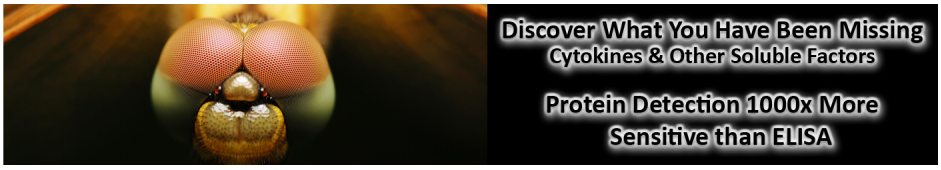

The Journal of Immunology

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Chemical Xenobiotics and Mitochondrial Autoantigens in Primary Biliary Cirrhosis: Identification of Antibodies against a Common Environmental, Cosmetic, and Food Additive, 2-Octynoic Acid[1]

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Emerging evidence has suggested environmental factors as causative agents in the pathogenesis of primary biliary cirrhosis (PBC). We have hypothesized that in PBC the lipoyl domain of the immunodominant E2 component of pyruvate dehydrogenase (PDC-E2) is replaced by a chemical xenobiotic mimic, which is sufficient to break self-tolerance. To address this hypothesis, based upon our quantitative structure-activity relationship data, a total of 107 potential xenobiotic mimics were coupled to the lysine residue of the immunodominant 15 amino acid peptide of the PDC-E2 inner lipoyl domain and spotted on microarray slides. Sera from patients with PBC ($n = 47$), primary sclerosing cholangitis ($n = 15$), and healthy volunteers ($n = 20$) were assayed for Ig reactivity. PBC sera were subsequently absorbed with native lipoylated PDC-E2 peptide or a xenobiotically modified PDC-E2 peptide, and the remaining reactivity analyzed. Of the 107 xenobiotics, 33 had a significantly higher IgG reactivity against PBC sera compared with control sera. In addition, 9 of those 33 compounds were more reactive than the native lipoylated peptide. Following absorption, 8 of the 9 compounds demonstrated cross-reactivity with lipoic acid. One compound, 2-octynoic acid, was unique in both its quantitative structure-activity relationship analysis and reactivity. PBC patient sera demonstrated high Ig reactivity against 2-octynoic acid-PDC-E2 peptide. Not only does 2-octynoic acid have the potential to modify PDC-E2 in vivo but importantly it was/is widely used in the environment including perfumes, lipstick, and many common food flavorings. ***The Journal of Immunology,*** **2005, 174: 5874–5883.**

Primary biliary cirrhosis has a long asymptomatic period and initiating events leading to loss of self-tolerance occur long before the onset of disease manifestations. This makes the initiating triggers of the autoimmune response difficult to identify. Several mechanisms have been postulated to explain loss of tolerance including that of a sequestered Ag (1), escape of autoreactive clones (2), and loss of immunoregulatory cells (3), but the thesis of molecular mimicry has become an actively investigated mechanism for etiology, particularly in multiple sclerosis (4, 5) and systemic lupus erythematosus (6). Both infectious organisms and environmental agents have been suggested to be the main drivers of molecular mimicry. The latter is well demonstrated in the induction of lupus in mice by mercuric chloride (7), and the human epidemics of L-tryptophan induced eosinophilic fasciitis and the toxic oil syndrome (8).

Recently, we have demonstrated that organic molecules can induce an anti-mitochondrial Ab response (AMA)[3] in rabbits virtually identical to that found in patients with primary biliary cirrhosis (PBC) (9). The autoantigens recognized by AMA are members of the 2-oxo-dehydrogenase complexes, particularly the E2 component of pyruvate dehydrogenase complexes (PDC-E2) (10). Several studies have shown that the immunodominant epitope of PDC-E2 is localized within the lipoyl domain of PDC-E2 (11, 12). Interestingly, AMA from patients with PBC are cross-reactive not only against native lipoylated PDC-E2 but also a number of chemically modified mimics conjugated to the same domain (13). In addition, rabbits and guinea pigs immunized with a laboratory synthesized organic chemical, 6-bromohexanoate conjugated to BSA, developed autoantibodies against not only lipoic acid (LA) but also PDC-E2 (14, 15).

We have continued the experimental work on guinea pigs and have preliminary histological data on liver lesions in xenobiotic immunized guinea pigs. A manuscript describing this work is in preparation. These findings suggest that the LA residue of PDC-E2 serves as a xenobiotic target, and an immune response against xenobiotics may result in the recognition of not only the modified or altered protein, but also the unmodified protein. To address this issue and to define the optimal structure of LA mimicking xenobiotics that is recognized by AMA, we performed a quantitative structure-activity relationship analysis over a wider array of compounds. We

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[3] Abbreviations used in this paper: AMA, anti-mitochondrial Ab response; PBC, primary biliary cirrhosis; PDC-E2, E2 component of pyruvate dehydrogenase; PSC, primary sclerosing cholangitis; NHS, *N*-hydroxysuccinimide; HSA, human serum albumin; LA, lipoic acid.

