

HLA DRB4 0101-restricted Immunodominant T Cell Autoepitope of Pyruvate Dehydrogenase Complex in Primary Biliary Cirrhosis: Evidence of Molecular Mimicry in Human Autoimmune Diseases

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Summary

We established six T cell clones specific for pyruvate dehydrogenase complex (PDC)-E2 peptides from four different patients with primary biliary cirrhosis using 33 different peptides of 17–20 amino acid residues corresponding to human PDC-E2 as stimulating antigens. The minimal T cell epitopes of these six T cell clones were all mapped to the same region of the PDC-E2 peptide 163–176 (GDLLAEIETDKATI), which corresponds to the inner lipoyl domain of PDC-E2. The HLA restriction molecules for this epitope were all identified as HLA DRB4 0101. The common essential amino acids of this epitope for these T cell clones were E, D, and K at positions 170, 172, and 173, respectively; other crucial amino acids for this epitope differed in each T cell clone. In addition, the alanine-substituted peptides at positions 170 and 173, but not 172, inhibited the proliferation of all T cell clones induced by the original peptide of human PDC-E2 163–176, indicating that amino acid D at position 172 is a critical MHC-binding site for all T cell clones tested. Interestingly, all T cell clones reacted to PDC-E2 peptide 36–49 (GDLIAEVETDKATV), which corresponds to the outer lipoyl domain of human PDC-E2. Furthermore, one T cell clone cross-reacted with exogenous antigens such as *Escherichia coli* PDC-E2 peptide 31–44/134–147/235–248 (EQLITVEGDKASM), which has an EXDK sequence. This is a definite demonstration of the presence of molecular mimicry at the T cell clonal level in human autoimmune diseases. It is also considered possible to design peptide-specific immunotherapy based on the findings of T cell autoepitopes in primary biliary cirrhosis.

Primary biliary cirrhosis (PBC)¹ is an autoimmune chronic cholestatic liver disease characterized by the presence of antimitochondrial antibodies (AMAs) and inflammation of interlobular bile ducts in the liver (1). PBC affects mainly women and ultimately leads to liver failure (2). The major mitochondrial antigens (Ags) recognized by AMAs have recently been identified as E2 components of 2-oxo acid dehydrogenase complexes; pyruvate dehydrogenase complex (PDC), 2-oxoglutarate dehydrogenase complex (OGDC), and branched chain 2-oxo acid dehydrogenase complex (BCOADC) that are localized in the mitochondrial inner membrane (3–9). Immunohistochemical examinations of the lymphocytes infiltrating the portal tracts of livers of PBC patients

revealed a predominance of activated CD8⁺ T cells, as well as CD4⁺ T cells, with B and NK cells (10–12). By fluorescence microscopy, it has been demonstrated that large amount of Ags immunoreactive with AMAs are present on the surface of biliary epithelial cells in the livers of PBC patients (13–15). Furthermore, bile duct epithelia and hepatocytes in PBC express large amounts of HLA class I and II molecules (15–17). These observations suggest that T cell-mediated mechanisms are involved in the pathogenesis of the destruction of bile duct epithelia and hepatocytes in PBC. However, thus far little has been reported on the autoepitopes of the mitochondrial Ags recognized by T cells in PBC (14, 18–20).

In other human organ-specific autoimmune diseases, such as multiple sclerosis (21–24), chronic thyroiditis (25–28), and myasthenia gravis (29–32), pathogenic T cell autoepitopes and their restriction molecules of MHC class I or II have recently been studied at the molecular level, and some pathogenic T cell autoepitopes have been identified. However, T cell autoepitopes remain to be elucidated in most other autoimmune diseases, because of the methodological limitations

¹ Abbreviations used in this paper: Ag, antigen; APC, antigen-presenting cell; AMA, antimitochondrial antibody; BCOADC, branched chain 2-oxo acid dehydrogenase complex; OGDC, 2-oxoglutarate dehydrogenase complex; PBC, primary biliary cirrhosis; PDC, pyruvate dehydrogenase complex; PDC-E2, E2 component of PDC; SI, stimulation index; SP, synthetic peptide.

in generating stable T cell clones specific for disease-relevant self Ags. Only some primitive studies concerning the T cell autoepitopes of U1 small nuclear ribonucleoprotein A protein (33) and glutamic acid decarboxylase (34, 35) have been previously reported for SLE and insulin-dependent diabetes mellitus, respectively.

In the present study, we established six T cell clones specific for PDC-E2 from the peripheral blood of patients with PBC. Surprisingly, the minimal T cell epitopes of these T cell clones from four different patients with PBC were all mapped to the same region of PDC-E2 peptide 163-176, which corresponds to the inner lipoyl domain of human PDC-E2. Furthermore, all T cell clones were restricted by HLA DRB4 0101. We also found that there are three common essential amino acid residues for this epitope, EXDK, and that one T cell clone cross-reacted with the peptide derived from exogenous Ags such as *E. coli* PDC-E2, which has an EXDK sequence.

This is not only a definite demonstration of the T cell autoepitope of mitochondrial Ags in PBC, but also a demonstration of "molecular mimicry" that is operative at the T cell clonal level in human autoimmune diseases. The possible mechanism of molecular mimicry at the T cell clonal level is also discussed.

Materials and Methods

Patients. 13 patients with PBC of various histological stages (stages I-IV) were studied (age 38-67 yr, mean age 58.7 yr; stage I: 4 patients, stage II: 7 patients, stage III: 1 patient, stage IV: 1 patient). The diagnosis of PBC was established based on the criteria of the autoimmune hepatitis/primary biliary cirrhosis subgroup, which is part of the Intractable Disease Study Group organized by the Ministry of Health and Welfare of Japan (36).

