy of Roche Laboratories, Nutley, NJ). These group 2 mixture-primed T cells were then subjected to peptide specificity analysis essentially as described above.

Limiting dilution analysis of human PDC-E2–specific T cell lines from peripheral blood, liver, or RLN. Standard limiting dilution assays using varying concentrations of lymphoid cells (96 wells/cell concentration) co-cultured with PDC-E2 peptide pulsed, including autologous EBV-transformed B cells were performed. Wells were scored positive if the mean cpm (mean cpm values of cells with antigen and mean cpm values of cells without antigen) was > 1,000 cpm, and SI was > 3. The estimation of the frequency of antigen-specific T cells was performed as described by Zhang et al. (20) using a Poisson formula (21, 22).

Establishment of antigen-specific T cell clones. The individual microcultures corresponding to the ones that gave positive proliferation in the precursor frequency analysis were expanded and maintained individually in culture in the presence of irradiated (8,000 rad) autologous EBV-transformed B cells, which were prepulsed with PDC-E2 peptide 163-176 (10 μg/mL) in X-Vivo 15 medium containing 20 U/ml of recombinant human IL-2. These cultured T cells were subsequently cloned at 0.5 cell per well in X-Vivo 15 medium containing 100 U/ml of recombinant human IL-2 in the presence of 5×10^4 irradiated autologous EBV-transformed B cells as feeders. The positively growing wells were expanded in a stepwise fashion, and the antigenic specificity of the cloned T cell lines was examined.

Analysis of the cell surface phenotypes of T cell clones by flow cytometry. The T cell clones were stained with FITC- or phycoerythrin-conjugated mAbs, including mAbs to CD3 (anti-Leu4), CD4 (anti-Leu32a), CD8 (anti-Leu2a), CD45RO (UCHL-1), and T cell receptor (TCR) (Becton Dickinson Immunocytometry Systems, Mountain View, CA). T cell clones were first incubated with an optimal concentration of these FITC- or phycoerythrin-conjugated mAbs at 4°C for 30 min. After washing the cells with PBS, the cells were resuspended in PBS containing 0.1% sodium azide and analyzed using a FACScan (Becton Dickinson).

Proliferation and cross-reactivity of T cell clones that respond to PDC-E2 peptide 163-176. A total of 5×10^4 T cells from each of the T cell clones were seeded in a 96-well round-bottom plate containing 3×10^4 irradiated (8,000 rad) autologous EBV-transformed B cells or 5×10^4 irradiated (3,000 rad) allogenic PBMCs from healthy subjects who were HLA DRB4 0101. The APCs were pulsed overnight with varying concentration of either PDC-E2 peptide 36-49, peptide 163-176, E2L1 (residues 1-98), E2L2 (residues 120-233), E2L1+L2 (residues 1-233), whole PDC-E2, OGDC-E2 peptide 100-113, whole OGDC-E2, ovalbumin (OVA), or unrelated control peptide mixture (group 4; final concentration of each antigen 10–50 μg/ml). Cultures were performed in triplicate. After 3 d of co-culture, a T cell proliferation assay was performed as described above.

Results

Proliferative response of T cells to mixtures of synthetic peptides corresponding to human PDC-E2. We studied the T cell proliferative response of mononuclear cell infiltrates from the liver of PBC patients co-cultured with autologous EBV-transformed B cells previously pulsed with groups 1–9 of the synthetic peptides corresponding to the deduced sequence of human PDC-E2 (Fig. 1). The liver-infiltrated T cells derived from four out of four patients with PBC gave a positive proliferative response to the synthetic peptides in groups 2 and 4 (Fig. 2) (pt. 1, 8 out of 96 wells were positive to group 2, and 3 out of 96 wells were positive to group 4; pt. 2, 8 out of 96 to group 2, and 5 out of 96 to group 4; pt. 3, 5 out of 96 to group 2, and 1 out of 96 to group 4; and pt. 4, 5 out of 96 to group 2, and 3 out of 96 to group 4). The frequency of responding wells in cultures pulsed with the other peptide groups was not considered significant (pt. 1; 1 out of 96 wells was positive to group 8; pt. 2; 1 out of 96 to group 3 and to group 8; pt. 3, 1 out of 96 to group 7; and pt. 4; 1 out of 96 to group 3, to group 6, and to group 9) (Fig. 2).

Identification of the epitope within the peptide mixture of group 2 of human PDC-E2. Microcultures that showed a positive proliferative response to the group 2 peptides were repeatedly co-cultured with the same peptide group 2 mixture-pulsed autologous APC as described in Methods. These group 2 mixed peptide–primed T cells were then subjected to co-culture with autologous APC individually pulsed with each of the