0022-1767/05/$02.00

Table I. *Name and number of xenobiotic compounds*

No.	Name	No.	Name
LA	Lipoic acid	58	2-Chlorocinnamic acid
1	2-(Trifluoromethyl)benzoic acid	59	6-Chloronicotinic acid
2	3-(Trifluoromethyl)benzoic acid	60	3-Chlorophenylacetic acid
3	4-(Trifluoromethyl)benzoic acid	61	3-(Chloromethyl)benzoic acid
4	2,5-Bis(trifluoromethyl)benzoic acid	62	3-Fluorophenylacetic acid
5	4-Bromobenzoic acid	63	4-Fluoro-3-methylbenzoic acid
6	2-Iodobenzoic acid	64	4-Fluorosalicylic acid
7	6-Bromohexanoic acid	65	2-Fluorocinnamic acid
8	5-Chlorovaleric acid	66	2,4-Difluorobenzoic acid
9	Chloroacetic acid	67	6-Heptenoic acid
10	Octanoic acid	68	Cyclohexanebutyric acid
11	(2,2,2-Trifluoroethoxy)acetic acid	70	4-(2-Thienyl)butyric acid
12	*trans*-2-(Trifluoromethyl)cinnamic acid	71	3-Phenoxypropionic acid
13	3-(Trifluoromethyl)cinnamic acid	72	*trans*-Styrylacetic acid
14	4-(Trifluoromethyl)cinnamic acid	73	4-Methylphenylthiolacetic acid
15	4-Chlorocinnamic acid	74	*S*-Benzylthioglycolic acid
16	3-Methylcinnamic acid	75	3-Bromocinnamic acid
17	3-Nitrocinnamic acid	76	4-(Bromomethyl)benzoic acid
18	3-[3-(Trifluoromethyl)phenyl]propionic acid	77	4-(Chloromethyl)benzoic acid
19	2-Trifluoromethyl-1,3-dithiane-6-pentanoic acid	78	3-Chlorocinnamic acid
20	2-Bromo-4-nitrobenzoic acid	79	4-Chlorophenylacetic acid
21	3-Fluorobenzoic acid	80	3-Fluorocinnamic acid
22	4-Fluorobenzoic acid	81	(3-Trifluoromethylphenyl)acetic acid
23	2-Fluorobenzoic acid	82	3-(1,1,2,2-Tetrafluoroethoxy)benzoic acid
24	Methacrylic acid	83	5-(2,4-Dichlorophenyl)-2-furoic acid
25	Cyclohexanecarboxylic acid	84	4-Bromophenylacetic acid
26	2-Methyl-3-nitrobenzoic acid	86	4-Propoxybenzoic acid
27	8-(Ethylthio)octanoic acid	87	4-(Methylthio)phenylacetic acid
28	Acetic acid	88	Cyclohexanepentanoic acid
29	6-(Ethylthio)hexanoic acid	89	8-Bromooctanoic acid
31	3-Methoxypropionic acid	90	4-Bromocinnamic acid
32	3-Pyridylacetic acid	91	4-Bromophenoxyacetic acid
35	4-Bromobutyric acid	92	p-Chlorophenoxyacetic acid
37	2,3-Dibromo-3-phenylproprionic acid	93	(4-Chlorophenylthio)acetic acid
38	4-Chlorobutyric acid	94	(4-Trifluoromethylphenyl)acetic acid
39	2-Chlorophenylacetic acid	95	(4-Fluorophenyl)anthranilic acid
40	4-Iodobutyric acid	96	3-(4-Fluorophenyl)propionic acid
41	3-Iodobenzoic acid	97	4-Fluorocinnamic acid
42	Pentadecafluorooctanoic acid	98	4-Fluorophenoxyacetic acid
43	3-Fluoro-4-hydroxybenzoic acid	99	4-Benzyloxybutyric acid
44	Hexanoic acid	102	4-(2,4-Dichlorophenoxy)butyric acid
45	*trans*-3-Hexenoic acid	103	3-(4-Fluorophenoxy)propionic acid
46	5-Hexenoic acid	104	4-(Trifluoromethoxy)phenylacetic acid
47	Sorbic acid	106	4-(Trifluoromethyl)hydrocinnamic acid
48	3-Cyclopentylpropionic acid	107	Decanoic acid
49	3-(2-Thienyl)acrylic acid	108	10-Bromodecanoic acid
50	*trans*-3-(3-Thienyl)acrylic acid	110	11-Bromoundecanoic acid
51	1-Piperidinepropionic acid	132	Benzenepropanoic acid
52	3-(*p*-Hydroxyphenyl)propionic acid	133	Cyclohexanepropanoic acid
53	3-(2-Hydroxyphenyl)propionic acid	134	6-Heptynoic acid
54	(Phenylthio)acetic acid	135	3-Ethoxybenzoic acid
55	(2-Pyrimidylthio)acetic acid	136	3-(4-Chlorophenyl)-2-propenoic acid
56	5-Bromovaleric acid	138	2-Octynoic acid
57	3-Bromophenylacetic acid	139	4-Pentylbenzoic acid

used a novel technique in which large numbers of small molecules were individually conjugated to the immunodominant peptide and spotted onto microarrays for high-throughput screening. Herein we report the presence of a previously unrecognized Ab in PBC, which is directed against 2-octynoic acid, a chemical with potential to modify PDC-E2 in vivo and widely found in the environment including perfumes, lipstick, and many common food flavorings.

Materials and Methods

Serum

A panel of well defined sera from our laboratory was used in the present study including samples from 41 AMA-positive PBC patients (histological stage 0 or 1 = 0, stage 2 = 6, stage 3 = 19, stage 4 = 15, unknown = 1), 6 AMA-negative PBC patients (stage unknown), 15 primary sclerosing cholangitis (PSC) patients and 20 healthy volunteers. The protocol was approved by the institutional review board of the University of California at Davis.

Preparation of peptide-agarose conjugates

Two peptidic amides (PDC peptide IETDKATIGFEVQEE and as a control, the human serum albumin peptide EENFKALVLIAFAQY) were synthesized on Rink amide MBHA resin by Fmoc chemistry (9, 15). Modification of agarose with methyl ketone groups was performed according to our published procedure (15, 16). As described below, 5 g of sodium carbonate was added to a solution of 3.2 g of agarose (type XI: low gelling temperature; Sigma-Aldrich) that was previously melted in 250 ml of deionized water. Then, 100 mg of 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) (Sigma-Aldrich) dissolved in 1 ml of DMSO (Sigma-Aldrich) and 0.2 g of sodium bromide (Sigma-Aldrich) were added (17, 18). While stirring at 4°C, 4.0 ml of sodium hypochlorite (1.3 M) (Sigma-Aldrich) solution was slowly added. The mixture was stirred overnight at 4°C. The

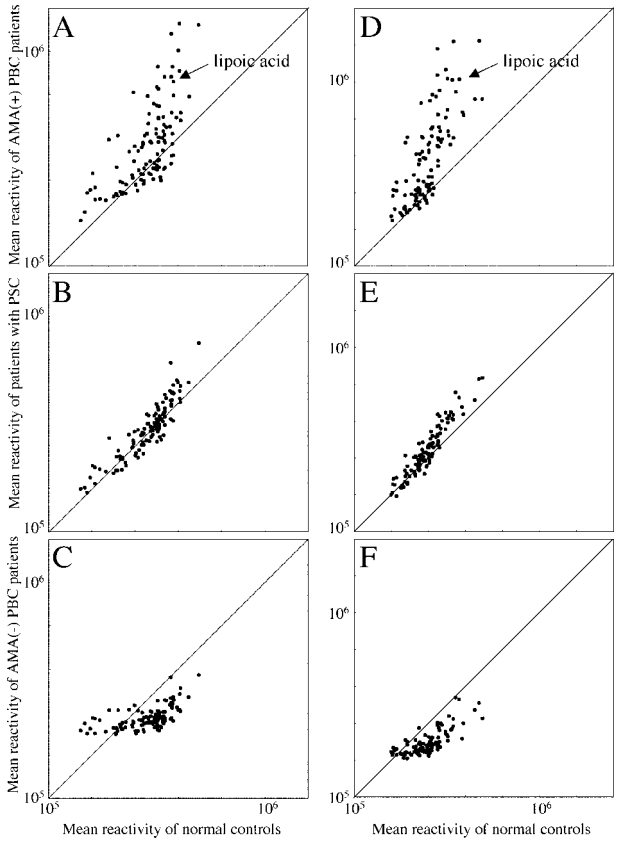


FIGURE 1. Sera Ig reactivity against xenobiotically modified PDC-E2 and lipoylated PDC-E2. Sera IgG reactivity of AMA (+) PBC patients (*A*), patients with PSC (*B*), AMA (−) PBC patients (*C*), and healthy volunteers was determined at 1/250 dilution by microarray. Each spot represents the mean IgG reactivity against each of 107 xenobiotically modified PDC-E2 or lipoylated PDC-E2. The mean IgG reactivity was plotted on the *y*-axis to compare with the mean IgG reactivity of normal control (*x*-axis). IgM reactivity of AMA (+) PBC patients (*D*), patients with PSC (*E*), and AMA (−) patients (*F*) were also determined. Mean reactivity is presented as the mean pixel counts of spots.