Preparation of Synthetic Peptides Corresponding to PDC-E2. 40 different peptides composed of 13-20 amino acid residues corresponding to the amino acid sequence of human PDC-E2 were synthesized via F-moc chemistry using a peptide synthesizer (model 432A Synergy; Applied Biosystems Inc., Foster City, CA) (9, 37). The peptides were purified by reverse-phase HPLC, and the purity of all peptides used in this study was >95%, as determined by HPLC analysis. The peptides were designed according to the analysis of the amphipathic regions of the antigenic molecules and the presence of consensus motifs based on the studies of Berzofsky et al. (38). Synthetic peptides (SPs) used in this study, indicated by the amino acid number from the NH₂ terminus of PDC-E2, were classified into seven groups as follows: group 1: SPs -40--21, -31--12, -17-2, 15-34, and 23-42; group 2: SPs 34-50, 38-56, 69-88, 80-99, and 137-156; group 3: SPs 144-163, 163-182, 168-187, 182-201, and 198-217; group 4: SP 208-227, 255-274, 261-280, 269-288, and 289-306; group 5: SPs 332-351, 341-360, 355-374, 363-382, and 373-392; group 6: SPs 392-411, 406-425, 449-466, 495-514, and 503-522; and group 7: SPs 521-540, 536-553, and 543-560 (Fig. 1). For fine T cell epitope mapping, SPs 161-177, 162-175, 163-175, 163-176, 163-177, 164-176, 164-177, and 163-182, as well as 14 different alanine- or serine-substituted peptides of SP 163-176 were synthesized. For the cross-reactivity experiments of T cell clones, five different peptides corresponding to human PDC-E2 peptide 36-49 (GDLIAEVETDKATV), *Pseudomonas putida* BCOADC 104-117 (DELLATIETDKIDI), *E. coli* PDC-E2 31-44/134-147/235-248 (EQLITVEGDKASM), human glycogen

phosphorylase β 354-367 (LVDLERMDWDKAWD), and HLA DR α chain 82-95 (QGALANIAVDKANL) were synthesized and used in a T cell proliferation assay. Purified porcine PDC and OGDG were purchased from the Sigma Chemical Co. (St. Louis, MO).

T Cell Proliferation Assay. PBMCs were separated from the peripheral blood by Ficoll-Isopaque gradient centrifugation. T cells were purified by rosetting PBMCs with sheep red blood cells. 1×10^5 T cells and 1×10^4 autologous irradiated (3,000 rad) non-T cells per well were cultured in the presence of peptide mixtures (groups 1-7; each synthetic peptide concentration was 10 μ g/ml) in a 96-well round bottom plate (Costar Corp., Cambridge, MA) in RPMI 1640 supplemented with 2 mM L-glutamine, 50 ng/ml of gentamycin, and 10% human AB-positive serum (complete RPMI medium) at 37°C in a humidified 5% CO₂ atmosphere. In each experiment, microcultures containing T cells and irradiated non-T cells, but no Ag served as negative controls. More than 30 wells were studied in each group. After 7 d of culture, the T cells were restimulated with the same concentration of Ag or without Ag in the presence of 5×10^4 freshly prepared, irradiated (3,000 rad) autologous PBMCs per well. After another 7 d of culture, half of the cells in the microculture wells were transferred to other plates and were pulsed with [³H]TdR (0.5 μ Ci/well; Amersham International, UK) for 10 h. [³H]TdR incorporation was measured with a scintillation counter (1205 Betaplate liquid scintillation counter; Pharmacia, Uppsala, Sweden). The stimulation index (SI) was calculated as follows: SI = the mean value of [³H]TdR incorporation (cpm) in wells containing Ag/the mean value of [³H]TdR incorporation (cpm) in wells not containing Ag. Usually 20-30 wells were studied for each group. The wells exhibiting an SI >2 were determined to be positive for T cell proliferation.

Establishment of Ag-specific T Cell Clones. The microcultures showing positive proliferation were kept in culture in the presence of freshly prepared autologous antigen-presenting cells (APCs) in complete RPMI medium containing the same peptide mixture (each peptide concentration was 10 μ g/ml) and 20 U/ml IL-2 (Takeda Medical Co., Tokyo, Japan). The cells were repeatedly stimulated with the same Ag in the presence of irradiated autologous PBMCs every week. After four to five repeated stimulations, these T cell lines were cloned at 0.5 cell per well in complete RPMI medium containing IL-2 (100 U/ml) in the presence of autologous irradiated (3,000 rad) PBMCs as feeders. The peptide mixture-specific T cell clones were expanded and studied in the following experiments.

Analysis of the Cell Surface Phenotypes of T Cell Clones by Flow Cytometry. T cell clones were stained with a variety of FITC- or PE-conjugated mAbs, including mAbs to CD3 (anti-Leu4), CD4 (anti-Leu32a), CD8 (anti-Leu2a), CD45RA (2H4), CD45RO (UCHL1), and TCR. All of these mAbs were purchased from Becton Dickinson Immunocytometry Systems (Mountain View, CA). T cell clones were first incubated with an adequate concentration of these FITC- or PE-conjugated mAbs at 4°C for 30 min. After washing the cells with PBS containing 0.1% sodium azide (Sigma Chemical Co.), the cells were analyzed by flow cytometry (FACScan[®]; Becton Dickinson).

Proliferation of T Cell Clones in Response to Different Concentrations of Synthetic Peptide in the Presence or Absence of Various mAbs to Cell Surface Molecules. Synthetic peptide was used at different concentrations (1-20 μ g/ml) to study the Ag specificity of the T cell clones. The role of cell surface molecules in the T cell proliferative response to Ag was also studied using various mAbs: mouse anti-CD3 (anti-OKT3), mouse anti-CD4 (anti-OKT4), mouse anti-CD8 (anti-OKT8), mouse anti-class I (W6/32), and mouse anti-HLA DP, DQ, and DR (B7/21, Hull, and L243) (all of these mAbs were

