

## Catalytic domain of PDC-E2 contains epitopes recognized by antimitochondrial antibodies in primary biliary cirrhosis

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### Abstract

**AIM:** To search for further immunodominant peptides of the pyruvate dehydrogenase complex E2-component (PDC-E2) recognized by antimitochondrial antibodies (AMA) in primary biliary cirrhosis (PBC).

**METHODS:** Sera from 95 patients with PBC were tested by enzyme-linked immunosorbent assay against 33 synthetic overlapping peptides (25 amino acids; aa) covering the entire length of the E2-subunit of PDC-E2. Furthermore, the inner lipoyl peptide 167-184 was used in an unlipoylated and a lipoylated form as well as coupled to ovalbumin. Sera from 11 AMA negative/ANA positive PBC patients, 63 patients with other liver disorders and 22 healthy blood donors served as controls.

**RESULTS:** Of the 95 PBC-sera, 74% reacted with the peptide 475-499 and 58% with the peptide 407-431 located within the catalytic domain of PDC-E2. Patients with other disorders or healthy controls were positive in only up to 18%. Antibodies to the unlipoylated

and lipoylated peptide 167-184 within the inner lipoyl domain were found in only 5% and 11% of the PBC sera, respectively; using ovalbumin-coupled peptides, the incidence increased up to 57% (unlipoylated form).

**CONCLUSION:** Peptides within the catalytic site of PDC-E2 rather than the previously reported lipoyl binding peptide 167-184 may represent major immunodominant epitopes recognized by AMA in PBC.

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**Key words:** Anti-M2; Epitope mapping; E2-subunit; Pyruvate dehydrogenase complex; Inner lipoyl domain; Active site; Catalytic domain; Primary biliary cirrhosis

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### INTRODUCTION

Antimitochondrial antibodies (AMA) are one of the most important criteria for the diagnosis of primary biliary cirrhosis (PBC), a chronic cholestatic liver disease of unknown etiology, which affects mainly middle-aged women and leads to a destruction of small bile ducts. Their target antigen named M2<sup>[1,2]</sup> is attached to the

inner mitochondrial membrane<sup>[3]</sup> and consists of five components<sup>[4]</sup>, which have been identified in the following years on molecular bases as subunits of the 2-oxo acid dehydrogenase complex of the inner mitochondrial membrane: the pyruvate dehydrogenase complex (PDC), the 2-oxoglutarate dehydrogenase complex and the branched-chain 2-oxo acid dehydrogenase complexes<sup>[5-7]</sup>. Each complex comprises multiple copies of three component enzymes termed E1 (a thiamine pyrophosphate-dependent decarboxylase), E2 (a coenzyme A-dependent decarboxylase), and E3 (a dihydrolipoyl dehydrogenase). The major M2-antigen which is recognized by nearly 90% of PBC sera is the E2 component of PDC.

The immunodominant epitope within PDC-E2 has been mapped to the region associated with the inner lipoyl domain wherein a lysine residue binds the lipoic cofactor for the enzyme with a minimum of 75 residues necessary for characteristic antibody recognition<sup>[6,8,9]</sup> suggesting that a conformational autoepitope may be primarily recognized<sup>[9]</sup>. A minor epitope is associated with the outer lipoyl domain<sup>[6,10]</sup>. Hitherto, epitope mapping with PBC sera has linked AMA-reactivity to the highly conserved amino acids surrounding the lipoyl-lysine K173, particularly linear peptides AEIETDKATIGFEVQEEG (corresponding to aa 167-184 within the human PDC-E2 but primarily labeled aa 81-100<sup>[6]</sup> according to its location within a subclone pRMIT-603 used to identify the immunodominant epitope) or LLAEIETDKATIGF (165-178)<sup>[11]</sup>. It was shown that these short peptides absorbed most reactivity with PBC sera by enzyme-linked immunosorbent assay (ELISA), albeit only at a serum dilution of 1:80000<sup>[6]</sup>. Binding of lipoic acid cofactor to K173 has been discussed to be necessary for antibody reaction<sup>[10,12]</sup>.

Until now, the large fragment of the catalytic domain of PDC-E2 (aa 331-560) has been regarded as 'immunologically silent' due to negative results in several studies<sup>[6,9,12-14]</sup>. In the present study we used peptides spanning the whole PDC-E2 enzyme, and it will be shown that sera from PBC patients recognize epitopes within the catalytic domain in an even higher incidence than those in the inner lipoyl domain.

## MATERIALS AND METHODS

### Patients

Sera from 95 patients with clinically well defined PBC were analyzed. All patients had been seen by one of the authors (Berg C), and all were AMA/anti-M2-anti-PDC-E2 positive. Detailed clinical, biochemical and histological data are given in Table 1.

Liver biopsy had been performed in 65 of the patients and was interpreted by Dr. Bianchi L, Professor, University of Basel, Switzerland. All patients had been followed for more than 5 years (range: 5-28 years). At time of first serum analysis, 65 had not yet received any therapy, 30 were already treated with ursodeoxycholic acid (at least 6 mo, doses 1000-1500 mg/d).