solid was removed by filtration. The filtrate was poured into 3 volume excess of ethanol. The agarose precipitate was obtained by filtration and washed with 70% ethanol. The powder was acidified by 1.0 M hydrochloric acid and precipitated in ethanol again. The white powder was thoroughly washed with 70% ethanol and dried in a lyophilizer. Oxidized agarose (0.4 g, 0.46 mmol of −COOH) was then dissolved in 50 ml of DMSO with heating. A solution of 2,2′-(ethylenedioxy)bis(ethylamine) (6.85 mmol) (Sigma-Aldrich) and *N*,*N*-diisopropylcarbodiimide (6.85 mmol) was added to the agarose solution. Thence the mixture was stirred at room temperature for 5 h and poured into 5-fold excess of cold (0°C) ethanol. The resulting precipitates were filtered and washed with ethanol. The occurrence of coupling was confirmed by the Chloranil test (19). Modified agarose was then dissolved in 50 ml of DMSO again. A solution of *N*,*N*-diisopropylcarbodiimide (6.85 mmol), levulinic acid (6.85 mmol), and 1-hydroxybenzotriazole (6.85 mmol) were added to the modified agarose solution. The mixture was stirred at room temperature overnight and poured into 200 ml of cold ethanol. The precipitates were filtered and washed with ethanol. Completion of coupling was confirmed using the ninhydrin test. Methyl ketone-modified agarose (47 mg, 0.04 mmol) was then dissolved in 10 ml of the appropriate aminooxyacetyl peptide solution (4 mM) in a 0.05 M NaAc/AcOH buffer (pH 4.5) containing 50% DMSO. The mixture was stirred for 5 h at 65–70°C. Ketones on modified agarose react selectively with aminooxy groups on peptides to form oximes at slightly acidic condition (20, 21). The conjugation solution was then dialyzed and subsequently lyophilized. Loading of each peptide was calculated by a quantitative ninhydrin test at 570 nm and determined to be: PDC peptide = 430 μmol/g, HSA peptide = 267 μmol/g.

Synthesis of mimeotopes and coupling with peptide-agarose conjugate

In addition to LA, 107 xenobiotic compounds were synthesized and used in this study (Table I). The compounds were chosen based upon the structural similarity to LA and were purchased from Sigma-Aldrich. Those 107 compounds and LA were coupled with *N*-hydroxysuccinimide (NHS) to give the corresponding NHS ester, which was subsequently coupled to the lysine residue on peptide-agarose conjugates (13, 15). Briefly, 40 μg of the peptide-agarose conjugates and 0.4 μmol of each of the NHS esters were mixed in 40 μl of DMSO. Mixtures were incubated at room temperature for 2 h. To ensure the complete coupling, a quantitative ninhydrin test was performed.

Preparation of microarray and detection of Ab against modified PDC-E2

Xenobiotic compounds-peptide-agarose mixtures were transferred to 384-well plates. Thereafter mixtures were spotted onto glass slides (Mercedes Medical) using the Affymetrix 417 Microarrayer. Six spots were blotted from each sample, and the means of these six spots were calculated to determine Ig reactivity. Spotted microarrays were stored at 4°C until use. Before use, microarrays were blocked with 3% nonfat dry milk in PBS buffer for 1 h at room temperature, and individual slides were thereafter incubated with diluted patient or control sera (1:250) in 1 ml of blocking buffer (3% nonfat dry milk in PBS with 0.05% Tween 20) (PBST) for 1 h at room temperature. After thorough washes with PBST, 1 ml of the Cy3- or Cy5-conjugated secondary Ab (1 μg/ml) (Zymed Laboratories) in blocking buffer was added to each slide and incubated at room temperature for 30 min. Subsequently slides were washed in PBST and water. Arrays were then dried and scanned using the Affymetrix 428 array scanner. Likewise, rabbit anti-LA Abs (1/5000 in blocking buffer) (Calbiochem) and mouse monoclonal anti-PDC-E2 Abs (1/5 in blocking buffer) (clone 2H4) (22, 23) were also assayed on the same microarray to confirm cross-reactivity between lipoylated PDC-E2 and xenobiotically modified PDC-E2.

Data analysis

Image data analysis was performed using the TIGR Spot Finder (The Institute for Genome Research) (24). Statistical analysis was performed using JMP software (SAS Institute) on all 107 compounds and LA individually. The intraassay error among six spots within a sample was <10% of the mean. Differences in the mean value of Ig reactivity against each of those compounds between PBC sera and control sera with $p < 0.05$ was considered significant as determined by an unpaired Student's *t* test. A paired *t* test was performed to evaluate the remaining Ig reactivity after absorption with lipoylated PDC-E2 peptide. Once again, a difference in the mean signal intensity given by sera following absorption with lipoylated PDC-E2 compared with compound 24-PDC-E2 was considered significant with $p < 0.05$.

Specificity of Ig reactivity against modified PDC-E2 peptide and lipoylated PDC-E2 peptide

Following our high-throughput analysis, sera from 5 representative patients with PBC were absorbed at a final dilution of 1:500 in PBST containing 3% milk with three different concentrations (10, 1, and 0.1 μg/ml) of xenobiotics (compounds 88, 138, or 24), PDC-E2 peptide-agarose conjugate, or lipoylated PDC-E2 peptide-agarose conjugate. After incubation at 4°C overnight, the mixture was centrifuged and then the supernatant saved. IgG and IgM reactivity of unabsorbed and absorbed sera against xenobiotic compounds-peptide-agarose conjugates was determined by the microarray assay.