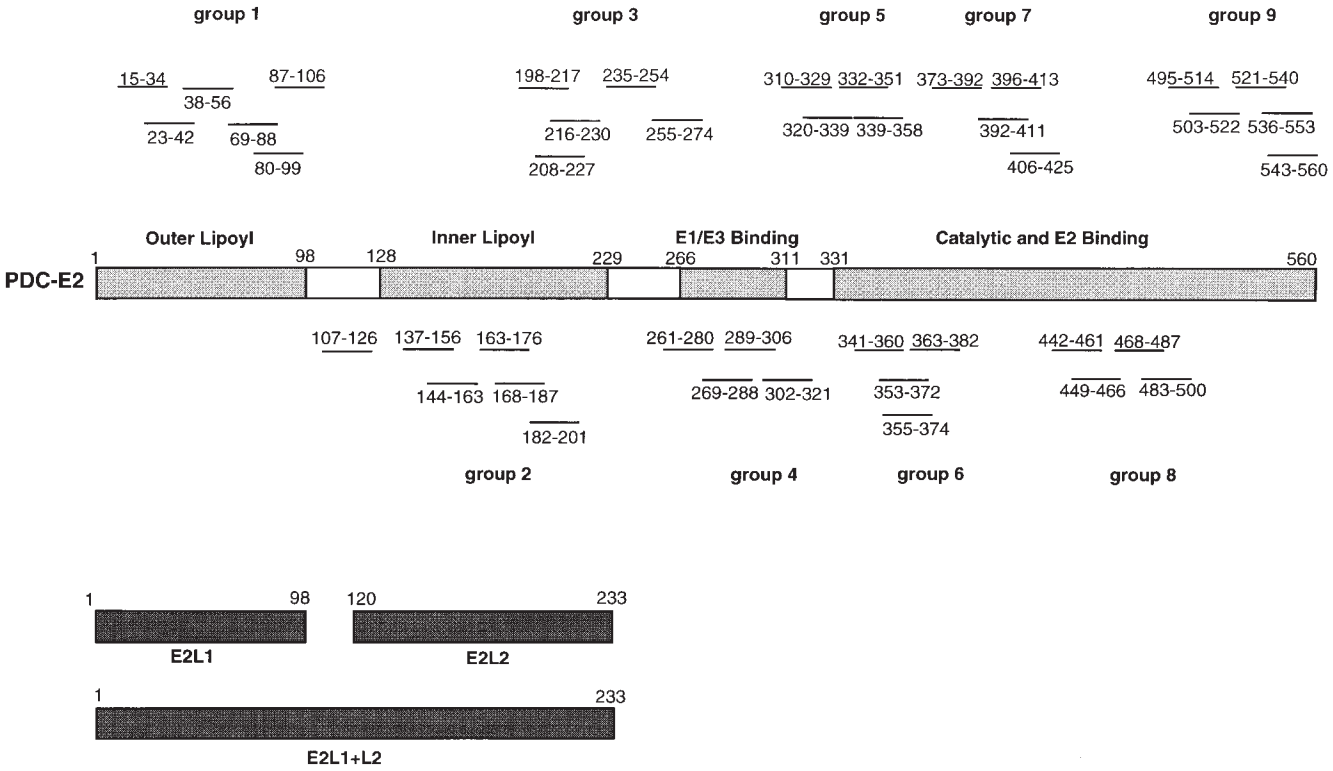

Figure 1. Synthetic peptides used for the proliferation assay. The amino acid sequences of PDC-E2 are based on the report by Coppel et al. (4). Synthetic peptides, which are indicated by the amino acid number from the N terminus of the PDC-E2, were classified into nine groups as described in Methods.