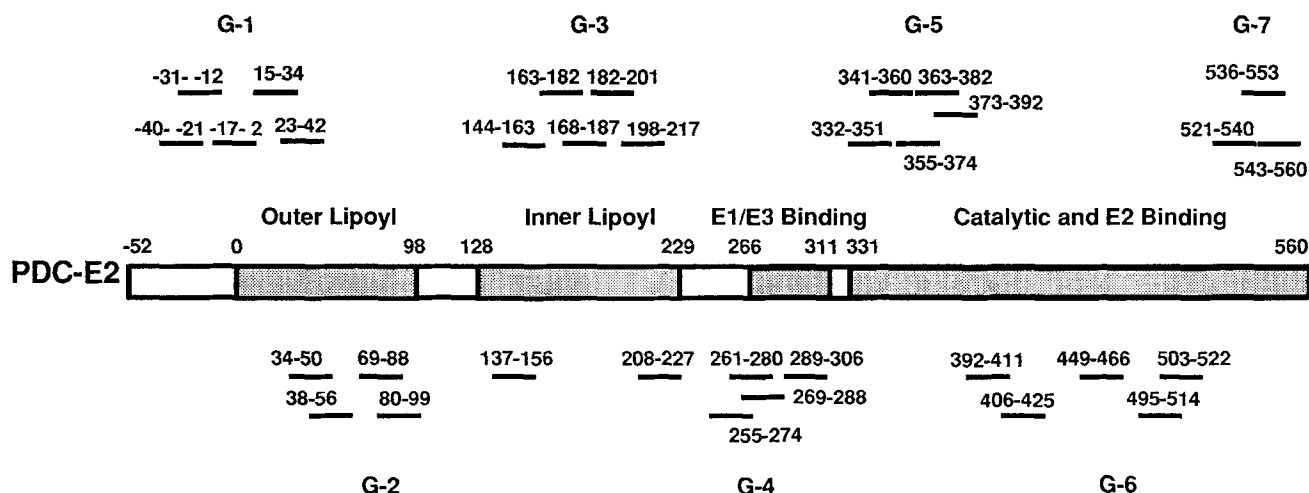


Figure 1. Synthetic peptides used for the generation of PDC-E2-specific T cell clones. The amino acid sequences of PDC-E2 are based on the report by Coppel et al. (5). Synthetic peptides, which are indicated by the amino acid number from the NH₂ terminus of PDC-E2, were classified into seven groups as described in Materials and Methods.

kindly provided by Dr. T. Sasazuki, Division of Genetics, Medical Institute of Bioregulation, Kyushu University, Kyushu, Japan). 5×10^4 T cells were seeded in a 96-well, round-bottom plate containing 5×10^4 irradiated (3,000 rad) autologous PBMCs and synthetic peptide in the presence or absence of various mAbs. All mAbs were used at a 1:100 dilution of ascites. After 3 d of culture, a T cell proliferation assay was similarly performed in triplicate as previously described.

Determination of the Critical Amino Acids of T Cell Epitopes for the Recognition by TCR or for the Binding to HLA DR Using Alanine- or Serine-substituted Peptides of SP 163-176. To determine the amino acids of SP 163-176 critical for the recognition by TCR (epitope) or for the binding to HLA (anchor), five T cell clones specific for SP 163-176 were studied for their proliferative response to alanine- or serine-substituted peptides of SP 163-176. In some experiments, alanine- or serine-substituted peptides were used as competitors for the original peptide 163-176 in a T cell proliferation assay.

PCR-based DNA Typing of HLA Class II Genes. The DNA typing of HLA class II genes was performed using a PCR/sequence-specific oligonucleotide probe (SSOP) analysis in 13 PBC patients and 7 healthy subjects as described elsewhere (39). Genomic DNA was extracted from peripheral granulocytes and subjected to PCR in 10 mM Tris-HCl (pH 8.4 at 24°C), 50 mM KCl, 2.0 mM MgCl₂, 0.1 mg/ml gelatin, 0.02% NP-40 to amplify the second exons of the DRB1, DQA1, DQB1, DPA1, or DPB1 genes, using thermostable DNA polymerase. Amplified DNA was spotted onto a nylon membrane (Hybond N⁺; Amersham International), and it was immobilized by alkaline denaturation in 0.4 N NaOH. The hybridization procedure with 140 types of SSOPs has been described previously (39). 11 positive hybridization signals were obtained by exposing the filters to x-ray film (XAR-5; Eastman Kodak Co., Rochester, NY) at room temperature for 30 min.

Results

Proliferative Response of T Cells to Synthetic Peptides Corresponding to Human PDC-E2. We studied the T cell prolifer-

ative response to synthetic peptides corresponding to putative T cell epitopes of human PDC-E2 (Fig. 1). The T cells derived from the majority of patients with PBC proliferated in response to synthetic peptide groups 2, 3, and 6 (the mean values \pm SD of stimulation indices ranged from 1.9 ± 0.4 to 2.6 ± 0.9), but not to groups 1, 4, 5, and 7 (the mean values \pm SD of stimulation indices ranged from 1.0 ± 0.2 to 1.4 ± 0.4).

Generation and Characterization of T Cell Clones Specific for Peptides Corresponding to Human PDC-E2. Microcultures that were stimulated by peptide mixture group 2, 3, or 6 and showed a value of [³H]TdR incorporation two times higher than the mean value of microcultures without any peptides were repeatedly stimulated with the same group of peptide mixtures (group 2, 3, or 6) in the presence of irradiated autologous PBMCs and IL-2 (20 U/ml) once every week. After four to five repeated stimulations, the T cell lines were cloned at 0.5 cell per well. Consequently, six T cell clones that proliferated in response to peptide mixture group 3 were obtained from four different patients (Table 1). These T cell clones responded to SP 163-182, but not to other peptides contained in the group 3 peptide mixture (Fig. 2). Whereas all T cell clones proliferated in a dose-dependent manner in response to SP 163-182 (Fig. 3), they did not proliferate in response to other Ags, such as OVA or OGDC. Furthermore, the proliferation of these peptide-specific T cell clones was inhibited by mAbs such as anti-CD3, anti-CD4, and anti-HLA DR (Fig. 3). The surface phenotypes of these T cell clones were CD3⁺, CD4⁺, CD45RO⁺, and TCR $\alpha\beta$ ⁺, as determined by a flow cytometric analysis (Table 1).

HLA Restriction of T Cell Clones Specific for SP 163-182. To identify the HLA molecules responsible for Ag presentation of SP 163-182 to T cell clones, we used allogeneic irradiated PBMCs with various HLA haplotypes as APCs. Briefly, the irradiated allogeneic PBMCs (5×10^4 per well) and each

Table 1. List of T Cell Clones Specific for Human PDC-E2 Generated in the Present Study

Donor	T cell clones	Peptide specificity	SI*	HLA restriction†	Surface Marker‡
Patient 2	T-pep-2-1	PDC-E2 163-182	48.2	DR	CD3 CD4 CD45RO TCR $\alpha\beta$
	T-pep-2-2	PDC-E2 163-182	49.1	DR	CD3 CD4 CD45RO TCR $\alpha\beta$
Patient 8	T-pep-8-1	PDC-E2 163-182	19.8	DR	CD3 CD4 CD45RO TCR $\alpha\beta$
Patient 10	T-pep-10-1	PDC-E2 163-182	15.1	DR	CD3 CD4 CD45RO TCR $\alpha\beta$
Patient 11	T-pep-11-1	PDC-E2 163-182	39.1	DR	CD3 CD4 CD45RO TCR $\alpha\beta$
	T-pep-11-2	PDC-E2 163-182	82.3	DR	CD3 CD4 CD45RO TCR $\alpha\beta$

* Stimulation index (SI) was calculated as described in Materials and Methods.

