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Immunization with a Xenobiotic 6-Bromohexanoate Bovine Serum Albumin Conjugate Induces Antimitochondrial Antibodies[1]

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The E2 subunit of pyruvate dehydrogenase complex (PDC-E2) is the major autoantigen recognized by antimitochondrial Abs (AMA) in primary biliary cirrhosis (PBC). Recently, we replaced the lipoic acid moiety of PDC-E2 with a battery of synthetic structures designed to mimic a xenobiotically modified lipoyl hapten on a 12-aa peptide that was found within the immunodominant autoepitope of PDC-E2 and demonstrated that AMA in PBC reacted against several organic modified mimotopes as well as, or sometimes significantly better than, the native lipoyl domain. Based on this data, we immunized rabbits with one such xenobiotic organic compound, 6-bromohexanoate, coupled to BSA. One hundred percent of immunized rabbits developed AMA that have each and every characteristic of human AMAs with reactivity against PDC-E2, E2 subunit of branched chain 2-oxo-acid dehydrogenase, and E2 subunit of 2-oxoglutarate dehydrogenase complex. The rabbit AMA also inhibited enzymatic function of PDC-E2 and, importantly, binds to peptide sequences not present in the xenobiotic carrier immunogen. In contrast, BSA-immunized controls did not produce such activity. Our observation that animals immunized with a xenobiotic BSA complex produce autoantibodies that react not only with the xenobiotic, but also with mitochondrial autoantigens recognized by autoimmune PBC sera, suggests that environmental xenobiotic agents can be a risk factor for the induction of PBC. ***The Journal of Immunology,*** **2003, 170: 5326–5332.**

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The presence of antimitochondrial autoantibodies is the serological hallmark of primary biliary cirrhosis (PBC),[3] a female predominant autoimmune liver disease characterized by chronic progressive destruction of intrahepatic small bile ducts with portal inflammation (1, 2). Although antimitochondrial Abs (AMA) recognize several members of the 2-oxo-acid dehydrogenase complexes (2-OADC), the predominant reactivity is directed against E2 subunit of pyruvate dehydrogenase complex (PDC-E2) (3, 4). A common feature of these proteins is the presence of lipoic acid-binding domains toward which patient autoantibodies are directed (5–8). Moreover, the major autoepitope recognized by both $CD4^+$ and $CD8^+$ T cells is localized to peptides that are part of the same inner lipoyl domain (9).

Because optimal reactivity of AMA requires the lipoyl domain as a component of the autoantigen (10), its structural configuration has been reasoned to be important for the breakdown of self tolerance in PBC. This view led us to hypothesize that exposure of genetically susceptible individuals to a xenobiotic agent, which mimics the lipoated peptide of PDC-E2, may be important in the etiology of PBC. Therefore, in a recent study, we synthesized the immunodominant 12-aa peptide epitope within the inner lipoyl domain of PDC-E2, replaced the lipoic acid moiety with a battery of synthetic structures designed to mimic a xenobiotically modified lipoyl hapten, and quantitated the reactivities of these structures with sera from PBC patients. Of importance was our finding that AMA from all patients with PBC, but not controls, reacted against several of the organic modified mimotopes as well as, or significantly better than, the native lipoyl domain (11). This observation prompted us to define the biological effect of these organic modified mimotopes in vivo, and to determine what effect these compounds may have when exposed to the immune system in another context, i.e., one that lacks the PDC-E2 peptide backbone. We report in this study that immunization of rabbits with bromohexanoate-BSA breaks tolerance to self mitochondrial proteins and induces AMA with each and every characteristic of the autoantibodies in patients with PBC.