Detection of Ab against modified PDC-E2 by ELISA

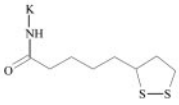
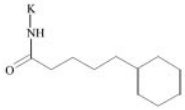
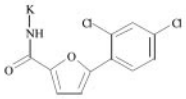
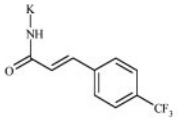
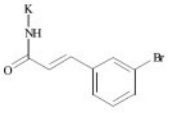
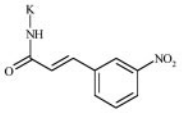
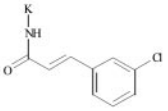
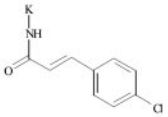
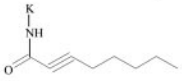
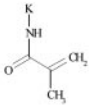
Microtiter plates were coated with 50 μl of each individual xenobiotic compound-peptide-agarose mixture in DMSO (1 mg/ml) for 2 h at room temperature, and plates were dried overnight. Dried ELISA plates were thereafter incubated with serially diluted (1/20, 1/60, 1/180, 1/540, and 1/1620) sera from five representative PBC patients for 1 h at room temperature. After washing, the plates were incubated with HRP-conjugated goat anti-human IgG + A + M Abs (Zymed Laboratories) for 30 min at room temperature. Subsequently plates were washed and incubated with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) containing hydrogen peroxide (Kirkegaard & Perry Laboratories). Likewise, Ig reactivity against recombinant human PDC-E2 was determined at 1/1000 sera dilution by standard ELISA as previously described (9).

Results

Identification of AMA-reactive xenobiotic compounds

Sera from 47 PBC patients (41 AMA positive and 6 negative), 15 PSC patients, and 20 healthy volunteers were assayed by microarray

Table II. *Serum IgG and IgM reactivity of patients with PBC and normal controls against lipoylated PDC-E2 peptide and xenobiotically modified PDC-E2 peptide by microarray*[a]

	Structure	Name	IgG		IgM	
			PBC	NC	PBC	NC
LA		Lipoic acid	718991*	378448	1030672**	370329
88		Cyclohexanepentanoic acid	1339822***	404633	1657028***	344074
83		5-(2,4-Dichlorophenyl)-2-furoic acid	1328219***	496957	1660965***	473981
110		11-Bromoundecanoic acid	1201246***	369687	1515634***	281940
14		2-(Trifluoromethyl)cinnamic acid	1004220**	398648	1159470***	311826
75		3-Bromocinnamic acid	846207**	321628	1089700***	281256
17		3-Nitrocinnamic acid	846198**	373955	1027486***	338052
78		3-Chlorocinnamic acid	809112**	405232	1037639***	320204
136		4-Chlorocinnamic acid	758836**	345287	893271*	290224
138		2-Octynoic acid	757314**	369752	884042**	354000
24		Methacrylic acid	216631	227983	201588	189025
		PDC peptide only	253417	305013	212115	219369

[a] Data presented here are mean intensity.
*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (Student *t*-test).

to detect relative Ab reactivity to lipoylated or xenobiotic-modified PDC-E2 peptide. Of 107 xenobiotics, AMA-positive PBC patient sera showed significantly ($p < 0.05$) higher IgG reactivity against lipoylated PDC-E2 peptide and 33 xenobiotic-modified PDC-E2 peptide than sera from PSC patients or normal controls (Fig. 1*A*). The compounds included nos. 12, 13, 14, 15, 17, 20, 27, 58, 68, 72, 75,

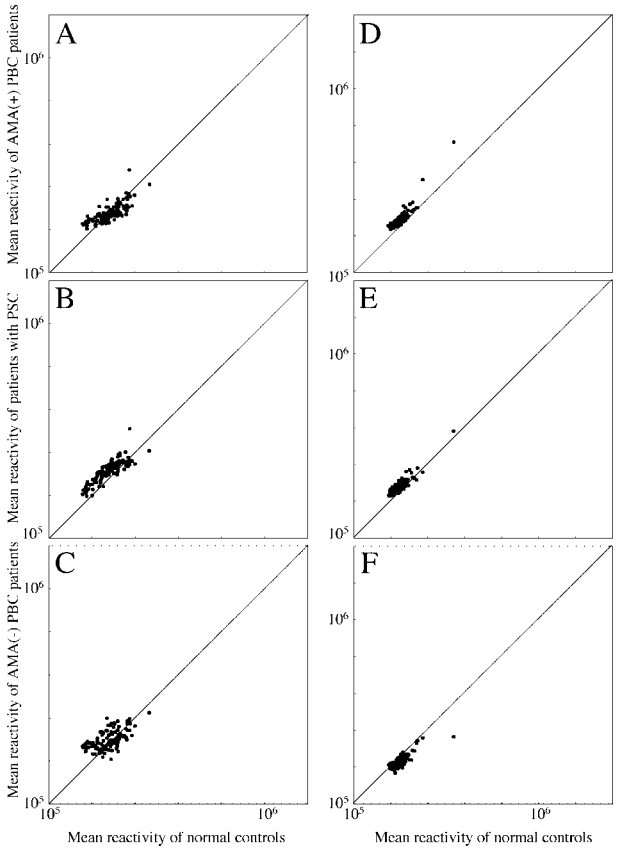


FIGURE 2. Sera Ig reactivity against xenobiotic mimeotopes or LA on HSA peptide. Sera IgG reactivity of AMA (+) PBC patients (*A*), patients with PSC (*B*), AMA (−) PBC patients (*C*), and healthy volunteers was determined at 1/250 dilution by microarray. Each spot represents the mean IgG reactivity against each of 107 xenobiotic mimeotopes or LA on HSA peptide. The mean IgG reactivity was plotted on *y*-axis to compare with the mean IgG reactivity of normal control (*x*-axis). IgM reactivity of AMA (+) PBC patients (*D*), patients with PSC (*E*), and AMA (−) patients (*F*) was also determined.

78, 81, 83, 86, 87, 88, 89, 92, 93, 94, 97, 102, 104, 106, 107, 108, 110, 133, 134, 136, 138, and 139. Of these 33 compounds, nine compounds showed significantly higher reactivity with PBC patient sera compared with LA-conjugated PDC-E2 (Table II). These compounds included nos. 14, 17, 75, 78, 83, 88, 110, 136, and 138.

There was also significantly higher IgM reactivity of PBC sera against 64 xenobiotic mimeotopes compared with normal control sera (Fig. 1D). Those compounds included nos. 2, 3, 5, 7, 10, 12, 13, 14, 15, 16, 17, 18, 20, 26, 27, 29, 41, 48, 55, 57, 58, 59, 60, 61, 64, 65, 67, 68, 70, 72, 75, 77, 78, 79, 80, 81, 82, 83, 84, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 97, 99, 102, 103, 104, 106, 107, 108, 110, 133, 134, 135, 136, 138, and 139. Of these 64 compounds, compound nos. 14, 17, 75, 78, 83, 88, and 110 showed higher reactivity than lipoylated PDC-E2. There was no detectable reactivity in sera from PSC patients or AMA-negative PBC patients (Fig. 1, *B*, *C*, *E*, and *F*).