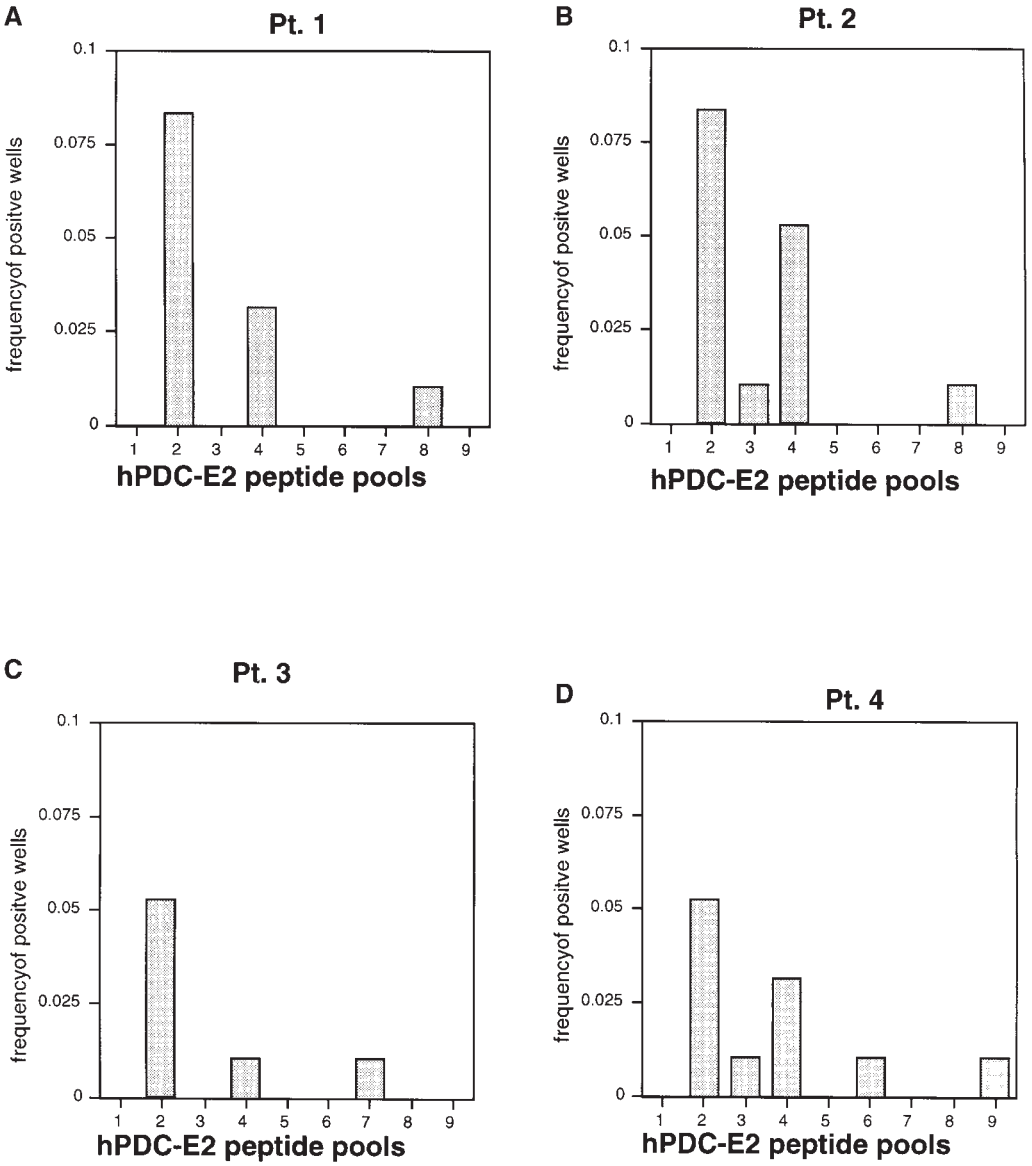

Figure 2. The heterogenic proliferation patterns to the peptide mixtures. Liver-infiltrated mononuclear cells from four patients with PBC were stimulated with peptide mixtures (groups 1–9) prepulsed with irradiated (8,000 rad) autologous EBV-transformed B cell lines every 10 d. On day 20, these T cells were tested for their proliferative response to each peptide mixture (groups 1–9) by a [^{3}H]thymidine incorporation assay. The proliferation patterns of all patients were heterogenic, but T cells from all four patients responded to peptide mixture group 2.

peptides contained within group 2 in efforts to define the peptide(s) that was inducing the proliferative response. As seen in Fig. 3, such peptide group 2–primed T cells only proliferated when co-cultured with the PDC-E2 peptide 163-176–pulsed autologous APC but not similar APC pulsed with the other peptides comprising group 2.

Differences in the frequency of T cells that respond to PDC-E2 peptide 163-176 within PBMC, liver-derived T cells, and T cells from lymph nodes. The frequency of T cells that respond to PDC-E2 peptide 163-176 was determined by limiting dilution analysis using a minimum of at least three cell concentrations. As shown in Table I and Fig. 4, the precursor frequency of T cells obtained from the liver that respond to PDC-E2 peptide 163-176 ranged from 1.66 to 4.13 $\times$ 10^{-5} in six patients with PBC; none of the liver-derived T cells from the three patients with PSC demonstrated a detectable response to PDC-E2 peptide 163-176. Furthermore, there were no detectable levels of T cells in the peripheral blood of the six patients with PBC (stage IV). However, the precursor frequency of T cells in the peripheral blood of six patients with earlier stage PBC (three were stage II, and three were stage III), which showed a detectable response to the PDC-E2 peptide 163-176 ranged from 2.49 to 4.85 $\times$ 10^{-7}. The precursor frequency of T cells in the peripheral blood from three healthy subjects that respond to PDC-E2 peptide 163-176 ranged from 1.41 to 1.71 $\times$ 10^{-7}. There were no marked differences in the frequency of peptide 163-176–specific T cells in the PBMC of PBC patients between stage II and III. We obtained T cells from RLN that responded to PDC-E2 peptide 163-176 in four out of four patients with PBC. The precursor frequency of RLN-derived T cells, which responded to PDC-E2 peptide 163-176 ranged from 1.96 to 5.46 $\times$ 10^{-5} (frequencies of liver vs. RLN were 1.92 $\times$ 10^{-5} vs. 1.96 $\times$ 10^{-5} in pt. 2, 1.66 $\times$ 10^{-5} vs. 5.46 $\times$ 10^{-5} in pt. 4, 4.13 $\times$ 10^{-5} vs. 2.02 $\times$ 10^{-5} in pt. 5, and 2.26 $\times$ 10^{-5} vs. 2.59 $\times$ 10^{-5} in pt. 6). As shown in Table I and Fig. 4, there were no differences in the frequencies between T cells derived from the liver compared with those from RLN.

Generation and characterization of the peptide specificity (cross-reactivity) and phenotype of the peptide 163-176 PDC-E2–specific T cell clones. A total of 28 stable T cell clones

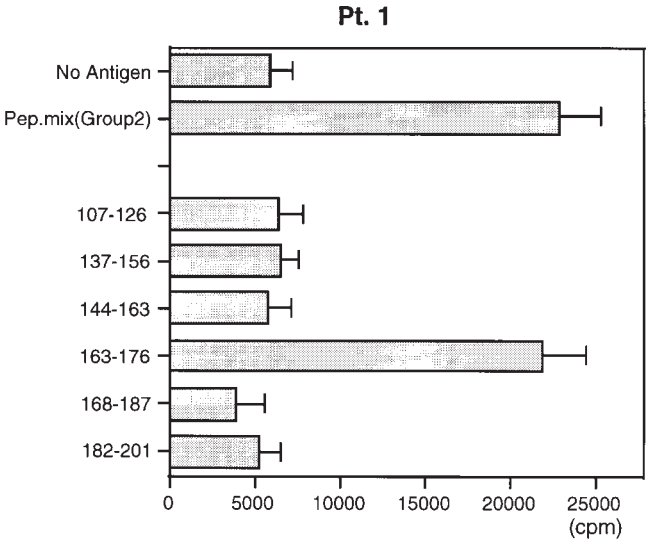

Figure 3. The peptide specificity of T cell lines specific for PDC-E2 peptide mixture group 2. T cells that showed a significant proliferation to peptide mixture group 2 were repeatedly stimulated with the same peptide mixtures in the presence of irradiated autologous EBV-transformed B cell lines and T-stim (4% vol/vol). After four to five repeated stimulations, the T cell lines specific for PDC-E2 peptide mixture group 2 were obtained from four different patients with PBC. These T cell lines were tested for their proliferative response to each peptide (SP 107-126, SP 137-156, SP 144-163, SP 163-176, SP 168-187, and SP182-201) by a [^{3}H]thymidine incorporation assay. All of the T cell lines reacted to SP 163-176 but none of the other peptides. The data are expressed as mean cpm±SEM.