**Table 1 Clinical, biochemical, histological and serological data in 95 patients with PBC (mean  $\pm$  SD)**

Parameters	
Females:males	88:7
Age (yr)	
mean $\pm$ SD	50 $\pm$ 9.8
Range	21-70
Biochemical parameters <sup>1</sup>	
AP (U/L) (normal < 120)	453 $\pm$ 451
GT (U/L) (normal < 50)	154 $\pm$ 143
ASAT (U/L) (normal < 35)	39.4 $\pm$ 42.6
ALAT (U/L) (normal < 35)	51 $\pm$ 49.3
Cholinesterase (kU/L) (normal > 5)	4.7 $\pm$ 1.7
Bilirubin (mg%) (normal < 1.5)	1.62 $\pm$ 0.8
Cholesterol (mg/dL) (normal < 200)	251 $\pm$ 69
Eosinophils (%) (normal < 4)	3.61 $\pm$ 2.36
IgG globulins (mg%) (normal < 1800)	1679 $\pm$ 855
IgA globulins (mg%) (normal < 400)	269 $\pm$ 158
IgM globulins (mg%) (normal < 280)	416 $\pm$ 255
Histological data <sup>2</sup>	
PBC stage I / II (n)	45
PBC stage III / IV (n)	20
Serological data	
Antimitochondrial antibodies (number positive)	
AMA (IFT) (titer > 1:160)	95
Anti-M2 (ELISA)	
Total	95
IgG type	87
IgM type	72
Anti-PDC-E2 (ELISA)	
Total	95
Antinuclear antibodies (number positive) (titer > 1:160)	
Nuclear dots (sp 100)	16
Nuclear membrane (gp 210)	10
Centromeres	8

<sup>1</sup>Referring only to the 65 untreated patients; <sup>2</sup>Liver biopsy had been performed in 65 patients; 42 of them were untreated, and of these patients 30 were in stage I / II and 12 in stage III / IV. IFT: Immunofluorescence test; ELISA: Enzyme-linked immunosorbent assay; PBC: Primary biliary cirrhosis; PDC-E2: Pyruvate dehydrogenase complex E2-component.

Furthermore, sera from 11 patients with clinically and histologically proven PBC being AMA negative but ANA positive were investigated. Five had antibodies to nuclear dots (sp 100), three to nuclear membranes (gp 210) and two to centromeres.

The investigations have been approved by the local Ethics committee.

### Control groups

As controls we used sera from 36 patients with untreated autoimmune hepatitis, 27 patients with alcoholic liver disease, and 32 patients with collagen disorders. All patients have been seen and followed either by Berg C or Klein R. Diagnosis was based on clinical, biochemical and serological parameters.

Sera from 22 healthy blood donors (kindly provided by Dr. Wernet D, Professor, Institute for Transfusion-medicine, University of Tübingen, Germany) were used as controls, all being negative for AMA/anti-M2 as shown by immunofluorescence test (IFT), ELISA and Western blotting. All sera were stored at -20°C.



**Figure 1** Amino acid (aa) sequence of the human pyruvate dehydrogenase complex E2-component (PDC-E2). For epitope mapping 25 mer peptides with 8 overlapping amino acids were constructed (see also Table 2). Overlapping amino acids are printed in bold; amino acid sequences of the immunodominant lipoyl binding epitopes in the outer (aa 41-53) and inner lipoyl domain (aa 167-183) are underlined. \*Lipoyl binding lysine in the outer and inner lipoyl domains; \*\*Active sites within the catalytic domain (S480 and H534).

## Antigens

The M2-antigen was released from beef heart submitochondrial particles by chloroform treatment as described<sup>[2]</sup>. Commercial PDC (from porcine heart) was obtained from Sigma-Aldrich (St. Louis, USA).

Thirty-three synthetic overlapping peptides covering the entire length of PDC-E2 were obtained from Biotrend (Cologne, Germany). Peptides were 25 amino acids (aa) long with 8 overlapping aa (Figure 1).

Peptide 11 contained the immunodominant decameric epitope consisting of aa IETDKATIGF as reported by Van de Water *et al*<sup>[6]</sup>, which had been extended to a 15 amino acid peptide by Amano *et al*<sup>[15]</sup> (IETDKATIGFEVQEE). All peptides were high-performance liquid chromatography (HPLC)-purified (more than 90% purity). An irrelevant peptide was used as background control. The peptides were reconstituted at 5 mg/mL DMSO and stored at -20°C.

The immunodominant peptide 167-184 (AEIETDKATIGFEVQEEG) was synthesized (Biotrend, Cologne, Germany) in an unlipoylated form and a form in which lipoic acid was coupled to lysine at position 173 (167-184-LA)<sup>[12]</sup>. Purity and conjugation was proven by HPLC (elution time 984.5 m/z vs 1076.7 m/z; purity > 95%). Since these peptides may be recognized only when attached to a carrier<sup>[12]</sup>, both forms of peptides were also coupled to ovalbumin (Biotrend, Cologne, Germany). For this purpose, at the C-terminal end a cysteine had to be added (AEIETDKATIGFEVQEEGC-OVA) which had to be substituted by a lysine for coupling the LA-

conjugated [AEIETDK<sub>(alpha-lipoic)</sub>ATIGFEVQEEGK-OVA]peptide. The presence of the hydrophobic lipoic acid moiety covalently bound to the peptide was again confirmed by the significant differences in HPLC-elution profiles when peptides without or with LA were analyzed (elution time 1.067.8 m/z vs 1.163.3 m/z; purity > 93%).