Role of PDC-E2 backbone in AMA reactivity with xenobiotics

To define the requirement and/or contribution of the PDC-E2 peptide backbone, the same compounds were coupled to the human serum albumin (HSA) peptide, and then the same sera were assayed on the microarray. The sera that previously showed unique reactivity to the PDC-E2 peptide did not show detectable reactivity when the same compounds were coupled to the HSA peptide (Fig. 2) demonstrating a requirement for the PDC-E2 peptide backbone for AMA recognition. There was no significant correlation between histological stage and Ig reactivity against any of the xenobiotic mimeotopes and lipoylated PDC-E2 peptide (data not shown).

Characterization of Abs with specificity for modified PDC-E2

To define the antigenic specificity of anti-modified PDC-E2 Abs, aliquots of sera from five representative PBC patients were absorbed with three different concentrations (10, 1, and 0.1 μg/ml) of lipoylated PDC-E2 peptide-agarose conjugate or compound 24-PDC-E2 peptide. Metacrylic acid (compound 24) was chosen as a negative control since sera from PBC patients did not react with compound 24-PDC-E2 peptide. Each aliquot was then analyzed for

Table III. *Inhibition of IgG and IgM reactivity from sera of patients with PBC against lipoic acid and xenobiotically modified PDC-E2 after absorption with lipoylated PDC-E2 peptide (LA) and compound 24-PDC-E2 peptide*[a]

Name		Preinhibition (%)		IgG[b]			IgM[b]		
				0.1	1	10	0.1	1	10
LA	Lipoic acid	24	100	100	100	78.0	100	100	79.4
		LA	100	50.5*	30.0**	23.7**	42.8*	20.9**	14.2*
88	Cyclohexanepentanoic acid	24	100	86.7	85.8	63.0	93.1	92.3	69.7
		LA	100	62.1*	35.4*	20.8**	61.0*	41.4**	19.1*
83	5-(2,4-Dichlorophenyl)-2-furoic acid	24	100	83.6	82.2	62.4	88.0	84.3	60.6
		LA	100	85.7	90.4	59.4	79.0	67.8	52.0
110	11-Bromoundecanoic acid	24	100	88.1	84.5	52.9	100	97.7	66.1
		LA	100	75.8	41.6	32.7	84.2	63.1	41.2
14	4-(Trifluoromethyl)cinnamic acid	24	100	86.5	83.0	56.0	93.4	92.4	60.2
		LA	100	73.7	43.6	30.7	76.9	57.8	34.6
75	3-Bromocinnamic acid	24	100	86.3	83.9	62.2	85.2	89.9	62.9
		LA	100	87.5	48.5	37.1	84.8	61.4	37.1
17	3-Nitrocinnamic acid	24	100	87.4	83.2	61.6	97.4	94.0	63.2
		LA	100	78.0	44.1	39.5	79.7	65.4	47.4
78	3-Chlorocinnamic acid	24	100	100	100	82.2	100	100	75.8
		LA	100	94.6	68.8	64.4	83.6	67.3	52.2
136	3-(4-Chlorophenyl)-2-propenoic acid	24	100	86.2	87.3	64.5	87.3	91.8	64.5
		LA	100	68.3	50.9*	38.6*	63.7	55.0*	35.6*
138	2-Octynoic acid	24	100	100	100	100	100	100	100
		LA	100	100	100	100	95.0	87.4	78.2

[a] Asterisks indicate significant difference ($p < 0.05$, paired *t*-test) compared with control 24.
[b] Antigen concentration (μg/ml).

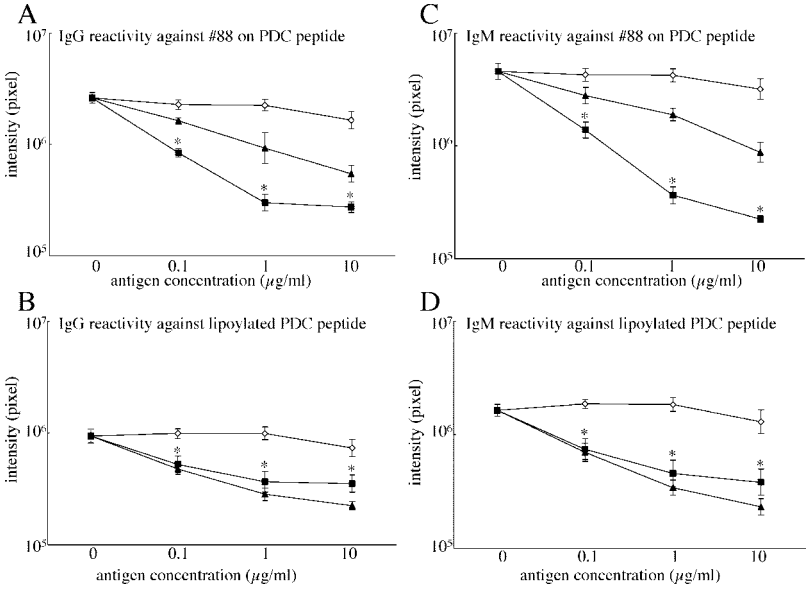


FIGURE 3. Cross-reactivity of PBC sera against compound 88-PDC-E2 peptide and lipoylated PDC-E2 peptide. Sera from five representative AMA (+) PBC patients were absorbed at a final dilution of 1/250 with three different concentrations (10, 1, and 0.1 μg/ml) of xenobiotics 24 (◇) or 88 (■)-PDC-E2 peptide-agarose conjugate or lipoylated PDC-E2 peptide-agarose conjugate (▲). Each aliquot was then analyzed for IgG and IgM reactivity against compound 88-PDC-E2 peptide (*A* and *C*) and lipoylated PDC-E2 peptide (*B* and *D*). *, Significant difference (paired *t* test) compared with control compound 24.

remaining reactivity, if any, against lipoylated PDC-E2 peptide and compounds 14, 17, 75, 78, 83, 88, 110, 136, and 138-PDC-E2 peptide by microarray. As expected, when sera were absorbed with lipoylated PDC-E2 peptide, both IgG and IgM reactivity against lipoylated PDC-E2 peptide was significantly decreased. Although IgG reactivity against compound 88 significantly decreased after lipoylated PDC-E2 absorption (Table III), weak but significant reduction of IgG reactivity was also noted against compound 136-PDC-E2 peptide. IgM reactivity against compounds 17, 78, 88, 110, and 136-PDC-E2 peptide was significantly decreased after lipoylated PDC-E2 peptide absorption. Importantly, IgG and IgM reactivity against compound 138 was not decreased after lipoylated PDC-E2 peptide absorption.