were established from nine different PBC patients (17 of these T cell clones were derived from the liver, 7 from RLN cells, and 4 from the peripheral blood) and subjected to antigenic specificity and phenotype characterization. Each T cell clone was co-cultured with autologous irradiated EBV-transformed B cells previously pulsed with either the truncated recombinant proteins E2L1 (outer lipoyl domain comprising a.a. residues 1–98), E2L2 (inner lipoyl domain comprising a.a. residues 120–233), E2L1+L2 (comprising a.a. residues 1–233), the unfractionated native PDC-E2 molecule (residues 1–560), or the peptide 36-49 of the outer lipoyl domain of PDC-E2. For purposes of control, each of the T cell clones was co-cultured with autologous irradiated EBV-transformed B cells previously pulsed with the priming peptide 163-176 (positive control) or OVA or unrelated control peptide mixture (group 4) (negative control). As seen in Table II, each of the cloned T cell lines examined proliferated in response not only to the PDC-E2 peptide 36-49 but also in response to each of the truncated construct and the entire PDC-E2 molecule. Profile of the proliferative response of one of the representative clone P-L1-1 is shown in Fig. 5. It should be noted that each of these cloned T cell lines was initially derived and identified by their response to a cocktail of PDC-E2 peptides. Subsequently, each of the cloned T cell lines was screened for its proliferative response to each of the individual peptides present in the cocktail and a minimal of three additional irrelevant non–PDC-E2–derived peptides to define and identify the peptide specificity of the cloned T cell line. Representative data of the specificity of the cloned T cell line is shown in Fig. 3. Such data emphasize the peptide specificity of the proliferative response shown in Fig.

Table I. Precursor-frequency Analysis of PDC-E2 163-176–Specific T Cells

		Precursors		
Subjects	Stage	PBMC	Liver	RLN
PBC				
Pt. 11	II	3.86×10^{-7} (1.76×10^{-7}-5.95×10^{-7})*		
Pt. 12	II	3.06×10^{-7} (1.24×10^{-7}-4.88×10^{-7})		
Pt. 13	II	4.57×10^{-7} (1.25×10^{-7}-7.87×10^{-7})		
Pt. 14	III	2.82×10^{-7} (1.53×10^{-7}-4.12×10^{-7})		
Pt. 15	III	4.00×10^{-7} (7.41×10^{-8}-7.25×10^{-7})		
Pt. 16	III	4.85×10^{-7} (1.98×10^{-7}-7.81×10^{-7})		
Pt. 17	III	2.49×10^{-7} (8.33×10^{-8}-4.15×10^{-7})		
Pt. 1	IV	$< 5.21 \times 10^{-8}$	3.32×10^{-5} (3.12×10^{-5}-3.52×10^{-5})	
Pt. 2	IV	$< 5.21 \times 10^{-8}$	1.92×10^{-5} (1.25×10^{-5}-2.58×10^{-5})	1.96×10^{-5} (1.61×10^{-5}-2.31×10^{-5})
Pt. 3	IV	$< 5.21 \times 10^{-8}$	1.86×10^{-5} (1.16×10^{-5}-2.56×10^{-5})	
Pt. 4	IV	$< 5.21 \times 10^{-8}$	1.66×10^{-5} (1.28×10^{-5}-2.04×10^{-5})	5.46×10^{-5} (2.37×10^{-5}-8.55×10^{-5})
Pt. 5	IV	$< 5.21 \times 10^{-8}$	4.13×10^{-5} (2.02×10^{-5}-6.21×10^{-5})	2.02×10^{-5} (1.36×10^{-5}-2.70×10^{-5})
Pt. 6	IV	$< 5.21 \times 10^{-8}$	2.26×10^{-5} (1.90×10^{-5}-2.62×10^{-5})	2.59×10^{-5} (2.14×10^{-5}-3.05×10^{-5})
Normal Controls				
HS.1		1.41×10^{-8} (6.99×10^{-8}-2.11×10^{-7})		
HS.2		1.51×10^{-7} (1.03×10^{-7}-1.98×10^{-7})		
HS.3		1.71×10^{-7} (1.28×10^{-7}-2.13×10^{-7})		
PSC				
PSC.1			$< 5.21 \times 10^{-7}$	
PSC.2			$< 5.21 \times 10^{-7}$	
PSC.3			$< 5.21 \times 10^{-7}$	

*95% confidence interval.