† HLA restriction was studied using mAbs: mouse anti-CD3 (anti-OKT3), mouse anti-CD4 (anti-OKT4), mouse anti-CD8 (anti-OKT8), mouse anti-class I (W6/32), and mouse anti-HLA DP, DQ, and DR (B7/21, Hull, and L243).

‡ Cell surface markers were studied using FITC- or PE-conjugated mAbs, including mAbs to CD3 (anti-Leu4); CD4 (anti-Leu32a), CD8 (anti-Leu2a), CD45RA (2H4), CD45RO (UCHL-1), and TCR.

T cell clone (5×10^4 per well) were cocultured in the presence of Ag for 3 d. [^3H]TdR (0.5 $\mu\text{Ci}/\text{well}$) was pulsed for the last 10 h and the [^3H]TdR incorporation was measured. Each assay was performed in triplicate. We also studied the genomic type of HLA class II in four patients with PBC who were the donors of peptide-specific T cell clones (patient 2: HLA DRB1 0410 1501 DRB4 0101 DRB5 0101; patient 8: HLA DRB1 0405 0803 DRB4 0101; patient 10: HLA DRB1 0405 0901 DRB4 0101; and patient 11: HLA DRB1 0901 1302 DRB3 0301 DRB4 0101), as well as in seven healthy subjects (Fig. 4). All T cell clones, including T-pep-2-1, responded to SP 163-182 presented by allogeneic PBMCs derived from S.S., T.M., K.H., E.B., N.F., and H.I., but not from K.Y. (Fig. 4). The common HLA class II genotype shared by patients 2, 8, 10, and 11, as well as healthy subjects, except for K.Y., was only HLA DRB4 0101 (data of DP or

DQ allele; not shown), indicating that these T cell clones recognized SP 163-182 in the context of HLA DRB4 0101.

Minimal Epitope Mapping of T Cell Clones Specific for PDC-E2. Next, minimal epitope mapping of T cell clones specific for SP 163-182 was performed using eight different peptides composed of 13–20 amino acids residues corresponding to PDC-E2 163-182 (SPs 162-175, 163-175, 164-177, 164-176, 163-176, 163-177, 161-177 and 163-182). As shown in Fig. 5, all T cell clones, including T-pep-2-1, proliferated in response to SP 163-176, but they did not proliferate in response to SP 164-176 or SP 163-175, indicating that the 14-amino acid sequence of PDC-E2 163-176 (GDLLAEIETDKATI) is the minimum T cell epitope in the context of HLA DRB4 0101.

Determination of Amino Acids Critical for Recognition by TCR or for Binding to HLA DRB4 0101 Using Alanine- or Serine-substituted Peptides of SP 163-176. As shown in Figs. 6 and 7, alanine- or serine-substituted SP 163-176 at position 165, 166, 167, 168, 170, 172, or 173 abrogated the proliferative response of the T cell clone T-pep-2-1. Alanine or serine substitution at position 166, 167, 168, 170, 171, 172, or 173 abrogated the proliferative response of clone T-pep-2-2. Alanine substitution at position 166, 170, 172, or 173 abrogated the proliferative response of clone T-pep-10-1. Whereas alanine substitution at only three positions—170, 172, or 173—abrogated the proliferative response of clone T-pep-11-1, alanine substitution at position 169, 170, 172, or 173 abrogated the proliferative response of clone T-pep-11-2. These results indicate that the common essential amino acids for MHC or TCR binding of SP 163-176 are E, D, and K at positions 170, 172, and 173, respectively. In addition, the results of the competition experiments for antigen presentation between nonstimulatory single alanine- or serine-substituted peptide analogues and SP 163-176 are shown in Fig. 8. The preincubation of T-pep-2-2 with alanine- or serine-substituted peptides of SP 163-176 at position 166, 167, 168, 170, 171, or 173 inhibited the proliferative response of clone T-pep-2-2 in response to the original peptide, SP 163-176. In contrast,

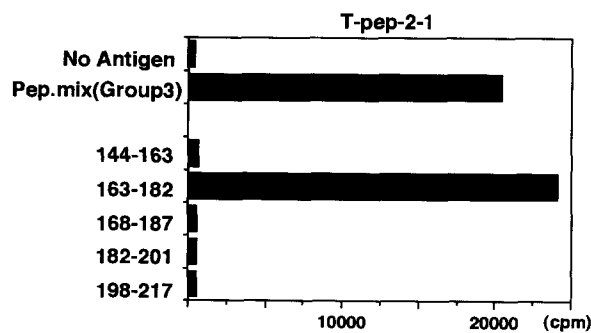


Figure 2. The peptide specificity of T cell clones specific for PDC-E2 peptide mixture group 3. T cells that showed a significant proliferation to peptide mixtures (group 2, 3, or 6) were repeatedly stimulated with the same peptide mixtures in the presence of irradiated autologous PBMCs and IL-2 (20 U/ml). After four to five repeated stimulations, the T cells were cloned at 0.5 cell per well. Six T cell clones specific for PDC-E2 peptide mixture group 3 were obtained from four different patients with PBC. These T cell clones were tested for their proliferative response to each peptide (SPs 144-163, 163-182, 168-187, 182-201, and 198-217) by a [^3H]TdR incorporation assay. All of the T cell clones reacted to SP 163-182, but to none of the other peptides.