Materials and Methods

Immunization of rabbits with organic modified mimotopes

Sixteen-week-old female New Zealand White rabbits were divided into three groups and immunized initially with a mixture of compounds (100 μg per compound/animal) s.c. in the presence of CFA and then boosted every 2 wk with the compound mixture and IFA. Group A was immunized with BSA-conjugated aromatic CF3 compounds; group B was immunized with BSA-conjugated halogenated aromatics; and group C was immunized with BSA-conjugated halogenated straight chain hydrocarbons (Fig. 1). The choice of these compounds was based on our earlier

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[3] Abbreviations used in this paper: PBC, primary biliary cirrhosis; AMA, antimitochondrial Ab; BCOADC-E2, branched chain 2-oxo-acid dehydrogenase; BEC, bile duct epithelial cell; OADC, oxo-acid dehydrogenase complex; OGDC-E2, E2 subunit of 2-oxoglutarate dehydrogenase complex; PDC-E2, E2 subunit of pyruvate dehydrogenase complex; PDC-E2-LA, lipoylated PDC-E2; RIg, rabbit Ig; RSA, rabbit serum albumin; STP, sodio 4-sulfotetrafluorophenoxy; Cpd, compound.

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work (11). Sera were collected beginning 8 wk after the initial immunization and every 4 wk thereafter for analysis of AMA reactivity by immunoblotting and ELISA against rOADC-E2 peptides. Based on the observation that group C halogenated straight chain hydrocarbons was the only group that produced AMAs, a second experiment was initiated. In this experiment, groups of New Zealand White rabbits at 16 wk of age were immunized with each of the three compounds in group C conjugated to BSA and followed once again for the production of AMAs. This second experiment demonstrated that animals immunized with either compound (Cpd) 7 or Cpd 8 conjugated to BSA, but not Cpd 9, produced AMAs. We then selected Cpd 7 for further study, and a final group of female New Zealand White rabbits at 16 wk of age was immunized s.c. with 100 μg/animal of 7-BSA (n = 10) or 100 μg/animal of BSA alone (n = 10) in the presence of CFA and then boosted every 2 wk in the presence of IFA. Sera were collected 4 wk after initial immunization and every 4 wk thereafter for analysis of AMA reactivity. Animal protocols were approved by the Institutional Review Board at the University of California.

Synthesis of 6-bromohexanoate BSA conjugate (7-BSA)

Water-soluble sodio 4-sulfotetrafluorophenoxy (STP) esters from 6-bromohexanoic acid (compound 7) were synthesized, as follows: 1,3-dicyclohexylcarbodiimide was added to a mixture of 4-sulfotetrafluorophenol sodium salt (2.4 mmol) and 6-bromohexanoic acid (2 mmol) in 1 ml dimethylformamide and 10 ml acetone. After stirring at room temperature overnight, the precipitate (dicyclohexylurea) was removed by Celite filtration and the solvent was removed by rotovap. The crude solid was purified by column chromatography with 25% dichloro methane in acetone to give the pure product as white crystals. Compound purity was verified by proton nuclear magnetic resonance and the presence of only one spot on TLC. The STP ester was then coupled to the lysine residues of BSA. There were no significant differences in the coupling efficiencies of all STP active esters (e.g., with 7, 8, and 9) with BSA relative to control, as monitored by appearance of the coupling byproduct (2, 3, 5, 6-tetrafluoro-4-sulfophenyl, sodium salt).

Detection of AMA reactivity and AMA isotype

AMA reactivity against recombinant proteins of PDC-E2, branched chain 2-oxo-acid dehydrogenase (BCOADC-E2), and OADC-E2 was analyzed by ELISA (7, 12) using recombinant proteins coated on 96-well microtiter plates at 5 μg/ml in carbonate coating buffer. After washing three times in PBS/0.05% Tween, the plates were blocked with 3% nonfat dry milk in PBS for 1 h, incubated with 100 μl of rabbit sera (1/100 dilution) for 1 h at room temperature, and then washed with PBS/0.05% Tween, as above. Then 100 μl of HRP-conjugated anti-rabbit Ig (anti-RIg) (BioSource International, Camarillo, CA) was added to each well, incubated for 1 h at room temperature, and washed as above. Immunoreactivity was detected by measuring the OD at 405 μm after incubating with 40 mM of 2.2′-azinobix(3-ethylbenzthiazoline sulfonic acid) containing 0.05 mM hydrogen peroxide.