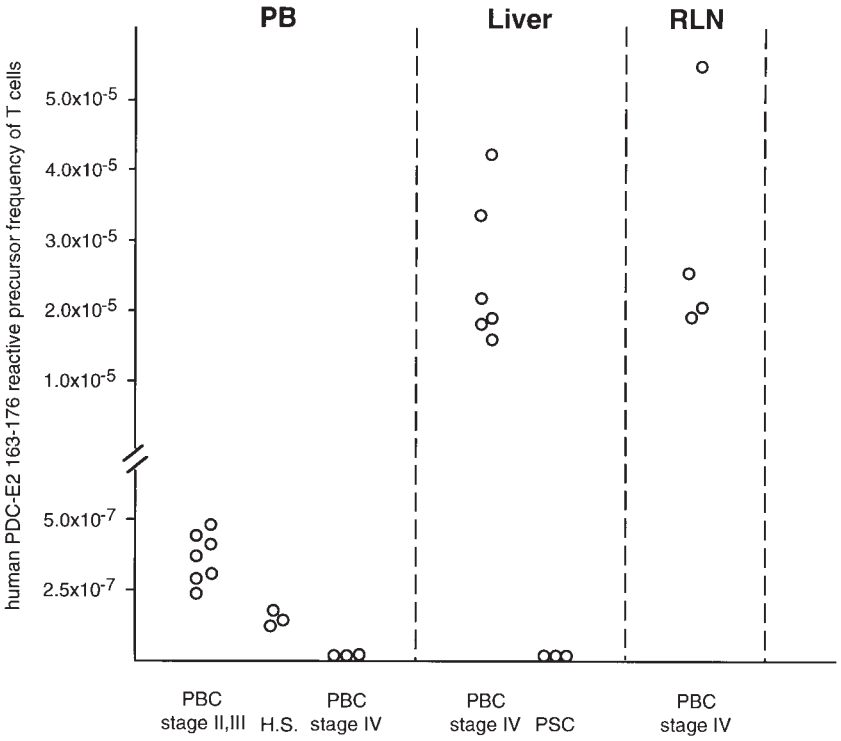

Figure 4. PDC-E2 peptide 163-176 reactive precursor frequency of T cells from peripheral blood (*PB*), liver, or regional lymph node (*RLN*). In PB, we examined seven samples from patients with PBC (*stage II* or *III*) three samples from healthy subjects (*H.S.*), and three samples from patients with PBC (*stage IV*). In liver, we examined six samples from patients with PBC and three samples from patients with PSC. In RLN, we examined four samples from patients with PBC. All samples are HLA DRB4 0101 positive.

5. This finding prompted us to examine the other mitochondrial autoantigens recognized by sera from PBC patients. A search revealed another sequence, the OGDC-E2 peptide 100-113 (DEVVCEIETDKTSV); a synthetic peptide comprising this sequence was synthesized. Three representative T cell clones (as listed under Table II, T cell clone P-L1-1 and P-L2-1 from the liver derived enriched mononuclear cells and P-R2-1 from the hepatic lymph node of PBC patients) that were repeatedly primed in vitro by co-culture with autologous APC pulsed with peptide 163-176 were analyzed for their potential cross-reactivity with OGDC-E2 peptide 100-113. Fig. 6 shows representative data derived from the T cell clone P-L1-1. Results show that this T cell clone not only proliferated in response to PDC-E2 peptide 163-176 and the native PDC-E2 but also to native OGDC-E2 and OGDC-E2 peptide 100-113. Again, peptide specificity of each of these cloned T cell lines is noted by their lack of proliferative response not only to OVA-pulsed autologous APCs but also APCs pulsed with a cocktail

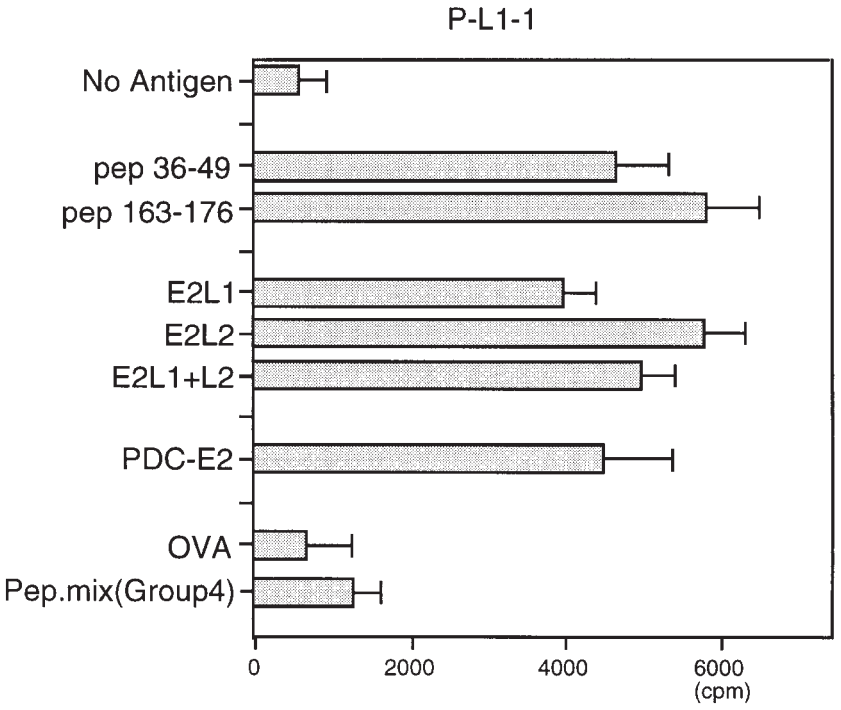

Figure 5. The cross-reactivity of T cell clones with PDC-E2 components. T cell clones were cultured with human PDC-E2 peptide 36-49, PDC-E2L1, PDC-E2L2, PDC-E2L1+L2, PDC-E2, OVA, or peptide mixture group 4 in the presence of autologous irradiated EBV-transformed B cell lines. After 3 d of culture, [^{3}H]thymidine was pulsed for 12 h, and [^{3}H]thymidine incorporation was measured. The data are presented as mean cpm±SEM.