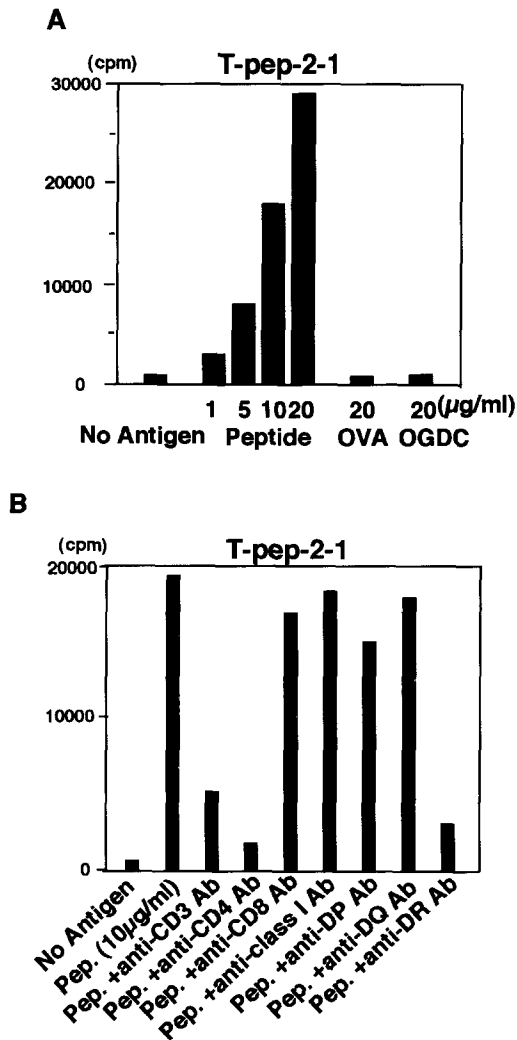


Figure 3. The Ag specificity of T cell clones specific for PDC-E2 peptide 163-182 and the effect of mAbs to cell surface molecules on the proliferative response of T cell clones. (A) T-pep-2-1 was cultured with PDC-E2 peptide 163-182 (1–20 μg/ml), OVA (20 μg/ml), and OGDC (20 μg/ml) in the presence of autologous irradiated PBMCs. After 3 d of culture, [³H]Tdr was pulsed for 10 h, and [³H]Tdr incorporation was measured. T-pep-2-1 proliferated in response to PDC-E2 peptide 163-182 in a dose-dependent manner, but not to OVA or OGDC. (B) T-pep-2-1 was similarly cultured with PDC-E2 peptide 163-182 (10 μg/ml) in the presence of various mAbs: anti-CD3, anti-CD4, anti-CD8, anti-HLA class I, anti-DP, anti-DQ, and anti-DR. After 3 d of culture, [³H]Tdr incorporation was similarly measured. mAbs such as anti-CD3, anti-CD4, and anti-HLA DR inhibited the proliferative response of T-pep-2-1 to PDC-E2 peptide 163-182. The proliferative response of other T cell clones specific for PDC-E2 peptide 163-182 were also studied, and their results were similar to those with T-pep-2-1.

only one peptide, SP 163-176 substituted with alanine at position 172, did not inhibit the proliferative response of clone T-pep-2-2. Similar competition experiment results were obtained for clones T-pep-11-1 and T-pep-11-2. These results indicated that amino acid D at position 172 is a critical MHC-binding site for all T cell clones tested, but that the amino acids critical for the recognition by TCR differed in each T cell clone.

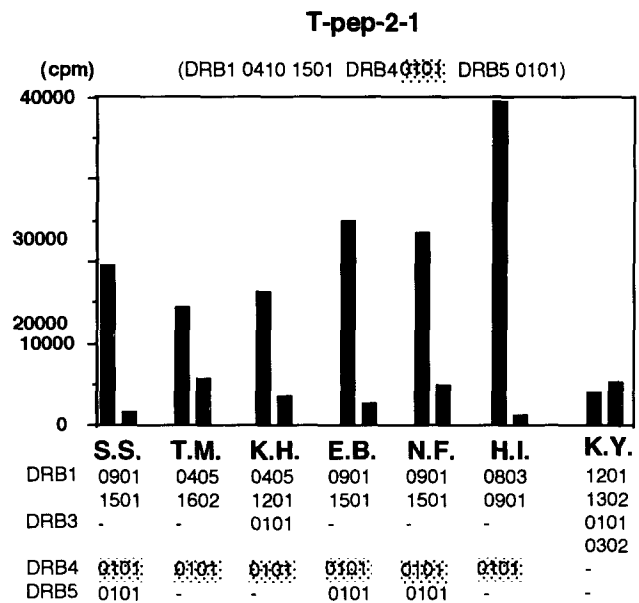


Figure 4. HLA restriction of T cell clones specific for PDC-E2 peptide 163-182. T-pep-2-1 was cultured with PDC-E2 peptide 163-182 (20 μg/ml) (left bar) or without any peptide (right bar) in the presence of irradiated allogeneic PBMCs derived from seven healthy controls with various HLA haplotypes: S.S., T.M., K.H., E.B., N.F., H.I., and K.Y. After 3 d of culture, [³H]Tdr was pulsed for 10 h, and [³H]Tdr incorporation was measured. All of the other T cell clones specific for PDC-E2 peptide 163-182 were similarly studied for their proliferative response to PDC-E2 peptide 163-182. All T cell clones proliferated in response to peptide 163-182 in the presence of allogeneic PBMCs having the HLA DRB4 0101 haplotype.

Proliferative Response of T Cell Clones to Other Peptides That Have Amino Acid Sequence Homology with Human PDC-E2 163-176. We next studied the proliferative response of T cell clones to five different self and nonself peptides: human PDC-E2 36-49 (outer lipoyl domain), *P. putida* BCOADC 104-117, *E. coli* PDC-E2 31-44/134-147/235-248, human glycogen phosphorylase β 354-367, and HLA DR α chain 82-95, which

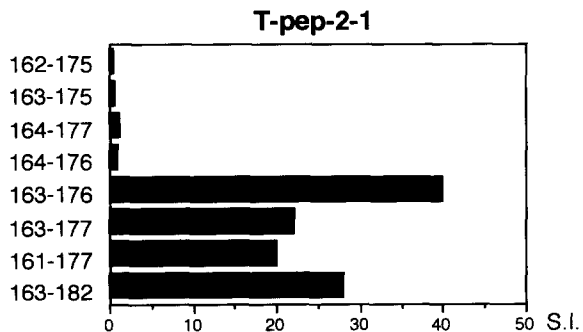


Figure 5. Minimal epitope mapping of T cell clones specific for PDC-E2 163-182. T-pep-2-1 was cultured with SP 162-175, 163-175, 164-177, 164-176, 163-176, 163-177, 161-177, or 163-182 in the presence of autologous irradiated PBMCs. After 3 d of culture, [³H]Tdr was pulsed for 10 h, and [³H]Tdr incorporation was measured. All of other T cell clones specific for PDC-E2 peptide 163-182 were similarly studied for their proliferative response to each peptide. The minimal epitopes of all T cell clones were mapped to 163-176.