## IFT

In the IFT cryostat sections from rat liver, kidney, heart, stomach and human thyroid were used to detect AMA and other autoantibodies<sup>[16]</sup>.

## ELISA

The ELISA for the detection of anti-M2/PDC-E2 antibodies was performed as described<sup>[17]</sup>.

Antibody reactivity with PDC-E2 peptides was determined using microtiter plates (Maxisorp, Nunc, Denmark) coated with 100 µL of each peptide at a concentration of 25 µg/mL in coating buffer (hydrogen bicarbonate buffer, 0.2 mol/L, pH 9.6) overnight at 4°C. After extensive washing and blocking with phosphate buffered saline (PBS) (60 mmol/L, pH 7.4) containing 1% bovine serum albumin (BSA) for 60 min they were incubated with 100 µL of patients' sera at a dilution of 1:500 for 90 min at room temperature. After washing with PBS containing 0.2% Triton X 100 and 0.5% BSA, the wells were incubated with peroxidase-conjugated monovalent anti-human IgG- and IgM-antibodies from goat (Dianova, Hamburg, Germany; dilution 1:3000) for 60 min at room temperature, washed as above, and AMA reactivity detected using orthophenyldiamine as substrate. Antibody reactivity was given as absorbance × 1000.

Optimal peptide concentrations (25 µg/mL) and serum dilutions (1:500) had been determined by serial dilutions prior to the study.

Normal ranges for antibody reactivities with all antigens/peptides were determined by analysis of 22 healthy donors. Mean value of their absorbance plus double the standard deviation was defined as cut off value.