*Table II. Reactivity of PDC-E2 163-176–Specific Cloned T Cell Lines**

				S.I. of Antigen			
	T cell clones	Peptide 36-49	Peptide 163-176	E2L1 (aa1-98)	E2L2 (aa120-233)	E2L1+L2 (aa1-233)	PDC-E2 (aa1-560)
Liver							
Pt. 1	P-L1-1	8.2	10.3	7.0	10.2	8.8	7.9
	P-L1-2	5.0	5.4	8.0	15.2	12.5	10.8
	P-L1-3	12.1	15.0	NT	NT	NT	NT
	P-L1-4	NT	12.8	NT	NT	NT	NT
Pt. 2	P-L2-1	11.9	12.3	9.0	8.9	14.8	8.2
	P-L2-2	5.4	6.4	NT	NT	NT	NT
Pt. 3	P-L3-1	8.1	7.5	5.2	8.2	7.6	9.1
	P-L3-2	8.0	8.7	6.2	9.2	7.7	6.8
	P-L3-3	12.1	10.1	6.6	11.6	13.5	NT
	P-L3-4	NT	7.9	NT	NT	NT	NT
	P-L3-5	16.9	15.0	NT	NT	NT	NT
Pt. 4	P-L4-1	5.9	4.6	5.9	10.4	10.7	8.2
	P-L4-2	18.0	21.7	NT	NT	NT	NT
	P-L4-3	NT	14.7	NT	NT	NT	NT
Pt. 5	P-L5-1	14.9	12.3	NT	NT	NT	NT
	P-L5-2	8.3	6.1	10.5	14.1	12.9	10.1
Pt. 6	P-L6-1	NT	9.2	NT	NT	NT	NT
RLN							
Pt. 2	P-R2-1	12.2	9.0	12.9	16.3	18.9	11.8
Pt. 4	P-R4-1	3.9	4.5	5.0	4.9	4.7	4.1
	P-R4-2	9.7	8.1	6.9	11.8	10.5	NT
	P-R4-3	5.9	4.1	NT	NT	NT	NT
	P-R4-4	NT	5.7	NT	NT	NT	NT
Pt. 5	P-R5-1	13.5	12.2	NT	NT	NT	NT
Pt. 6	P-R6-1	5.9	5.7	6.7	11.4	13.4	8.8
PBMC							
Pt. 11	P-P11-1	10.1	9.8	NT	NT	NT	NT
Pt. 12	P-P12-1	NT	7.3	NT	NT	NT	NT
Pt. 13	P-P13-1	7.0	7.9	NT	NT	NT	NT
	P-P13-2	NT	11.6	NT	NT	NT	NT

*These cloned T cell lines were all $CD3^+CD4^+CD45RO^+TCR\alpha\beta^+$. NT, not tested.

of peptides from group 4. In addition, each cloned T cell line has been previously screened (see comment above) denoting that such proliferative response while showing cross-reactivity between PDC and OGDC-E2 peptides are not promiscuous peptide responders and have an element of very defined peptide specificity. Similar sets of data were observed with the other two T cell clones and confirmed by repeated testing of each of these three T cell clones (data not shown). Among three T cell clones, a dose response to PDC-E2 peptide 36-49, 163-176, and OGDC-E2 100-113 was seen in the representative T cell clone P-LI-1. As seen in Fig. 7, P-LI-1 had dose-dependent proliferation to all three different antigens, PDC-E2 peptide 36-49, 163-176, and OGDC-E2 100-113.

Each of the 28 T cell clones was also subjected to cell surface phenotype analysis by flow microfluorometry. These T cell clones express $CD3^+$, $CD4^+$, $CD45RO^+$, and TCR $\alpha\beta^+$ (data not shown) denoting that each of these T cell clones is a memory helper T cell. Based on the sequence of these three peptides (PDC-E2 peptide 36-49, 163-176, and OGDC-E2 100-113), a motif comprising of ExETDK appears to be the motif with the appropriate anchor residues to bind HLA DRB4 0101 or the appropriate cognate residues of the TCR.

Discussion

The concept that degenerate peptides that bind to the same MHC molecules and induce the activation of the same clonal population of T cells with different functional consequences takes into account several assumptions. First, the self-peptide/MHC expressed by APCs during normal healthy life is a required stimulus for the maintenance and survival of T cells. Second, the cognitive requirements for specific peptide residues by the TCR is not as stringent as once assumed (23, 24). Third, there must exist a library of peptides that are larger than previously believed that bind to the same MHC molecule with the obvious requirement for the presence of specific anchor residues. Thus, T cells have the potential to be activated essentially by two complementary mechanisms. These mechanisms induce the increased density of MHC and costimulatory molecules on local cells, which increases the avidity of the interaction between T cells and APCs followed by the induction of T cell activation. Also, alterations in the affinity of single bimolecular complexes will modulate this activity. The high degree of degeneracy in antigen recognition by TCR (23) suggests that activation of T cells normally responding to self-antigens occurs after exposure of these clones to foreign pathogen-derived peptides. Recent data support the view that self-peptides are required as a source to maintain T cell clones in the peripheral circulation. The data reported herein can be discussed with relevance to these points and to PBC (25, 26).