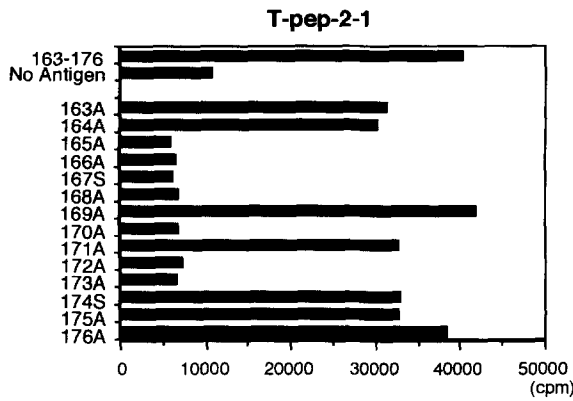


Figure 6. Proliferation of the T cell clones in response to alanine- or serine-substituted peptides of PDC-E2 163-176. T-pep-2-1 was cultured with alanine-substituted peptide 163-176 at positions 163, 164, 165, 166, 168, 169, 170, 171, 172, 173, 175, and 176, and with serine-substituted peptide 163-176 at positions 167 and 174. After 3 d of culture, [³H]TdR was pulsed for 10 h, and the [³H]TdR incorporation was measured.

share amino acid sequence homology with human PDC-E2 163-176 in ratios of 11:14, 9:14, 5:14, 4:14, and 6:14, respectively (40). Human PDC-E2 36-49, *P. putida* BCOADC 104-117, and *E. coli* PDC-E2 31-44/134-147/235-248 have EXDK sequences, and the other two peptides have DXDK or AXDK sequences. As shown in Fig. 9, all T cell clones proliferated in response to human PDC-E2 36-49, which has EXDK sequences and corresponds to the outer lipoyl domain of human PDC-E2. One T cell clone, T-pep-11-2, proliferated in response to *E. coli* PDC-E2 31-44/134-147/235-248, but not *P. putida* BCOADC 104-117, which have EXDK sequences. The other two T cell clones, T-pep-2-2 and T-pep-11-1, did not proliferate in response to these two peptides with EXDK sequences. None of the T cell clones proliferated in response to human glycogen phosphorylase β 354-367 or HLA DR α chain 82-95.

Discussion

Although mitochondrial Ags are present in the inner membranes of mitochondria in every cell type in the body, the autoimmune process in PBC is restricted to the liver; the

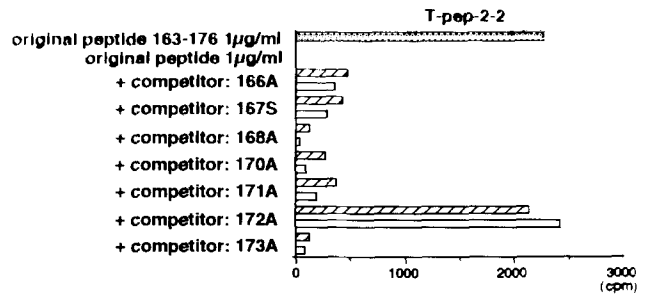


Figure 8. Competition for antigen presentation between nonstimulatory single alanine- or serine-substituted peptide analogues and original PDC-E2 peptide 163-176. T-pep-2-2 was first cultured with 40 or 80 μ g/ml nonstimulatory peptide analogues (alanine-substituted peptides at positions 166, 168, 170, 171, 172, and 173 and serine-substituted peptide at position 167) in the presence of autologous irradiated PBMCs for 1 h. The original PDC-E2 peptide 163-176 was then added to the culture at a final concentration of 1 μ g/ml. After 3 d of culture, [³H]TdR was pulsed for 10 h, and [³H]TdR incorporation was measured. [ZZZ], competitor 40 μ g/ml; [□], competitor 80 μ g/ml.

infiltration of T cells, B cells, and macrophages is distributed mainly around the biliary ductular tissues in the liver (2, 10–12). This organ-specific autoimmune process in PBC, though non-organ-specific target self Ags are present, may be explained by the specific expression of mitochondrial Ags in addition to HLA DR and DQ molecules on or near the luminal surface of biliary epithelial cells in the liver (13–17). B and T cells infiltrating the liver produce AMAs and proliferate in response to PDC or OGDC in vitro, respectively (14, 18–20). E2, E1 α , and E1 β components and protein X of PDC and E2 components of OGDC and BCOADC have also been identified as autoantigens recognized by AMAs (3–9).

Based on these observations, it is very likely that the immune response to mitochondrial Ags, such as PDC, OGDC, and BCOADC in the liver, plays a central role in the pathogenesis of PBC. Therefore, the identification of the immunodominant autoepitopes and HLA molecules involved in the immune response to mitochondrial Ags could possibly clarify the pathogenesis of PBC and establish therapeutic strategies to prevent the occurrence or the progression of PBC. However, thus far, there have been no reports on the T cell

	163	164	165	166	167	168	169	170	171	172	173	174	175	176
	G	D	L	L	A	E	I	E	T	D	K	A	T	I
T-pep-2-1				■		■		■		■	■			
T-pep-2-2				■					■	■				
T-pep-10-1				■										
T-pep-11-1														
T-pep-11-2							■	■		■	■			

Figure 7. The amino acids of PDC-E2 163-176 critical for the recognition by TCR or for the binding to HLA DRB4 0101. Shaded squares indicate the amino acids that abolish the proliferative response of T cell clones by alanine or serine substitution.