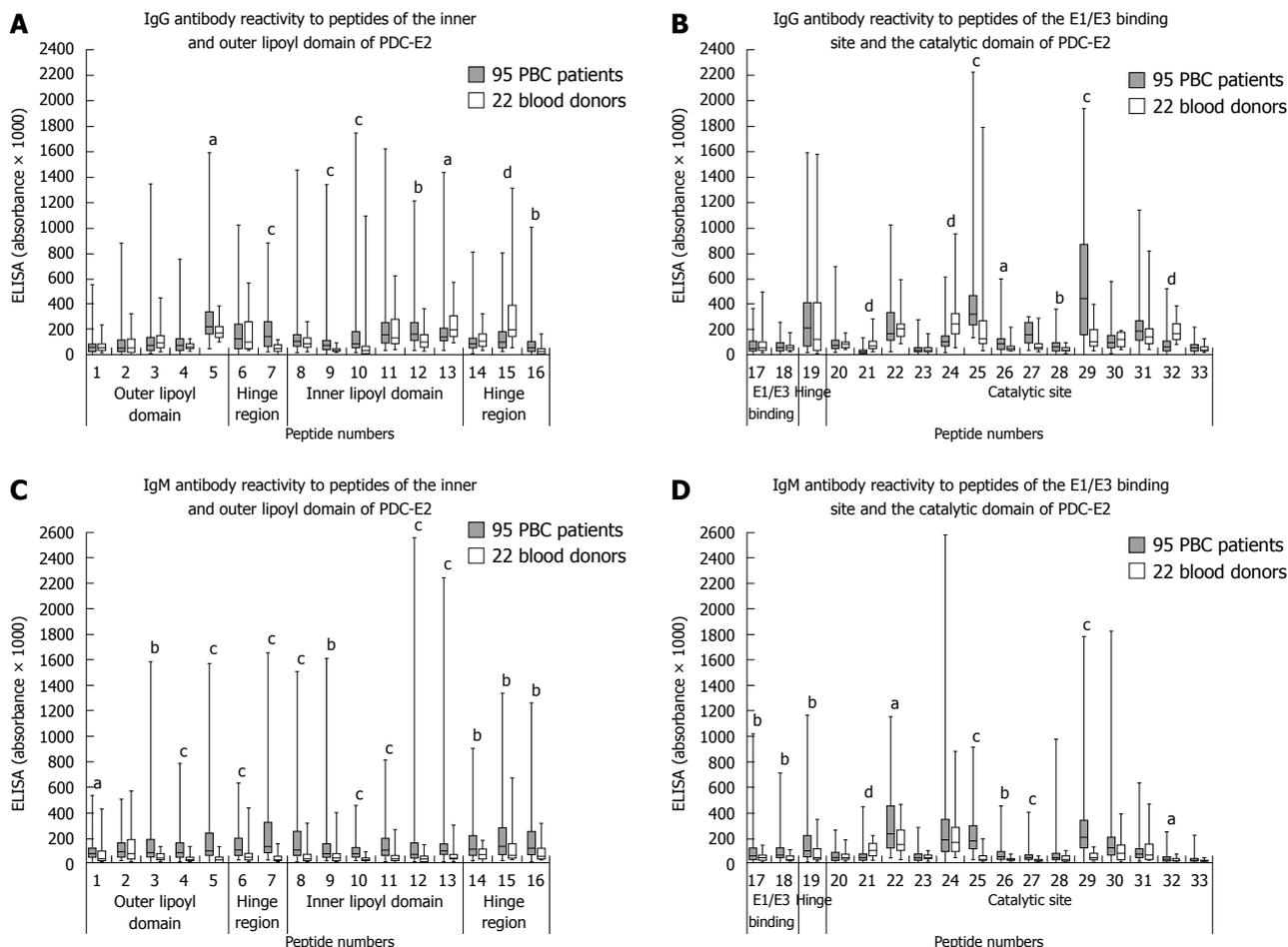
## Statistical analysis

For the comparison of antibody reactivity in different groups of patients, SPSS version 15.0 was used applying the non parametric Mann-Whitney test. For analysis of paired data the Wilcoxon signed rank test was used. Differences with  $P < 0.05$  were considered statistically significant.

## RESULTS

### High incidence of antibodies to epitopes within the catalytic site of PDC-E2 in PBC sera

Sera from 95 patients with PBC all being anti-M2/PDC-E2 positive in the ELISA using the purified M2-antigen and the commercially available PDC were tested



**Figure 2** Box plots showing the reactivity of sera from 95 primary biliary cirrhosis (PBC) patients (grey bars) and 22 blood donors (white bars) with 33 overlapping peptides spanning the whole PDC-E2 sequence. IgG antibody reactivities. A: Peptide 1-16; B: Peptide 17-33; IgM antibody reactivities. C: Peptide 1-16; D: Peptide 17-33. Solid bars extend from the 25th to 75th percentile. The line in the middle is the median. The whiskers extend down to the lowest value and up to the highest value. Significantly higher antibody reactivity with PBC sera than with sera from healthy individuals: <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01, <sup>c</sup>*P* < 0.001. Significantly lower antibody reactivity with PBC sera than with sera from healthy individuals: <sup>d</sup>*P* < 0.001. ELISA: Enzyme-linked immunosorbent assay.

against 33 peptides (25 mers) spanning the whole sequence of PDC-E2.

Surprisingly, the reactivity of the PBC sera with the peptides 3, 10 and 11 containing the immunodominant lipoyl binding epitopes in the outer (aa 41-53) and inner lipoyl domain (aa 167-183)<sup>[6,10]</sup> was rather low although significantly higher than that of healthy controls (Figure 2A and C). Only 11% had IgG-antibodies to peptide 3, 29% to peptide 10, and 12% to peptide 11; IgM-antibodies were observed in 28%, 37%, and 39%, respectively. In contrast, up to 74% of the PBC sera reacted with the two peptides 25 and 29 within the catalytic domain and the peptide 7 (aa 101-125) in the first hinge region (Tables 2 and 3), and IgG and IgM reactivity towards these three peptides was significantly higher in PBC patients than in controls (Figure 2).

The peptides 15, 21 and 24 were the only ones whose reactivity was lower with PBC sera as compared to control sera (Figure 2A, B and D).

Most PBC sera recognized several peptides in parallel. IgM-antibodies generally reacted with a higher diversity of peptides than IgG-antibodies (IgG: mean ± SD: 5.3 ± 7.7 peptides, median: 3 peptides, range: 0-30

peptides; IgM: mean ± SD: 9 ± 7.1 peptides, median: 7 peptides, range: 0-28 peptides). Two PBC sera had neither IgG- nor IgM-antibodies to any of the 33 peptides although both sera showed high antibody reactivities towards PDC and M2 in the ELISA.

Sera from patients with AMA negative/ANA positive PBC hardly reacted with any of the peptides (Tables 2 and 3).

Incidence and reactivity of antibodies to the different peptides in sera from patients with autoimmune hepatitis, alcoholic liver disease and collagen disorders resembled that in healthy controls (data not shown).

**Reactivity of PBC-sera with the inner lipoyl domain epitope aa 167-184 using different conjugates**

In view of the fact that the 25 mers peptide 10 (aa 152-176) and 11 (aa 169-193) used in the epitope mapping above did not completely respond to the published sequence 167-184 (AEIETDKATIGFEVQEEG), we also synthesized the latter peptide in an unlipoylated form and a form containing lipoic acid (LA) at aa K173 (peptide 167-184-LA) and applied both forms in the ELISA. In accordance to a previous study<sup>[12]</sup>, also with

**Table 2** Reactivity and incidence of IgG-antibodies to 33 PDC-E2 peptides in PBC patients as compared to healthy individuals *n* (%)