To determine the cross-reactivity among compounds 88, 138, and LA, aliquots of sera from five representative PBC patients were absorbed with either compound 88 or 138-PDC-E2 peptide, and then analyzed for reactivity against lipoylated PDC-E2 peptide and compounds 88 and 138-PDC-E2 peptide on microarrays. When sera were absorbed with compound 88, IgG reactivity against lipoylated PDC-E2 was significantly decreased (Fig. 3*B*). Ig reactivity against compounds 14, 17, 75, 78, 83, 88, 110, and 136 was also significantly decreased after absorption with compound 88, but Ig reactivity against compound 138 was not decreased after absorption with compound 88 (data not shown). In contrast, when sera were absorbed with compound 138, no detectable decrease in Ig reactivity against lipoylated PDC-E2 was noted (Fig. 4, *B* and *D*). IgG reactivity against compound 88-PDC-E2 peptide was also decreased after either lipoylated PDC-E2 peptide or compound 88-PDC-E2 peptide absorption, and a significantly greater reduction was observed after compound 88-PDC-E2 peptide absorption compared with lipoylated PDC-E2 peptide absorption (Fig. 3*A*). A similar pattern of IgM reactivity reduction was also observed after either compound 88-PDC-E2 peptide absorption or lipoylated PDC-E2 peptide absorption (Fig. 3, *C* and *D*).

In addition to 2-octynoic acid (compound 138), two other structurally similar compounds—compound 134, which also has a C-C triple bond but at C6 and compound 10 (octanoic acid)—were studied in parallel to determine their cross-reactivity with compound 138. Preincubation of sera with compound 138 and an irrelevant control compound 24 were not able to remove either IgG

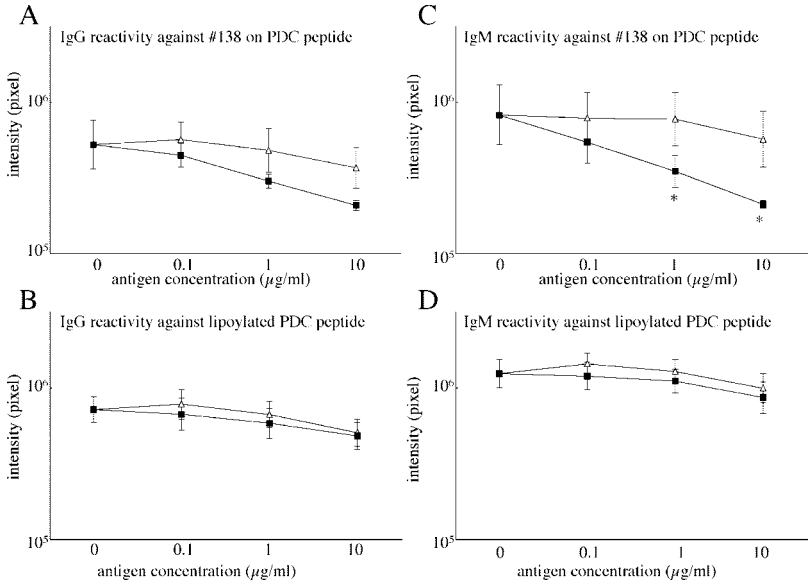


FIGURE 4. Lack of cross-reactivity of PBC sera against compound 138-PDC-E2 peptide and lipoylated PDC-E2 peptide. Sera from five representative AMA (+) PBC patients were absorbed at a final dilution of 1/250 with three different concentrations (10, 1, and 0.1 mg/ml) of xenobiotics 24 (△) or 138 (■)-PDC-E2 peptide-agarose conjugate. Each aliquot was then analyzed for IgG and IgM reactivity against compound 138-PDC-E2 peptide (*A* and *C*), and lipoylated PDC-E2 peptide (*B* and *D*). *, Significant difference (paired *t* test) compared with control compound 24.

Table IV. *Ig specificity of compound 138 with PBC sera and structure-Ig reactivity relationship analysis of aliphatic compounds*

	Name	IgG	IgM	Pre-inhibition	(%)	IgG[a] 0.1	IgG[a] 1	IgG[a] 10	IgM[a] 0.1	IgM[a] 1	IgM[a] 10
PBC sera											
LA	Lipoic acid	718991	1030672	24	100	100	92.7	70.3	100	100	80.0
				138	100	92.9	81.0	66.9	96.6	89.4	69.5
88	Cyclohexanepentanoic acid	1339822	1657028	24	100	100	90.0	77.1	100	99.6	87.6
				138	100	100	96.4	66.7	100	97.1	64.7
138	2-Octynoic acid	757314	884042	24	100	100	91.7	70.4	95.4	94.1	69.3
				138	100	85.1	57.5	39.7	66.3	42.5[b]	25.7[b]
10	Octanoic acid	423051	496419	24	100	98.2	84.7	71.0	97.3	87.3	69.1
				138	100	94.3	78.0	60.6	89.8	74.8	53.5
134	6-Heptynoic acid	490819	501511	24	100	100	100	99.6	100	100	100
				138	100	100	100	88.0	100	100	100
Aliphatic compounds											
LA	Lipoic acid	718991	1030672	24	100	100	100	78.0	100	100	79.4
				LA	100	50.5[b]	30.0[b]	23.7[b]	42.8[b]	20.9[b]	14.2[b]
88	Cyclohexanepentanoic acid	1339822	1657028	24	100	86.7	85.8	63.0	93.1	92.3	69.7
				LA	100	62.1[b]	35.4[b]	20.8[b]	61.0[b]	41.4[b]	19.1[b]
68	Cyclohexanebutyric acid	219127	257299	24	100	87.2	87.7	71.7	93.1	96.1	77.2
				LA	100	58.1[b]	33.9[b]	21.3[b]	54.0[b]	35.9[b]	18.9[b]
133	Cyclohexanepropanoic acid	459970	568402	24	100	80.0	83.7	70.2	82.7	89.1	72.2
				LA	100	59.2	32.9[b]	24.0[b]	42.7[b]	24.2[b]	15.0[b]
25	Cyclohexanecarboxylic acid	286096	270130	24	100	100	100	100	100	100	100
				LA	100	100	100	100	100	100	100
107	Decanoic acid	666031	789153	24	100	90.2	91.3	74.7	91.8	96.9	76.8
				LA	100	63.8	48.8[b]	32.7[b]	59.2	49.6[b]	30.2[b]
10	Octanoic acid	423051	496419	24	100	100	100	100	100	97.6	79.4
				LA	100	87.9	67.0	64.9	56.4	44.3[b]	39.3
44	Hexanoic acid	220873	201875	24	100	100	100	100	93.8	94.6	100
				LA	100	100	100	100	100	99.8	100

[a] Antigen concentration (μg/ml).
[b] Significant difference ($p < 0.05$, paired *t*-test) compared with control 24.

or IgM reactivity against compound 134 or compound 10 (Table IV). Likewise, preincubation with either compound 24 or 138 did not significantly reduce either IgG or IgM reactivity against LA or another LA mimic compound 88.