Rabbit mitochondrial preparations were resolved on 8% SDS-PAGE at 30 mA, transferred onto nitrocellulose filters, blocked with 3% nonfat dry milk in PBS, pH 7.4, and probed with immunized rabbit sera (1/100 dilution) for 1 h. After three 10-min washes with PBS/0.05% Tween, the filters were incubated with HRP-conjugated anti-RIg, washed with PBS Tween, and developed by chemiluminescence. Immunoreactivity against recombinant 2-OADC-E2 polypeptides was performed, as described (7). Positive and negative sera from patients with PBC were used throughout as controls. The isotype of the rabbit AMA was determined by immunoblotting against mitochondrial preparations using isotype-specific anti-rabbit Abs.

PDC enzyme activity assay

To determine whether the 7-BSA-immunized sera inhibit PDC enzyme activity, a predetermined dilution of the sera was incubated with PDC (Sigma-Aldrich, St. Louis, MO) for 10 min at room temperature and added to a PDC reaction mixture containing 5 mM of sodium pyruvate, 2.5 mM NAD^+, 0.2 mM of thiamine pyrophosphate, 0.1 mM of coenzyme A, 0.3 mM of DTT, 1 mM of magnesium chloride, and 50 mM of potassium phosphate buffer, pH 8.0. The change in absorbency per minute at 340 nm was monitored for 5 min. Enzyme activity of preimmunized sera was run in parallel, and the values were defined as 100% activity. The inhibition of PDC enzyme activity by PBC and control human sera was analyzed in parallel.

Ag specificity of 7-BSA-immunized rabbit sera

To determine whether Abs to 7-BSA are cross-reactive to the OADC enzymes, affinity-purified sera against 7-BSA were prepared. Briefly, 7-BSA was resolved on 8% SDS-PAGE and transferred to nitrocellulose filters, and the band was excised. The band was then blocked with 1% BSA, incubated with 1/10 dilution of sera for 1 h at room temperature, and washed with PBS containing 0.05% Tween 20, and the bound Ab was eluted with elution buffer (0.1 M glycine, 20 mM $MgCl_2$, 0.5% Tween, pH 2.5). Eluted Ab was immediately neutralized with 1 M Tris, and stored at −20°C until use. Affinity-purified Abs were then tested for their reactivity against recombinant peptides PDC-E2, BCOADC-E2, and E2 subunit of 2-oxoglutarate dehydrogenase complex (OGDC-E2) and an irrelevant negative control peptide by immunoblotting (12, 13). Furthermore, 6-bromohexanoate BSA conjugate-immunized rabbit sera (1/1000) were incubated with 100 μg of Ags (including BSA, 7-BSA, 8-BSA, 9-BSA, rPDC-E2, rBCOADC-E2, rOGDC-E2, and an irrelevant protein control each at 100 μg/ml) separately overnight and analyzed for their reactivities against the above panel of Ags by ELISA. Percent reactivity was calculated as 1 − (OD of absorbed sera)/(OD of unabsorbed sera) × 100. Finally, the specificity of the carrier protein (BSA) was determined by incubating rabbit sera (1/1000) with 100 μg of 6-bromohexanoate coupled to different carriers, including OVA, rabbit serum albumin (RSA), histones, and RIg, and tested for their reactivity against BSA, 7-BSA, 7-RSA, PDC-E2, BCOADC-E2, and OGDC-E2 by immunoblotting.