	Peptide No.	Amino acid	PBC patients		Healthy individuals ( <i>n</i> = 22)
			Anti-M2+ ( <i>n</i> = 95)	AMA-/ANA+ ( <i>n</i> = 11)	
Outer lipoyl domain (aa 1-80)	1	-2-23	4 (4)	0	1 (5)
	2	16-40	6 (6)	0	2 (9)
	3	33-57	10 (11)	0	2 (9)
	4	50-74	15 (16)	0	0
	5	67-91	26 (27)	0	2 (9)
Hinge	6	84-108	17 (18)	0	3 (14)
	7	101-125	42 (44) <sup>1</sup>	0	0
Inner lipoyl domain (aa 133-217)	8	118-142	14 (15)	0	1 (5)
	9	135-159	32 (34)	0	0
	10	152-176	28 (29)	0	2 (9)
	11	169-193	11 (12) <sup>2</sup>	0	2 (9)
	12	186-210	16 (17)	0	1 (5)
Hinge	13	203-227	6 (6)	0	3 (14)
	14	220-244	4 (4)	0	2 (9)
	15	237-261	3 (3)	0	4 (18)
	16	254-278	20 (21)	0	1 (5)
E1/E3-binding site (aa 272-303)	17	271-295	3 (3)	0	1 (5)
	18	288-312	7 (7)	0	1 (5)
Hinge	19	305-329	19 (20)	0	3 (14)
Catalytic site (aa 331-561)	20	322-346	12 (13)	2 (18)	1 (5)
	21	339-363	0	0	1 (5)
	22	356-380	19 (20)	0	1 (5)
	23	373-397	2 (2)	1 (9)	1 (5)
	24	390-414	1 (1)	1 (9)	2 (9)
	25	407-431	53 (56) <sup>3</sup>	0	4 (18)
	26	424-448	10 (11)	0	1 (5)
	27	441-465	5 (5)	0	2 (9)
	28	458-482	18 (19)	0	0
	29	475-499	55 (58) <sup>4</sup>	0	1 (5)
	30	492-516	9 (9)	0	0
	31	509-533	17 (18)	0	2 (9)
	32	526-550	2 (2)	0	1 (5)
	33	543-561	11 (12)	0	1 (5)

<sup>1</sup>Twelve patients had antibodies only of the IgG-, 28 only of the IgM-, and 30 of the IgG- and IgM-type; in total, 70 (71%) reacted with peptide 7; <sup>2</sup>Four patients had antibodies only of the IgG-, 30 only of the IgM-, and 7 of the IgG- and IgM-type; in total, 41 (43%) reacted with peptide 11; <sup>3</sup>Seventeen patients had antibodies only of the IgG-, 18 only of the IgM-, and 37 of the IgG- and IgM-type; in total, 72 (76%) reacted with peptide 25; <sup>4</sup>Two had antibodies only of the IgG-, 16 only of the IgM-, and 53 of the IgG- and IgM-type; in total, 71 (75%) reacted with peptide 29. Similar data as for healthy controls were obtained with sera from patients with autoimmune hepatitis, alcoholic liver disease and collagen disorders. Peptides 3, 10, 11, 25 and 29 are previous and novel immunodominant epitopes. +: Positive; -: Negative.

these peptides reactivity of the 95 PBC sera was low (Table 4).

In order to find out whether the antibodies may recognize a conformational epitope the peptides were coupled to ovalbumin (OVA-peptide 167-184 and OVA-peptide 167-184-LA)<sup>[12]</sup>. With these conjugated peptides reactivity was stronger, but most sera showed a high reactivity already with OVA alone (Figure 3). IgG- and IgM-reactivity towards the unlipoylated OVA 167-184 was significantly higher than that towards the lipoylated form (OVA 167-184-LA), reactivity towards both forms of peptides was significantly higher than that against OVA alone.

**Table 3** Reactivity and incidence of IgM-antibodies to 33 PDC-E2 peptides in PBC patients as compared to healthy individuals *n* (%)

	Peptide No.	Amino acid	PBC patients		Healthy individuals ( <i>n</i> = 22)
			Anti-M2+ ( <i>n</i> = 95)	AMA-/ANA+ ( <i>n</i> = 11)	
Outer lipoyl domain (aa 1-80)	1	-2-23	7 (7)	0	1 (5)
	2	16-40	10 (11)	0	2 (9)
	3	33-57	27 (28)	0	0
	4	50-74	33 (35)	1 (9)	1 (5)
	5	67-91	42 (44)	0	1 (5)
Hinge	6	84-108	31 (33)	0	1 (5)
	7	101-125	52 (55) <sup>1</sup>	1 (9)	2 (9)
Inner lipoyl domain (aa 133-217)	8	118-142	34 (36)	0	1 (5)
	9	135-159	35 (37)	0	2 (9)
	10	152-176	35 (37)	0	1 (5)
	11	169-193	37 (39) <sup>2</sup>	0	2 (9)
	12	186-210	27 (28)	0	1 (5)
Hinge	13	203-227	23 (24)	0	2 (9)
	14	220-244	26 (27)	0	0
	15	237-261	43 (45)	0	3 (14)
	16	254-278	33 (35)	0	1 (5)
E1/E3-binding site (aa 272-303)	17	271-295	15 (16)	0	0
	18	288-312	24 (25)	0	1 (5)
Hinge	19	305-329	36 (38)	0	3 (14)
Catalytic site (aa 331-561)	20	322-346	1 (1)	0	0
	21	339-363	4 (4)	0	0
	22	356-380	24 (25)	0	1 (5)
	23	373-397	10 (11)	0	0
	24	390-414	23 (24)	1 (9)	3 (14)
	25	407-431	55 (58) <sup>3</sup>	0	1 (5)
	26	424-448	21 (22)	0	0
	27	441-465	16 (17)	0	0
	28	458-482	15 (16)	0	0
	29	475-499	70 (74) <sup>4</sup>	0	1 (5)
	30	492-516	14 (15)	0	2 (9)
	31	509-533	6 (6)	0	2 (9)
	32	526-550	4 (4)	0	0
	33	543-561	9 (9)	0	1 (5)