Cross-reactivity of anti-LA Ab and anti-PDC-E2 Ab

Cross-reactivity between LA and compound 88 was also confirmed with the use of a rabbit anti-LA Ab and an anti-PDC-E2 mAb (clone 2H4). As expected, the rabbit anti-LA Ab strongly bound to LA. Interestingly, the rabbit anti-LA Ab showed weak reactivity to compounds 88, 110, and 17 (Fig. 5). In contrast, anti-PDC-E2 mAb, clone 2H4, showed high reactivity against LA and compound 88 on the PDC peptide (data not shown).

Detection of Ab against modified PDC-E2 by ELISA

To confirm the Ab reactivity against lipoylated PDC-E2 and xenobiotically modified PDC-E2, an ELISA assay was performed with lipoylated PDC-E2, compound 138-PDC-E2, non-lipoylated PDC-E2 and negative control compound 24-PDC-E2. Sera from five representative patients with PBC demonstrated Ig reactivity against compound 138-PDC-E2 peptide, the non-lipoylated PDC-E2 peptide and the lipoylated PDC peptide (Fig. 6).

Discussion

PBC is a chronic progressive cholestatic liver disease associated with AMA in >95% of the patients (25). Interestingly, AMA can be found on routine screening many years before the clinical appearance of disease (26, 27), suggesting that the etiologic events

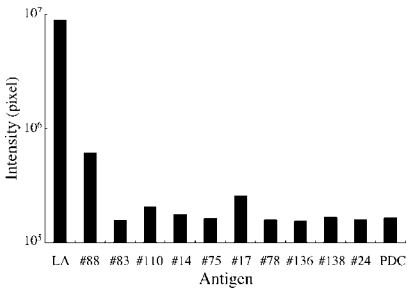


FIGURE 5. Cross-reactivity of rabbit anti-LA Ab against xenobiotically modified PDC-E2 peptide. Rabbit anti-LA Abs (1/5000) were assayed on the microarray to detect cross-reactivity between lipoylated PDC-E2 and xenobiotically modified PDC-E2. Note that rabbit anti-LA Abs reacted with LA, compound 17, 88, and 110 but the specificity of the rabbit anti-LA sera was more directed at LA in contrast to the data above with PBC sera.

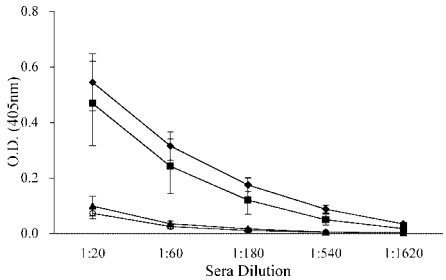


FIGURE 6. Detection of Ig reactivity against lipoylated PDC peptide and xenobiotically modified PDC peptide by ELISA. Sera from five representative patients were serially diluted (1/20, 1/60, 1/180, 1/540, and 1/1620) and Ig reactivity against lipoylated PDC peptide (♦), compounds 24 (▲), and 138 (■) on PDC peptide and non-lipoylated PDC peptide (○) were determined by standard ELISA.

Table V. *IgG and IgM reactivity of PBC and control sera with aliphatic and unsaturated compounds*

	Name	IgG		IgM	
		PBC	NC	PBC	NC
Aliphatic compounds					
LA	Lipoic acid	718991*	378448	1030672*	370329
7	6-Bromohexanoic acid	443916	351462	444426*	286624
8	5-Chlorovaleric acid	284488	304548	237876	239879
9	Chloroacetic acid	283378	312993	243138	235643
10	Octanoic acid	423051	339570	496419*	283859
28	Acetic acid	413667	286346	685898	385004
35	4-Bromobutyric acid	251977	269342	223067	224995
38	4-Chlorobutyric acid	491530	392963	803010	492367
40	4-Iodobutyric acid	284987	317769	263684	268374
44	Hexanoic acid	247792	277605	214662	211421
56	5-Bromovaleric acid	287368	345977	243631	264395
89	8-Bromooctanoic acid	269738*	292714	243834*	227546
107	Decanoic acid	220873*	215395	201875*	199870
108	10-Bromodecanoic acid	266643*	298170	237787*	241149
110	11-Bromoundecanoic acid	568295*	308191	664141*	282746
Unsaturated compounds					
LA	Lipoic acid	718991*	378448	1030672*	370329
45	trans-3-Hexenoic acid	290512	320195	281646	263462
46	5-Hexenoic acid	257831	329267	218927	247560
67	6-Heptenoic acid	204826	172854	202842*	165496
134	6-Heptynoic acid	490819*	324508	501511*	232600
138	2-Octynoic acid	757314*	369752	884042*	354000

*, Significant difference ($p < 0.05$, Student's t test) compared with normal control (NC).

that generate AMA play an important role in the pathogenesis of PBC. We hypothesized that molecular mimicry between self-Ag (lipoylated PDC-E2) and xenobiotically modified PDC-E2 may initiate AMA production. This thesis is supported by the observation that anti-PDC-E2 Abs from patients with PBC were able to recognize xenobiotically modified PDC-E2 peptides, mimicking LA (13). Moreover, rabbits and guinea pigs immunized with the LA mimic 6-bromohexanoate, conjugated to BSA, produced AMA (9, 14, 15).

To investigate potential structures of LA mimics, which are recognized by AMAs present in PBC patients, we screened 107 different organic compounds by a microarray assay. We found that 33 (IgG) or 64 (IgM) compounds had a significantly higher reactivity against PBC sera compared with normal control sera. The higher IgM reactivity could be explained by the pentameric nature of IgM molecules enabling it to bind Ag more effectively. This is supported by the fact that all compounds that were reactive with IgG also reacted with IgM Abs. In addition, nine or seven of these IgG- or IgM-reactive compounds, respectively, were more reactive than the LA-PDC-E2 peptide itself against PBC sera.