Immunoreactivity against rabbit epithelial cells

To determine whether anti-PDC-E2 Abs from 7-BSA-immunized rabbits recognize self Ags, PDC-E2 affinity-purified Abs from 7-BSA-immunized rabbits were tested for their reactivity against rabbit epithelial cells by immunohistochemistry. Briefly, sera from three 7-BSA-immunized rabbits and three other BSA-only-immunized rabbit sera (as controls) were used for affinity purification of Abs against PDC-E2 and BSA, respectively. Nitrocellulose membrane (2 cm^2) was cut and soaked in recombinant protein PDC-E2 and BSA, 100 μg/ml, in PBS. The membranes were then dried and incubated in blocking solution (5% of dried skim milk powder in PBS) for 1 h at room temperature. Optimally diluted rabbit sera in blocking solution were added to the membrane and thereafter incubated for 1 h at room temperature and washed three times with PBST (0.05% Tween 20 in PBS) for 5 min of each time. Membrane-bound Abs were then eluted with elution buffer (0.1 M glycine, pH 2.6) for 30 min at room temperature and immediately neutralized with 1:10 vol of 1 M Tris-Cl, pH 9.6, buffer. Each of the affinity-purified Abs was used to stain rabbit epithelial cells; the affinity sera reduced the background seen with whole sera. Rabbit cells (CCL-7; American Type Culture Collection (ATCC), Manassas, VA), 1 × 10^5 cells/ml, were seeded to four-well glass plates and cultured in Eagle's minimal essential medium (ATCC) containing 10% FCS overnight at 37°C in a humidified 5% CO_2 atmosphere. Immunohistochemical staining was performed using the Vectastain ABC kit. Rabbit cells were washed three times in PBS and fixed with 4% paraformaldehyde in PBS for 20 min. The cells were then washed briefly with PBS, treated with permeabilization solution (0.3% of Tween 20 and Triton X-100 in PBS) for 10 min, and blocked with blocking solution (1% of normal goat serum in PBS) for 30 min. Thereafter, the cells were incubated with Abs to PDC-E2 for 2–4 h in the humidified chamber and washed three times in PBS. After that, the cells were incubated with biotin-conjugated anti-rabbit Ab for 30 min and washed three times in PBS before incubating with avidin and biotinylated HRP complex (Vector Laboratories, Burlingame, CA) solution. Reactivity was visualized by the addition of alkaline phosphatase substrate. The slides were mounted with crystal mounting medium and analyzed by fluorescent microscopy using the Olympus Provis Microscope (Olympus America, Melville, NY). BSA-purified Abs and preimmune sera were used as controls.

Inhibition of PDC-E2 reactivity of 7-BSA-immunized rabbit sera by PBC sera

A competitive ELISA was conducted to determine whether Abs from 7-BSA-immunized rabbits and AMA from patients with PBC recognize the same PDC-E2 epitope. Briefly, recombinant proteins of human PDC-E2 lipoyl domain were resuspended in carbonate coating buffer (5 μg/ml) and coated onto 96-well ELISA plates. After blocking with 3% BSA in PBS for 1 h, the wells were incubated with either optimally diluted PBC sera (n = 5), or normal control sera (n = 5), or blocking buffer for 1 h and then washed with PBS Tween three times. Sera from 7-BSA-immunized rabbits (n = 5) and BSA-immunized rabbits (n = 5) at 1:100 were added to the plates. After 1 h, the plates were washed and incubated with HRP-conjugated anti-rabbit Abs (Zymed Laboratories, San Francisco, CA) for 1 h.