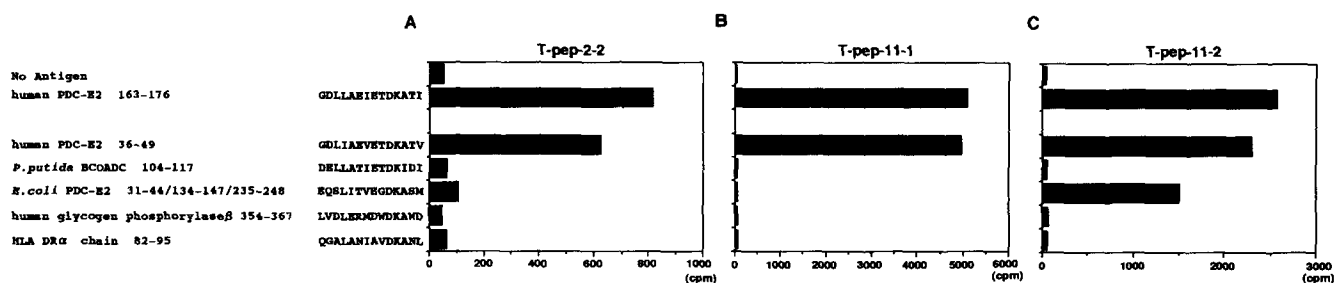


Figure 9. The cross-reactivity of T cell clones specific for PDC-E2 peptide 163-176 with other self or non-self peptides. (A) T-pep-2-2 was cultured with various self or non-self peptides (human PDC-E2 36-49, *P. putida* BCOADC 104-117, *E. coli* PDC-E2 31-44/134-147/235-248, human glycogen phosphorylase β 354-367 and HLA DR α chain 82-95) (20–80 $\mu\text{g/ml}$) in the presence of autologous irradiated PBMCs. After 3 d of culture, [^3H]TdR was pulsed for 10 h, and [^3H]TdR incorporation was measured. T-pep-11-1 (B) and T-pep-11-2 (C) were similarly studied for their proliferative response to various self and non-self antigens.

autoepitopes of mitochondrial Ags at the molecular level or their MHC restriction molecules (14, 18–20).

We previously found that PDC-specific T cells are circulating in the peripheral blood of patients with PBC, and that all T cell lines specific for PDC proliferate in response to PDC-E2, indicating that PDC-E2 contains the immunodominant T cell autoepitopes of PDC (data not shown). Therefore, in the present study, we tried to generate PDC-E2-specific T cell clones by using various peptides covering PDC-E2 as selecting Ags. The characterization of six PDC-E2-specific T cell clones successfully generated in the present study showed the following: (a) minimal T cell autoepitopes of T cell clones specific for PDC-E2 were all located on the same region of PDC-E2 peptide 163-176 (GDLLAEIETDKATI), which corresponds to the inner lipoyl domain of human PDC-E2; (b) all T cell clones specific for human PDC-E2 163-176 are restricted by the same MHC molecule, HLA DRB4 0101; (c) E, D, and K at positions 170, 172, and 173 are common essential amino acid residues for this T cell epitope; (d) all T cell clones specific for human PDC-E2 163-176 react to PDC-E2 peptide 36-49 (GDLIAEVETDKAT), which corresponds to the outer lipoyl domain of human PDC-E2; (e) one T cell clone (T-pep-11-2) cross-reacts with exogenous Ags, such as *E. coli* PDC-E2 peptide 31-44/134-147/235-248 (EQSLITVEGDKASM), which has EXDK sequences, but none of the T cell clones cross-react with *P. putida* BCOADC peptide 104-117 (DELLATIETDKIDI), which has EXDK sequences; and (f) none of the T cell clones cross-react with self-peptides derived from human glycogen phosphorylase β or HLA DR α chain, which share amino acid homology with human PDC-E2 163-176 but do not have EXDK sequences.

It has been hypothesized that PBC may have a bacterial etiology because of the high incidence of recurrent urinary tract infection in patients with PBC (1, 2, 8). Furthermore, the sera from patients with PBC react with both human PDC-E2 and *E. coli* PDC-E2 (8, 9). This is because PDC-E2 peptides are evolutionarily highly conserved among different species, especially in their lipoyl acid-binding sites (4–6). Thus, a mechanism of molecular mimicry has been proposed as a possible cause of PBC (40). In this hypothesis, the T cells first recognize the lipoyl domain of *E. coli* PDC-E2 containing

EGDKA sequences. Then, these T cells cross-react with self peptides containing AVDKA sequences from HLA DR α chain or ETDKA sequences from human PDC-E2 by aberrantly expressed class II HLA molecules on biliary epithelial cells (13–17). Finally, this initiates the autoimmune cascade leading to the destruction of the bile ducts in which AMAs and/or autoreactive T cells specific for mitochondrial Ags may play a pathogenic role. At this stage, the autoimmune process takes place in the absence of the exogenous Ags, such as *E. coli* PDC-E2, which initiated the immune response. In some animal models of autoimmune diseases, such as experimental allergic encephalomyelitis and autoimmune oophoritis, the mechanism of molecular mimicry has been proven to be operative in vivo (41–47).

In this study, we demonstrated the presence of a T cell clone specific for human PDC-E2 163-176 (GDLLAEIETDKATI) that cross-reacts with the lipoyl acid-binding site of *E. coli* PDC-E2 (EQSLITVEGDKASM). This is the proof that molecular mimicry between human and *E. coli* PDC-E2 can be operative at the T cell clonal level in PBC. On the other hand, none of T cell clones specific for PDC-E2 cross-reacted with HLA DR α chain peptide 82-95 (QGALANIAVDKANL) or human glycogen phosphorylase β peptide 354-367 (LVDLERMDWDKAWD), which share amino acid homology with PDC-E2 but have no EXDK sequences. This finding indicates that the presence of amino acid homology per se is not sufficient to provoke the cascade of molecular mimicry. Moreover, it is important that clone T-pep-11-2 cross-reacted with *E. coli* PDC-E2 (EQSLITVEGDKASM), but not with *P. putida* PDC-E2 (DELLATIETDKIDI), which not only has EXDK sequences, but also has greater amino acid homology with human PDC-E2 163-176 than *E. coli* PDC-E2 (EQSLITVEGDKASM). To explain this phenomenon of cross-reactivity at the T cell clonal level, it will be necessary to dissect the molecular mechanism of the MHC class II-peptide-TCR interaction.