<sup>1,2,3,4</sup>Same as Table 2. Peptides 3, 7, 10, 11, 25 and 29 are previous and novel immunodominant epitopes.

After subtracting the reactivities with OVA from that with the OVA-coupled peptides, 42 (44%) of the 95 PBC sera had IgG-antibodies to the unlipoylated (OVA 167-184) and 21 (22%) to the lipoylated peptide (OVA 167-184-LA) (Table 4). IgM antibodies were found in 57% and 31%, respectively.

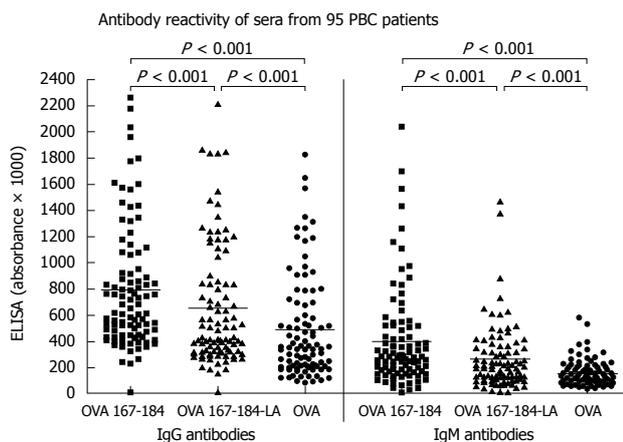
#### **Influence of UDCA-treatment on anti-peptide reactivities**

In order to see whether UDCA-treatment may influence the reactivity with distinct peptides we compared the reaction of 65 patients who were without any therapy at the time of serological analysis with that of 30 patients who received UDCA for at least 6 mo. However, no significant differences in antibody titers were observed for any of these antigens/peptides between the two groups (data not shown), and also mean number of peptides recognized by each patient did not differ [IgG: untreated group: mean  $\pm$  SD 5.7  $\pm$  6 peptides, median 4 peptides, treated group: 4.5  $\pm$  5.1 peptides, median 2 peptides ( $P$  = 0.11); IgM: untreated group: 9.2  $\pm$  7 peptides, median

**Table 4** Reactivity of sera from 95 anti-M2 positive PBC patients with different peptide conjugates containing the reported immunodominant epitope of PDC-E2<sup>[6,12]</sup>

Peptides of the inner lipoyl domain	IgG-antibodies			IgM-antibodies		
	Positive n (%)	mean absorbance × 1000 ± SD (median)	Cut off value	Positive n (%)	mean absorbance × 1000 ± SD (median)	Cut off value
152-176 (present study peptide 10)	28 (29)	153 ± 243 (87)	150	35 (37)	95 ± 77 (73)	80
169-193 (present study peptide 11)	11 (12)	210 ± 219 (157)	300	37 (39)	143 ± 120 (101)	120
167-184	0	113 ± 63 (93)	100	5 (5)	53 ± 79 (20)	100
167-184-LA	3 (3)	135 ± 71 (111)	100	10 (11)	78 ± 107 (40)	100
OVA-167-184 <sup>1</sup>	42 (44)	316 ± 260 (271)	100	54 (57)	254 ± 318 (117)	100
OVA-167-184-LA <sup>1</sup>	21 (22)	175 ± 280 (148)	100	29 (31)	113 ± 217 (52)	100

<sup>1</sup>After subtraction of reactivity to OVA alone.



**Figure 3** IgG- and IgM-reactivity of sera from 95 PBC patients with the OVA coupled unlipoylated and lipoylated peptide 167-184 (OVA-167-184 and OVC-167-184-LA) (without subtraction of the OVA-values) and OVA alone.

7 peptides, treated group: 8.5 ± 7 peptides, median 6 peptides (*P* = 0.65)].

## DISCUSSION

The inner lipoyl domain of PDC-E2 and especially the peptide aa167-184 has been previously considered the prominent immunodominant epitope recognized by sera from PBC-patients, although there is no general consensus<sup>[6,10,11,15,18-20]</sup>. In these analyses sera from PBC patients were incubated with the peptide, and it was shown that it absorbed the anti-M2 activity, albeit only at high serum dilutions. In other studies fusion proteins were used containing the peptide and tested by ELISA against PBC sera revealing positive results. However, using this peptide in the ELISA, no significant reactivity with PBC sera was observed<sup>[5,12]</sup>. There was evidence that antigenicity could be improved after coupling the peptide to ovalbumin and/or lipoylation of K173<sup>[12]</sup>, but this is also still controversially discussed. Accordingly, the uncertainty on reactivity with the linear epitope and an absolute requirement for lipoylation of PDC-E2 for antibody reactivity strongly suggests that rather a conformational epitope within the inner lipoyl domain may

represent the immunodominant epitope as outlined by several authors<sup>[9,11]</sup>.