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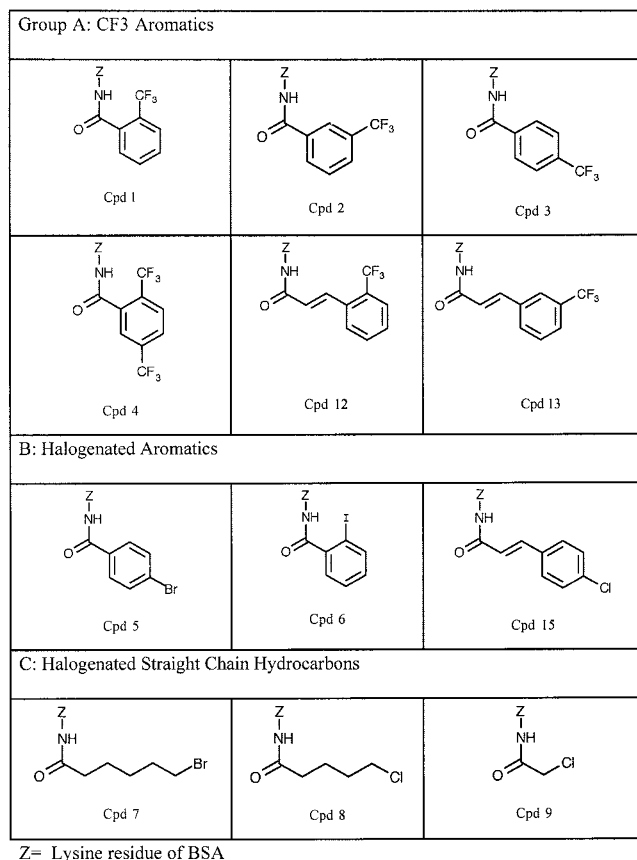

FIGURE 1. Structural configuration of lipoic acid mimics for rabbit immunization. Female New Zealand white rabbits at 16 wk of age were divided into three groups. Each group was immunized with a mixture of compounds (100 μg per compound/animal) s.c. in the presence of CFA and then boosted every 2 wk in the presence of IFA. Group A was immunized with BSA-conjugated aromatic CF3 compounds, group B was immunized with BSA-conjugated halogenated aromatics, and group C was immunized with BSA-conjugated halogenated straight chain hydrocarbons.

After washing, immunoreactivity of rabbit sera was determined by measuring the OD at 405 μm after incubating with 40 mM of 2,2′-azinobix(3-ethylbenzthiazoline sulfonic acid) containing 0.05 mM hydrogen peroxide. OD_{405} from wells of 7-BSA-immunized rabbits only was taken as 100% reactivity. Percent reactivity was defined as the mean OD_{405} of 7-BSA-immunized sera wells that were preincubated with human sera/mean OD_{405} of wells incubated by 7-BSA-immunized sera × 100%. The mean percent inhibition ± SEM of each group was determined.

Liver function analysis

Serum samples from 7-BSA- and BSA-immunized rabbits at 4 (n = 7), 7, and 10 mo postimmunization (n = 10) were analyzed for liver function, including alkaline phosphatase, γ-glutamyl transpeptidase, alanine aminotransferase, aspartate aminotransferase, cholesterol, total bilirubin, and total protein using the Roche COBAS Mira Classic Instrument (Englewood, NJ).

Results

Identification of 6-bromohexanoate as an AMA-inducing xenobiotic agent

Initially, female New Zealand White rabbits were immunized with mixtures of three groups of organic modified lipoyl mimotopes designated as groups A, B, and C (Fig. 1), which were each conjugated to the lysine residues of BSA. Among these groups, only those belonging to the mixture of BSA-conjugated compounds (7-BSA, 8-BSA, and 9-BSA) in group C induced readily detectable levels of AMAs against rOADC-E2s. In efforts to determine whether sera from the group C rabbits show cross-reactivity to OADC-E2, and to identify which of the individual group C compounds induced such AMAs, a competitive inhibition assay was performed. Thus, aliquots of the sera from rabbits immunized with the group C mixture of compounds were first incubated with 7-BSA, 8-BSA, or 9-BSA, and were then screened for their ability to react with the OADC-E2 enzymes by immunoblotting. Our data demonstrated that incubation of the AMA-reactive rabbit sera with either 7-BSA or 8-BSA inhibited the reactivity of such sera against the OADC-E2 enzymes. This observation is intriguing because AMAs, the hallmark of PBC, were induced by a xenobiotic compound conjugated to an irrelevant carrier (BSA) that mimicked only the lipoic acid moiety and was independent of the PDC-E2 peptide.