The antigen and peptide specificity of each of the 28 T cell clones described demonstrates a proliferative response to PDC-E2 peptide 163-176–pulsed autologous APC. This response is restricted in each case to HLA DRB4 0101 (data not shown). In efforts to further define the specificity and/or cross-reactivity for each of these T cell clones, a search was made for peptides that show similarity both within the PDC molecule and the other major autoantigens of PBC. Two other sequences were identified, peptide 36-49, located on the outer lipoyl domain of PDC-E2, and peptide 100-113 of oxo-glutarate acid dehydrogenase (OGDC)–E2. In addition, autologous APC pulsed with the recombinant PDC-E2–truncated fragments (E2L1, outer lipoyl domain, amino acids 1–98, E2L2, inner lipoyl domain, amino acids 120–233, E2L1+L2, and amino acids 1–233) and unfractionated native beef PDC-E2 and rat OGDC-E2 were used in co-culture experiments with each of the T cell clones. This study supports previous findings that indeed multiple T cell epitopes for the PDC-E2 autoantigen exist in PBC and, in addition, has led to the identification of a new cross-reactive epitope encoded by another distinct but related autoantigen, OGDC-E2 for PBC. Such findings led us to define a common T cell autoantigen motif with a signature sequence encompassing ExETDK. Thus, it appears that there are certain minimal requirements for the binding of these peptides to HLA DRB4 0101 associated with a certain degree of plasticity for the remaining residues, including PDC-E2 peptide 163-176 (GDLLAEIETDKATI), PDC-E2 peptide 36-49 (GDLIAEVETDKATV), and OGDC-E2 peptide 100-113 (DEVVCEIETDKTSV).

Although our estimates of precursor frequency, using the limiting dilution assays used herein, may be underestimates

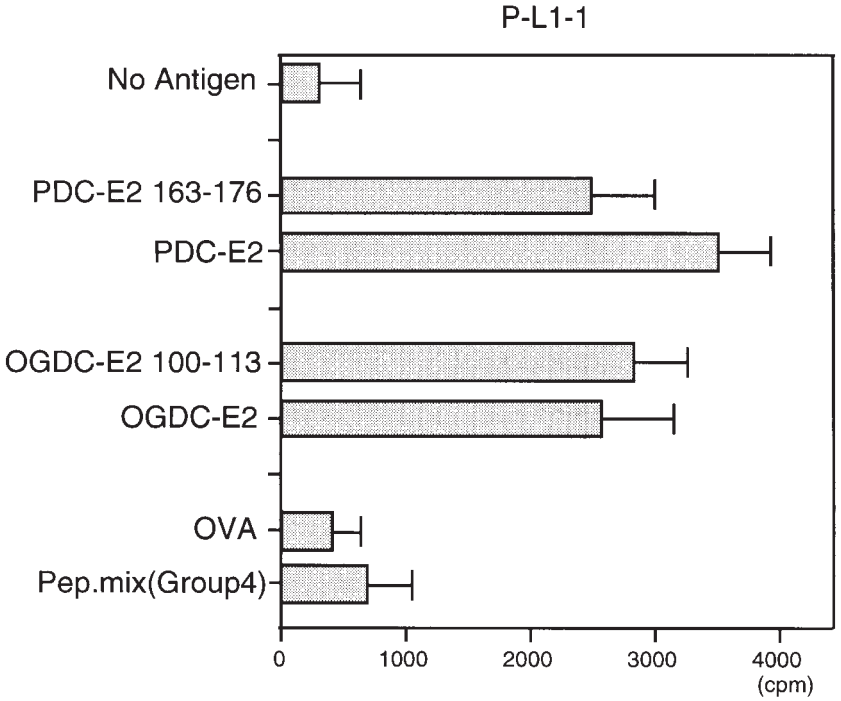

Figure 6. The cross-reactivity of T cell clones with OGDC-E2 components. In addition to PDC-E2 (50 μg/ml) and PDC-E2 peptide 163-176 (10 μg/ml), T cell clones were cultured with human OGDC-E2 peptide 100-113 (10 μg/ml), OGDC-E2 (50 μg/ml), OVA (50 μg/ml), and each peptide of group 4 (10 μg/mL), in the presence of autologous irradiated EBV-transformed B cell lines. After 3 d of culture, [^{3}H]thymidine was pulsed for 12 h, and [^{3}H]thymidine incorporation was measured. The data are presented as mean cpm±SEM.

because of antigen-induced apoptosis, the values obtained are clearly significant (27). Recent experiments have indicated that not all organ-specific autoreactive T cells are deleted within the thymus; a certain frequency of such autoreactive cells circulates at a low frequency in the periphery (28). This is supported by the frequency of $CD4^+$ T cells that recognize sequestered autoantigens in the circulation of normal individuals as well as in the blood of patients with autoimmune diseases, including PBC (29–31). Common to T cells reactive to other autoantigens, autoreactive T cells specific for human PDC-E2 peptide 163-176 are present in both patients with PBC and healthy subjects. The mean frequency of PDC-E2 peptide 163-176–reactive peripheral T cells was 3.66×10^{-7} in patients with PBC (except at end stage disease) and 1.54×10^{-7} in healthy subjects. This difference in the precursor frequencies between PBC patients and healthy controls is observed in patients with other human organ–specific autoimmune disease, including multiple sclerosis (MS) (32–34). Thus, in two such studies, the mean frequency of autoantigen myelin basic protein (MBP)–reactive T cells was 3.2×10^{-6} in patients with MS as com-