The recognition of peptide fragments in the context of class II MHC molecules by T cells is a central event in the development of the immune response (48–50). Proteolytic fragments of peptides processed by APCs that match the physicochemical character of the peptide-binding grooves formed by α and β chains of class II MHC molecules are expressed

on the surface of APCs and are recognized by T cells (48–50). The structural motifs of peptides capable of binding to HLA DR have been determined based on the peptide sequences eluted from purified DR complexes (51, 52) or phage peptide libraries (53, 54). These reports indicated that only certain residues are involved in allele-specific peptide binding, whereas the other residues are promiscuous (51–54). An analysis of the crystal structure of HLA DRB1 0101 protein complexed with an influenza virus peptide demonstrated the exact structural explanation for the MHC class II–peptide–TCR interaction at the molecular level (50). Because polymorphic residues of DR alleles are scattered within the peptide-binding grooves, different DR molecules are capable of binding peptides with different structural motifs, and this phenomenon contributes to the HLA-linked polymorphism of the immune response: DRB1 0405 confers increased susceptibility to rheumatoid arthritis, but not to insulin autoimmune syndrome, whereas DRB1 0406 conversely confers susceptibility to the latter but not to the former.

To date, motifs for binding peptides have been reported in various DR molecules, including DR1 (DRB1 0101), DR2 (DRB1 1501), DR3 (DRB1 0301), DR4 (DRB1 0401, DRB1 0405, DRB1 0406), DR11 (DRB1 1101), DR7 (DRB1 0701), and DR8 (DRB1 0801) (24, 52–55), indicating that two or three hydrophobic amino acids are necessary for the binding of the peptide to HLA hydrophobic pockets. However, no peptide-binding motif of HLA DRB4 0101 has been reported. Although the precise analysis of the peptide-binding motif of HLA DRB4 0101 is now underway using HLA DRB4 0101–transfected L cells and various modified peptides based on PDC-E2 peptide 163-176 (GDLLAEIETDKATI), our preliminary study indicates that LXXIXXD is an HLA DRB4 0101-binding motif. If this is the case, the E and K at positions 170 and 173 may be crucial TCR-binding sites, and the amino acid at position 169 (I for human and *P. putida* PDC-E2 and V for *E. coli* PDC-E2) may have an important effect on the structure of the TCR-binding site. The rationale for the development of molecular mimicry at the T cell clonal level is now under investigation using T cell clones specific for human PDC-E2 163-176 (GDLLAEIETDKATI) and/or *E. coli* PDC-E2 (EQSLITVEGDKASM) and various peptides that share amino acid homology with these peptides.

In Caucasians, PBC has been reported to be associated with HLA DR3 (56), HLA DRw8 (57), and possibly HLA DR4 (58). An association with HLA DR2 (59) and DP B1 0501 (60) has been reported in Japanese patients with PBC. In other studies, no such associations were found with HLA Ags (61). Thus, no consensus seems to exist concerning the associa-

tions between PBC and HLA. In the present study, all T cell clones from four different patients with PBC recognized human PDC-E2 163-176 in the context of the same HLA allele, HLA DRB4 0101, indicating that PDC-E2 peptide 163-176 plays a central role in at least all patients with PBC who have the HLA DRB4 0101 allele. The ratio of HLA DRB4 0101 positivity among patients with PBC is ~77%. This is similar to the control Japanese population (62), indicating that the possession of HLA DRB4 0101 may not be a genetic predisposition for PBC. However, in contrast to the highest degree of polymorphism of HLA DRB1, polymorphism of HLA DRB4 0101 has not been reported. Therefore, it is possible that the HLA DRB4 0101-binding PDC-E2 peptide identified in the present study could provide important information for the development of peptide-specific immunotherapy in the majority of patients with PBC who have an HLA DRB4 0101 allele.

Interestingly, the T cell epitope of PDC-E2 peptide 163-176 almost completely overlaps with the B cell epitopes that were identified in the inner lipoyl domain of PDC-E2 (PDC-E2 165-184 with lipoic acid located at K at 174) (7–9). In addition, all T cell clones specific for PDC-E2 peptide 163-176 cross-reacted with PDC-E2 peptide 36-49, which corresponds to the outer lipoyl domain of PDC-E2. This PDC-E2 outer lipoyl domain was also shown to be the immunodominant B cell epitope of PDC-E2 that cross-reacts with the B cell epitope of the inner lipoyl domain of PDC-E2 (7–9). Recently, “universal” T cell epitopes of certain viruses have been found to be located near the B cell epitopes, indicating that the regions containing both T and B cell epitopes can be highly immunogenic (37, 63–66). Although there have been no reports theoretically showing the exact relationship between T and B cell epitopes of self Ags, our results may indicate the possibility that peptides of self Ags containing both T and B cell epitopes can be highly immunogenic autoepitopes that cause various autoimmune responses. In this aspect, we speculate that the region corresponding to or overlapping PDC-E2 peptide 163-176 can also be a T cell autoepitope in the context of MHC class II molecules other than the HLA DRB4 0101 allele. We are now trying to identify the T cell autoepitopes in patients with PBC who have no HLA DRB4 0101 allele.

In conclusion, the present study not only is a definite demonstration of an immunodominant T cell autoepitope of PDC-E2, but it is also a demonstration of the molecular mimicry that is operative at the T cell clonal level in PBC. Based on these findings, it is considered possible to develop a strategy for the peptide-specific immunotherapy in PBC.

We thank Dr. S. Matsushita of the Division of Immunogenetics, Department of Neuroscience and Immunology, Kumamoto University; Dr. N. Kamikawaji of the Division of Genetics (Director: Prof. T. Sasazuki); and Dr. M. Nakashima of the Division of Molecular Immunology (Director: Prof. T. Watanabe), Medical Institute of Bioregulation, Kyushu University, for their helpful comments and discussion. We also thank Dr. T. Mukai of the First Department of Internal Medicine, Faculty of Medicine, Kyushu University,

and Dr. A. Kimura and Dr. T. Sasazuki of the Division of Genetics, Medical Institute of Bioregulation, Kyushu University, for the DNA typing of HLA class II genes.

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (06670488).

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Received for publication 24 August 1994 and in revised form 22 November 1994.

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