To confirm the above findings, a second series of experiments was conducted in which each of the three compounds in group C was assessed for its ability to independently induce AMA. Again, we immunized individual rabbits, each with a single compound (i.e., 7-BSA, 8-BSA, or 9-BSA) at 100 μg/animal. Rabbits immunized with 7-BSA and 8-BSA, but not 9-BSA, produced AMA at 4 and 8 wk, respectively, after the initial boost and continued to

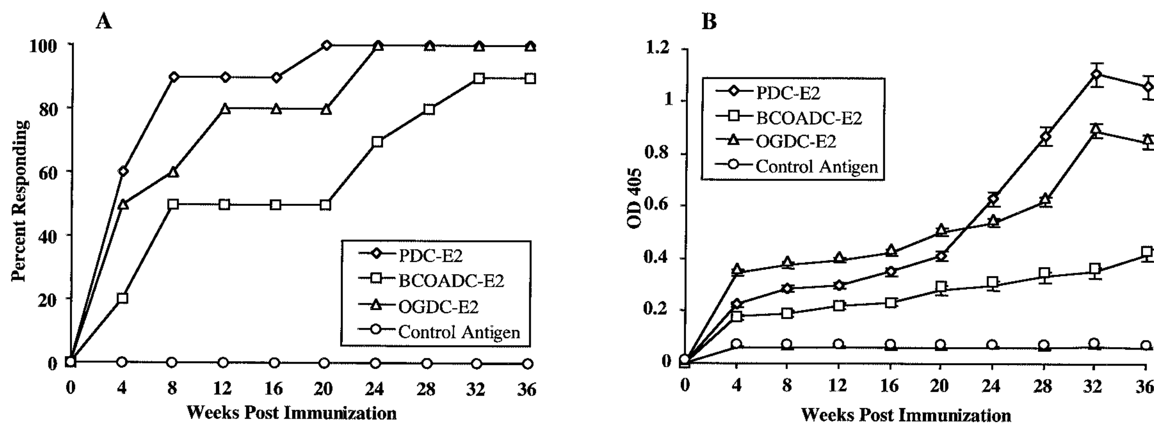

FIGURE 2. Temporal expression of 6-bromohexanoate BSA conjugate-immunized rabbit serum Ig reactivity against mitochondrial autoantigens and control Ag. Percent responsiveness (*A*) and ELISA (OD ± SEM) (*B*). Rabbit sera up to 36 wk postimmunization were assayed for AMA reactivity against PDC-E2, BCOADC-E2, OGDC-E2, and a negative control Ag by ELISA. All assays were performed in triplicate. In 6-bromohexanoate BSA conjugate-immunized rabbits, AMA to PDC-E2, BCOADC-E2, and OGDC-E2 were detected as early as 4 wk after initial immunization; by 20 wk postimmunization, 100% of rabbits were PDC-E2 positive.

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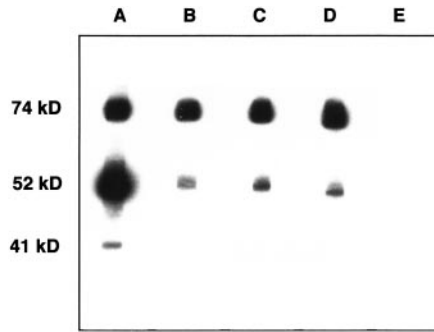

FIGURE 3. Immunoblot of sera (1/100 dilution) from rabbits immunized with BSA-conjugated 6-bromohexanoate and control (BSA) immunogens. Sera from 7-BSA-immunized rabbits reacted to a 74-kDa protein band (PDC-E2) and 52-kDa protein bands (BCOADC-E2 and OGDC-E2) (*B–D*). Sera from patients with PBC reacted to the same proteins and also to a 41-kDa PDC-E1α (*A*). Sera from BSA-immunized animals (*E*) were run as a negative control.