To define a correlation between the chemical structure of the compounds and their ability to bind AMAs, we analyzed the compounds that were either IgG or IgM reactive according to their chemical structure by several parameters. First, an analysis of the length of the aliphatic chain showed that there is increased binding of Ig with an increasing number of carbon atoms. Moreover, a minimum of eight carbon atoms in the aliphatic acid was required for IgM binding and ten carbon atoms for significant binding to IgM and IgG Abs from patient sera (Table V). A similar result was obtained comparing aliphatic acids of different chain length carrying a cyclohexyl group on the terminal carbon atom (Table IV). The cross-reactivity of LA-specific AMAs with the carbon chain of aliphatic acids could also be confirmed in absorption assays with LA. When sera were pre-absorbed against LA-conjugated PDC-E2 peptide there was a significant reduction in Ig reactivity against compounds carrying long aliphatic chains (Table IV). This data suggests a primarily hydrophobic interaction between aliphatic chain attached to the lysine of the PDC-E2 peptide and the Ag binding site of the Ig molecule. However, the Ig reactivity is not limited to aliphatic side chain. Also, acids containing ethylene (e.g., 6-heptenoic acid) or acetylene bonds (e.g., 6-heptynoic acid and 2-octynoic acid) reacted with PBC sera (Table V). Another interesting finding is that all compounds that were derived from cinnamic acid were significantly more reactive with PBC sera than with control sera. Among those compounds were cinnamic acid derivatives carrying hydrophobic halogen or trifluoromethyl substitutions on the aromatic ring. However, aromatic structures derived from benzoic acid did not bind AMAs, unless they were substituted predominantly in para-position with halogens, trifluoromethyl, or alkyl groups, such as compounds 3, 5, or 139. We therefore hypothesize that the acid moiety attached to the lysine residue of the immunodominant PDC-E2 peptide must preferably be of hydrophobic character and of a certain size to be successfully recognized by the Ag-binding site of AMAs.

Of importance is the finding that, in fact, some of these xenobiotics are more reactive than the lipoylated PDC-E2 peptide (Table II). Importantly, our data from the inhibition study demonstrates that compounds 14, 17, 75, 78, 83, 88, 110, and 136-PDC-E2 peptide were cross-reactive with the lipoylated PDC-E2 peptide (Table III). However, the inhibition study with LA and compound 138 demonstrated that compound 138-PDC-E2 peptide is unique in that it did not cross-react with the lipoylated PDC-E2 peptide (Table IV). Sera Ig reactivity against 138 was not decreased after compound 88-PDC-E2 peptide absorption (remaining IgG reactivity against compound 138-PDC-E2 peptide after compound 88-PDC-E2 peptide absorption vs compound 138-PDC-E2 peptide absorption (0.1, 1, and 10 μg/ml, respectively); 98, 92, and 99% vs 85, 57, and 40%). Also, rabbit anti-LA Ab was not cross-reactive with compound 138 even though this Ab showed some cross-reactivity to compounds 88, 110, and 17 (Fig. 5). These findings suggest that anti-2-octynoic acid (compound 138) Abs are a new distinct Ab population of PBC. Furthermore, it should be noted that we observed significant IgG or IgM recognition to compound 138 in 35/41 (85%) of PBC sera compared with control

sera. We note that identification of hapten PDC-E2-specific Abs in patients with PBC warrants further work on determining the presence of T cell reactivity to modified PDC-E2, e.g., octynoic acid modified PDC-E2.

The lack of specific inhibition of Ig reactivity by compound 138 against LA, compounds 88, 10, and 134 indicates that they are not cross-reactive to compound 138. It is interesting to note that although both compounds 10 and 134 are recognized by IgG and IgM from patients with PBC, compound 134 is more reactive than compound 10. The chemical structures of compounds 10, 134, and 138 are similar in being aliphatic straight chains but are different in their carbon chain length and presence/position of the C-C triple bond. A higher degree of inhibition of Ig reactivity by compound 138 against compound 10 when compared with compound 134, suggests that the number of carbon atoms in the aliphatic chain might be important with regard to Ab binding of anti-compound 138. Further studies are necessary to define the significance of the C-C triple bond and the aliphatic carbon chain length in Ig recognition of this unique Ab population in PBC. Nevertheless, the distinct inhibition of Ig reactivity between compounds 10, 134, and 138 by compound 138 and the lack of cross-reactivity between LA and compound 138 supports the thesis that anti-2-octynoic acid Abs are a distinct Ab population in sera of patients with PBC.

2-Octynoic acid (compound 138) is a member of the acetylenic fatty acids. Although only a few acetylenic fatty acids are found in nature, they are chemically synthesized and widely used in human products. For example, 2-octynoic acid methyl ester, one of the oldest artificial flavorings, has been used since the 1900s in perfumes, soaps, detergents, lipsticks, toilet waters, facial creams, and perfumed cosmetics because of its violet scent (28–30). 2-Octynoic acid methyl ester also finds some use in flavor compositions mainly for cucumber, berry complexes, fruit blends, peach imitation, liqueur flavorings, and various "floral" and "muscatel" flavors. The concentration of 2-octynoic acid methyl ester in those products is normally low (0.1–2 ppm), but in chewing gum it may reach 10–20 ppm (31), There are also reports of an allergic reaction against 2-octynoic acid methyl ester (32, 33).

Our findings are interesting in light of recent data demonstrating Ab reactivity in patients with PBC against a ubiquitous microorganism, *Novosphingobium aromaticivorans*, which is able to metabolize xenobiotics similar to the chemical compounds identified in this study (34, 35).

Although PDC-E2 is the major autoantigen in PBC, the lipoyl domain of the other members of the 2-oxoacid dehydrogenase complexes are also targets of AMA, including the E2 subunit of the branched chain 2-oxoacid dehydrogenase complex, the E2 subunit of the 2-oxoglutarate dehydrogenase complex and the E3BP subunit of PDC (11, 36–41). In mammals, attachment of LA to the lipoyl domain is achieved by the lipoate-protein ligase in a two-step reaction (42, 43). Lipoic acid is activated by ATP (or GTP) and the lipoyl residue from the resulting lipoyl-AMP (or GMP) intermediate is transferred to the lysine residue on the lipoyl domain. It is important to note that the lysine residue of the PDC can accept a variety of carboxylic acids aberrantly even without the dithiolane ring (44–46). We submit that humans are exposed to a large number of xenobiotics and that such exposure commonly occurs in the liver. Many of these compounds have the potential to modify a lysine residue of PDC. In addition to environmental factors, there is growing evidence that genetic susceptibility also plays an important role in the etiology of PBC. An analysis of clinical characteristics in twins within a 1400-family cohort showed that concordance of PBC in identical twins is among the highest reported for patients with autoimmune diseases (47). Also, a recent report of a significantly higher frequency of X monosomy in female patients with PBC suggests that haplo insufficiency for specific X-linked genes may be an important factor leading to female susceptibility to PBC (48). In genetically susceptible individuals, these modified PDC-E2 molecules will lead to a loss of tolerance and the initiation of the AMA response. Because AMAs, when tested with recombinant Ags, are only found either in patients with PBC or in those with preclinical disease, we further submit that the clinical onset of disease will subsequently depend on a second insult. This second insult, perhaps a liver-specific oxidant stress will be a relatively common event since we do not find AMA positivity in the absence of clinical disease (49, 50).

Disclosures

The authors have no financial conflict of interest.

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