In the present study analyzing a large group of 95 patients with clinically, serologically and histologically defined PBC we could largely confirm these data. Thus, testing the sera by ELISA against 25 mer peptides spanning the whole sequence of PDC-E2, we found only a weak antibody reactivity to peptide 10 (aa 152-176) or peptide 11 (aa 169-193) containing the lipoyl binding K173 and a low incidence of positive results. Since these peptides derived from the peptide scan did not completely correspond to the published peptide 167-184, we also synthesized this epitope but again did not obtain significant reactions. In accordance with a previous report, antigenicity could be increased by coupling with OVA<sup>[12]</sup> while lipoylation had no additional effect. However, already antibody reactivity to OVA itself was rather high indicating that this protein is not suitable for coupling autoantigens to detect conformational autoantibodies in sera from patients with autoimmune disorders. The wide distribution of antibodies to OVA in the general population is a well known phenomenon<sup>[21]</sup>.

In contrast, we found strongly reactive linear epitopes in the first hinge region (peptide 7, aa 101-125) and in the catalytic domain of PDC-E2 - until now regarded as ‘immunologically silent’<sup>[9,14]</sup>. Thus, up to 55% of the 95 PBC sera had antibodies of the IgG- or IgM-type to peptide 7 and up to 74% to the peptides aa 407-431 (peptide 25) or aa 475-499 (peptide 29) in the catalytic domain. The specificity of this reaction was underlined by the finding that sera from patients with AMA negative PBC or other disorders did not react with these peptides. Although there have been several studies analyzing the catalytic domain they failed to detect relevant antibody reactivities<sup>[6,9,12]</sup>, but most of them used larger peptide sequences indicating that - in contrast to antibodies to the inner lipoyl domain - antibodies to the catalytic domain may be rather directed against linear and not against conformational peptides.

The reaction of PBC sera with the catalytic domain is of interest since it has been reported by several authors that anti-PDC-E2 antibodies inhibit the PDC-E2 enzyme activity, which has been attributed until now to their binding to the inner lipoyl domain<sup>[22-25]</sup>. However,

since the catalytic domain contains the active site an enzyme inhibition by interference with this region seems even more likely. Thus, the catalytic centre is formed by a long channel across the interface between the catalytic domains of two neighbored E2-subunits. The opening of the channel pointing towards the outer face of the molecule forms the lipoamide binding site, whereas the opposite entrance corresponds to the coenzyme A (CoA) binding site. In the middle of the catalytic centre there is a pair formed by H534 and Ser480 from neighboring E2-subunits (nomenclature according to human PDC) which separates these two substrate binding sites and is directly involved in the transacetylase reaction<sup>[26-32]</sup>. Our observation in the present study that highest antibody reactivity in PBC sera was obtained with peptide 29 (aa 475-499) which contains Ser480, might, therefore, also explain the inhibitory potency of PBC sera on PDC-E2-enzyme activity. The inner catalytic domain as a whole shares less than 35% sequence identity in prokaryote and human PDC. In contrast, the structures at the active sites are highly conserved in pro- and eukaryotes. The reactivity of autoantibodies with evolutionary highly conserved enzymes and their interaction with enzyme function *in vitro* is a quite frequently observed phenomenon in autoimmune disorders in general<sup>[33,34]</sup>.

The etiopathogenetic role of anti-M2/PDC-E2 antibodies for PBC is still a matter of debate. Coupling of the inner lipoyl domain with 2-octynoic acid instead of lipoic acid has been discussed to increase antigenicity of the enzyme PDC-E2, and common environmental, cosmetic and food additives containing this 2-octynoic acid have, therefore, be postulated to play a role in their induction by formation of an altered PDC-E2 in the sense of a neoantigen<sup>[15,35,36]</sup>. Our observation that anti-PDC-E2 antibodies react preferentially with the active site of the catalytic domain of PDC-E2 is strongly suggestive for a further mechanisms including molecular mimicry or defects in apoptosis<sup>[5,37,38]</sup>. Interestingly, similarity alignment (<http://www.expasy.org>) revealed that a sequence consisting of the five aa TFFTIS within peptide 29 containing the active site S480 shares an identity of 80% with a five-aa-peptide within an envelope protein of human  $\beta$ -retrovirus previously cloned from patients with PBC<sup>[39]</sup>. The relevance of this observation is, of course, still rather speculative.