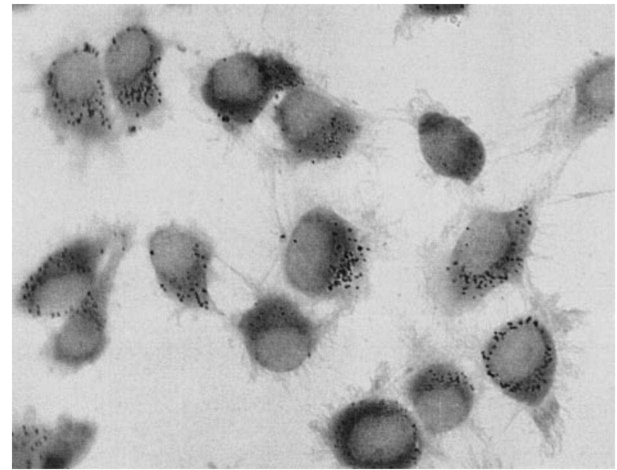

FIGURE 4. Immunohistochemical staining of rabbit epithelial cells. Note the typical AMA staining pattern of anti-PDC-E2 Abs from 7-BSA-immunized rabbits.

produce AMA thereafter. AMA was not detected from rabbit immunized with 9-BSA throughout. Results from these in vivo studies led to the identification of the BSA conjugate of 6-bromohexanoate (7-BSA) as the optimal AMA-inducing xenobiotic agent.

The 6-bromohexanoate BSA conjugate-induced AMA resembles human PBC

We then performed more detailed studies on 7-BSA using a larger group of rabbits. Thus, we immunized 10 rabbits with 7-BSA, and 10 rabbits with BSA only. Serum Abs to PDC-E2, BCOADC-E2, and OGDC-E2 were detected in the 7-BSA-immunized rabbits as early as 4 wk after the initial immunization (Fig. 2). By 20 wk postimmunization, 100% of the animals responded to PDC-E2. Of interest was the finding that the frequency of rabbits showing an autoantibody response was greatest for PDC-E2, followed by OGDC-E2 and finally BCOADC-E2, the identical order of frequency noted in cross-sectional studies of sera from groups of patients with PBC. These Abs are true autoantibodies, as they recognize rabbit OADC-E2 enzymes by immunoblotting (Fig. 3). These Abs were IgG. Furthermore, sera from immunized rabbits inhibited PDC enzyme activity (32 ± 3.8%), a finding also noted with human PBC sera (56 ± 9%). No AMA or enzyme inhibition was seen using preimmune sera and/or sera from the BSA-immunized control rabbits. In addition, affinity-purified rabbit Abs against 7-BSA reacted not only against 7-BSA, but also recombinant PDC-E2, OGDC-E2, and BCOADC-E2 proteins, but not to irrelevant protein control. These observations supported the view that 7-BSA indeed induced AMA by molecular mimicry.

Ag specificity and cross-reactivity hierarchy of the 6-bromohexanoate BSA conjugate induced AMA

To characterize the antigenic specificity of the serum Abs present in the 7-BSA-immunized rabbit sera, aliquots of these sera were preabsorbed with a panel of reagents, including BSA, 7-BSA, 8-BSA, 9-BSA, rPDC-E2, rBCOADC-E2, rOGDC-E2, and an irrelevant protein control. Each aliquot was then analyzed for reactivity against the above panel of Ags. Interestingly, when 7-BSA rabbit sera were absorbed (at 1/1000 dilution) with 7-BSA, reactivity to recombinant PDC-E2, BCOADC-E2, and OGDC-E2 (by immunoblotting and ELISA) was markedly decreased. Unrelated control Ags failed to absorb such reactivity (Table I). In contrast, when 7-BSA-immunized sera were absorbed with recombinant mitochondrial Ags (PDC-E2, BCOADC-E2, and OGDC-E2), their reactivity to 7-BSA remained virtually unchanged. The selective absorption of anti-PDC-E2 reactivity only by PDC-E2, of BCOAD-E2 reactivity by PDC-E2 or BCOADC-E2, and to OGDC-E2 by either PDC-E2, BCOADC-E2, or OGDC-E2, suggests that there is an important hierarchy in the cross-reactivity of the immune response reminiscent of the concept of determinant spreading.