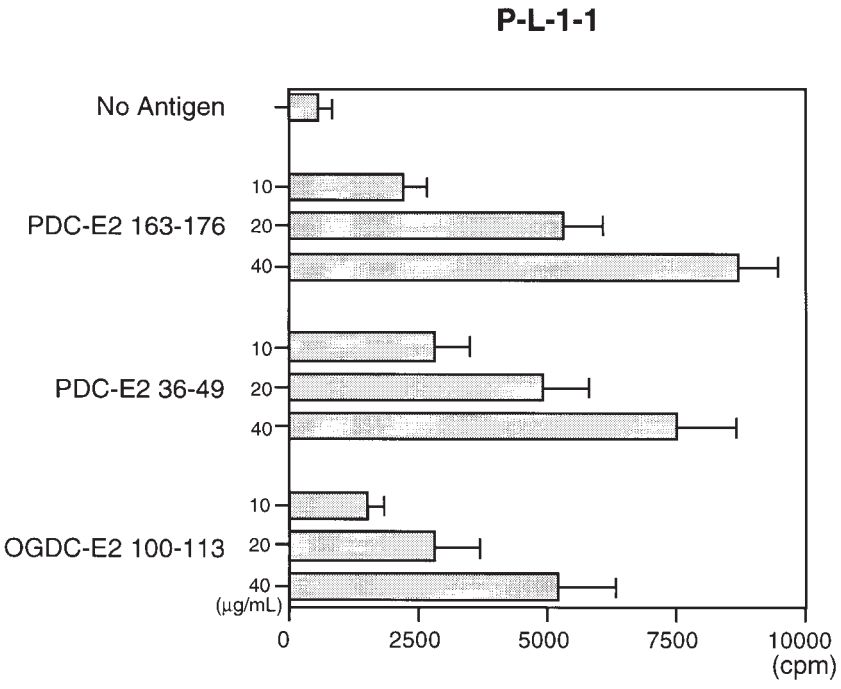

Figure 7. The dose dependency of T cell clones. T cell clones were cultured with human PDC-E2 peptide 36-49, 163-176, or human OGDC-E2 peptide 100-113 in the presence of allogenic PBMC from healthy subjects who were HLA DRB4 0101. After 3 d of culture, [^{3}H]thymidine incorporation was measured. The data are presented as mean cpm±SEM.

pared with 1.2×10^{-6} in healthy subjects. Similarly, the mean frequency of autoantigen proteolipid protein–reactive T cells was 1.3×10^{-6} in patients with MS as compared with 0.3×10^{-6} in healthy subjects (20, 35). In the data reported herein, there was an $\sim$ 1.5–3.5-fold increase in the frequency of PDC-E2 residues 163-176–reactive T cells in the blood of patients with PBC as compared with healthy controls. It is important to note that in the early or moderate stage of PBC, the frequency of peripheral T cells that respond to peptide 163-176 is significantly higher than that in the end stage of PBC. Whether this represents the disappearance of cells during the course of the disease or a progressive homing of cells to the liver or the RLN is not currently known. In the end stage of PBC, even though there are no detectable T cells that respond to peptide 163-176 in the peripheral blood, there is a significant frequency of T cells that respond to peptide 163-176 in the livers (1.66×10^{-5} to 4.13×10^{-5}) or RLN (1.96×10^{-5} to 5.46×10^{-5}) of these patients. These differential frequencies of autoantigen-reactive T cells in disease target organs as compared with the recirculating pool in the PBMC of PBC patients is similar to the findings seen in MS. The mean frequency of MBP-reactive T cells in cerebrospinal fluid from patients with MS was 5.4×10^{-5} and the mean frequency of proteolipid protein synthetic peptide–reactive T cells was 9.5×10^{-5} in the cerebrospinal fluid of MS (20, 36). The frequency of peptide 163-176–reactive T cells in the liver of PBC is less than the frequency of MBP-reactive T cells in MS. One possible reason for this difference is that T cells in PBC were derived from the end stage and T cells in MS were from the active stage patients. Interestingly, the frequencies of autoreactive T cells in RLN are essentially equal to that found in the liver. This could reflect migration of lymphocytes and dendritic cells from the portal tract of the liver to the hilar lymph node (37). Dendritic cells are the most efficient APC in presenting endogenous naturally processed self-epitopes (38). On the other hand, high numbers of B cells that produce PDC autoantibodies exist in the liver tissue in PBC (39). Antigen-specific B cells are efficient APCs for primed T cells due to specific uptake of antigen via surface immunoglobulin (40, 41).

We note that autoreactive T cells restricted by HLA DRB4 0101 recognize both the inner and outer lipoyl domains, two regions that share $\sim$ 63% identity (42). Furthermore, PDC-E2 peptide 36-49 (GDLIAEVETDKATV), peptide 163-176 (GDLLAEVETDKATI), and OGDC-E2 peptide 100-113 (DEVVCEIETDKTSV). The second position of each peptide consists of acidic hydrophilic amino acids (D or E), the third position of each peptide consists of hydrophobic amino acids (L or V), the fourth, seventh, and fourteenth position have hydrophobic amino acids (I, L, or V at position 4, V or I at position 7, V or I at position 14), the sixth is E, the eighth is E, the ninth is T, the tenth is D, and the eleventh is K. Thus, the motif D/E OOXEOETDKxxO (O is a hydrophobic amino acid) is obtained. However, the MHC/TCR contact residues of PDC-E2 36-49, 163-176, or OGDC-E2 100-113 have not yet been defined in detail.

In previous studies by our laboratory, patient sera were tested for antibody activity against two lipoic acid–binding regions (E2L1 and E2L2) of PDC-E2, and sera from 80–90% of patients showed reactivity against both domains. Thus, the T and B cell epitopes appear to be localized to the same region of the autoantigen. This is similar to MS, where MBP-specific autoantibodies react with an immunodominant MBP peptide, which is also the epitope recognized by MBP-specific T cell clones (43). This may be a result of not only more efficient uptake and processing of the autoantigen by autoimmune B cells as stated above but also could be due to the fact that the epitopes may be protected from degradation during antigen processing. These data provide evidence for a role of the immunodominant PDC-E2 peptide 163-176 and/or peptides with a similar motif in the immunobiology of PBC. These results also allow for additional questions, which should be addressed, including analysis of the TCRs expressed by the clones. The shared epitope in the autoantigenic peptides contain several charged residues. These may be responsible for degenerate recognition by the T cells that may express TCRs with reciprocally charged residues.

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