Another peptide strongly recognized by PBC sera (up to 58%) was the peptide 25. However, 18% of healthy controls also reacted with this peptide indicating that probably natural autoantibodies towards this region exist. Also the fact that antibody reactivity to some peptides within the catalytic domain did not differ significantly between healthy individuals and PBC-patients (i.e. peptide 22, 30, 31) or were even lower in the PBC sera than in sera of the controls (peptides 15, 21 and 24) may point towards the existence of natural autoantibodies to several PDC-E2 epitopes as also outlined by others<sup>[5,13]</sup>. It is well known that natural autoantibodies, which play an important role in the 'first line defense' react preferentially with archaic enzymes, and this would fit with our observation. Interestingly, we

also observed antibodies to the PDC-E2 complex in sera from healthy family members of PBC-patients, but these seem to recognize other epitopes than the patients' sera (manuscript in preparation).

In general, most sera recognized several epitopes on the entire PDC-E2 molecule, and diversity of IgM antibodies was even higher than that of IgG antibodies. Using overlapping octameric peptides representing the inner lipoyl domain of PDC-E2 similar observations were made by Mackay *et al.*<sup>[5]</sup>, and they also noted that normal human sera reacted with multiple peptides, although levels of reactivity were generally lower than those observed using PBC sera<sup>[5,40]</sup>.

Two of the 95 PBC sera did not react with any of the 33 peptides although they were strongly anti-M2/PDC-E2 positive in ELISA and Western blotting again underlining the importance of conformational epitopes. Our preliminary findings that anti-M2/PDC-activity could not be significantly absorbed with any of the linear 167-184 peptides or with peptide 25 or 29 (data not shown), fit to this concept. Based upon molecular modeling of antibodies reacting with antigens, over 90% of B-cell epitopes are thought to be conformational<sup>[41,42]</sup>. Attempts to map B-cell epitopes have, therefore, met with mixed success depending largely on the system under investigation. Since there are so many different types of autoepitopes, linear, conformational, cryptic, *etc.*<sup>[43]</sup>, no universally applicable method is available that allows for the identification of all autoepitopes. Performing those analyses one has, therefore, to be aware of the techniques' advantages and disadvantages. Peptide scan as used in the present study does not allow determining conformational epitopes but it has the advantage that even cryptic epitopes can be detected. This method has been proven useful in the identification of immunodominant epitopes of several autoantigens for instance in collagen disorders<sup>[43]</sup>; and considering the concept of "epitope-spreading" it is not unlikely that the multiple immunoreactivity towards several linear and conformational epitopes in an autoimmune disease starts with cross-reactivity to a single (linear) peptide which may lead us to the initiating agent.

In conclusion, we have firmly documented that PBC sera react preferentially with peptides of the catalytic domain of PDC-E2. However, it still has to be proven whether this catalytic domain is also targeted by T-cells. Furthermore, the data again underline the importance of conformational epitopes for AMA-reactivity in PBC.

## COMMENTS

### Background

Antimitochondrial antibodies (AMA) reacting with the 2-oxo acid dehydrogenase complex of the inner mitochondrial membrane are highly specific for the serological diagnosis of primary biliary cirrhosis (PBC), a chronic cholestatic liver disorder of unknown etiology.

### Research frontiers

The M2-antigen consists of five components which have been identified as subunits of the 2-oxo acid dehydrogenase complex: the pyruvate

dehydrogenase complex (PDC), the 2-oxoglutarate dehydrogenase complex and the branched-chain 2-oxo acid dehydrogenase complexes. Each complex comprises multiple copies of three component enzymes termed E1, E2, and E3. The major M2-antigen which is recognized by nearly 90% of PBC sera is the E2 component of PDC.

### Innovations and breakthroughs

Within PDC-E2 an immunodominant epitope has been identified which is associated with the inner lipoyl domain (aa 167-184) wherein a lysine residue (K173) binds the lipoic cofactor for the enzyme. Binding of lipoic acid cofactor to K173 has been discussed to be necessary for antibody reaction. Replacement of this lipoic acid by 2-octynoic acid seemed even to enhance antibody binding. It was, therefore, speculated that xenobiotics may lead to an accumulation of 2-octynoic acid hereby preventing binding of lipoic acid to the PDC-E2 and generation of neoantigen with the consequence of generation of autoreactive B- and T-cells. The present study contradicts this concept to some extent indicating that epitopes within the catalytic site of PDC-E2 are even stronger reactive with AMA.

### Applications

The identification of immunodominant epitopes recognized by autoreactive T- or B-cells may improve our understanding with respect to their generation (for instance as consequence of crossing reactivity with infectious agents or induction by neoantigens) and their possible pathogenetic role in PBC.

### Terminology

The 2-oxoacid dehydrogenase complex is a multi enzyme complex located at the inner membrane of mitochondria which catalyzes the oxidative decarboxylation of 2-oxo acids to the corresponding acyl-coenzyme A. It is not yet known why this non-organ specific protein complex becomes an autoantigen recognized by autoantibodies in a disease affecting mainly bile ducts such as PBC.

### Peer review

The authors analyse immunodominant targets of PDC-E2 in sera of about 100 patients with PBC. Interestingly, they show that the catalytic site of PDC-E2 contains several immunodominant epitopes. The group has an outstanding expertise in the field of clinical immunology. The data presented in the manuscript add to the understanding of immunological events in PBC.

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