The difference in the degree of absorption of BSA reactivity by 7-BSA, 8-BSA, and 9-BSA in our data is most likely due to differences of structural changes in BSA upon modification by each of these compounds. The data that incubation with 7-BSA removed most of the Ab reactivity to BSA when compared with 8-BSA and 9-BSA suggest that BSA is least modified in 7-BSA compared with 8-BSA and 9-BSA. In contrast to animals immunized with 7-BSA (a six-carbon aliphatic halogenated hydrocarbon) and 8-BSA (a five-carbon aliphatic halogenated hydrocarbon), no AMA were generated when the animals were immunized with 9-BSA (a two-carbon halogenated hydrocarbon). Therefore, the length and nature of the hydrocarbon side chain appear to be critical in breaking tolerance to the mitochondrial self Ags. To address the question of whether the hapten 6-bromohexanoate itself or the carrier protein BSA plays a role in the induction of

Table I. *Percent reactivity following absorption of rabbits immunized with 6-bromohexanoate BSA*[a]

		ABSORBENT							
Ag	Unabsorbed	BSA	7-BSA	8-BSA	9-BSA	PDC-E2	BCOADC-E2	OGDC-E2	Control
BSA	100	32.2	17.4	57.3	63.4	87.6	77.1	79.8	86.1
7-BSA	100	97.1	13.6	61.6	90.3	85.4	83.2	87.6	85.7
PDC	100	83.3	29.1	33.5	73.3	20.6	83.1	82.1	93.3
BCOADC	100	86.4	31.1	37.4	71.1	26.7	27.5	68	89.0
OGDC	100	89.6	35.5	43.2	77.9	30.1	33.7	30.1	92.2

[a] Expressed as remaining reactivity (%) determined by ELISA. SEM of all assays were <8.5%.

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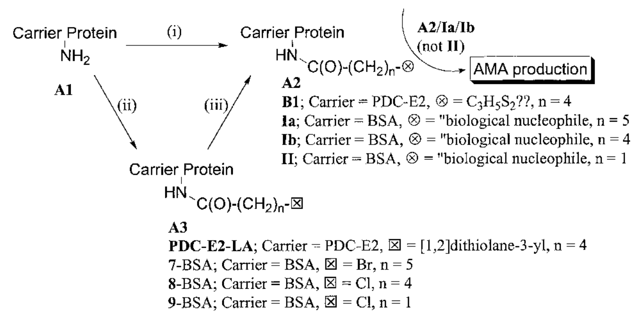

i. exposure to functional xenobiotic; ii. (for **A1** → **A3** only) exposure to pre-functional xenobiotic; iii. post conjugation modification (e.g. thiol addition)

FIGURE 5. Postulated mechanisms of AMA production in xenobiotic immunized rabbits. Mechanism A, Systemic protein modification in which A2 = HSA with a xenobiotic ($C(O)(CH_2)_n - \otimes$) covalently attached via lysine. A3 = lysine-functionalized HSA ($C(O)(CH_2)_n - \boxtimes$); postconjugation modification gives A2. Mechanism B, Lipoic acid modification in which PDC-E2-LA = PDC-E2 with lipoic acid on lysine and B1 = PDC-E2 with a xenobiotic modified (⊗) lipoic acid on lysine. Postulated mechanism of AMA generation from immunization with 7- and 8-BSA in which 7-BSA = BSA conjugate of compound 7, 8-BSA = BSA conjugate of compound 8, and 9-BSA = BSA conjugate of compound 9. Ia, Ib, and II = modified BSA conjugates (⊗ = undefined, presumably X (Br or Cl) is displaced by a biological nucleophile (e.g., glutathione)).

AMA in the xenobiotic immunized rabbits, 6-bromohexanoate were coupled to various carriers, including OVA, RSA, histones, and RIg, and tested for their ability to inhibit AMA reactivity of 7-BSA-immunized rabbits. Interestingly, similar to 7-BSA, 7-OVA, 7-RSA, 7-histone, and 7-RIg inhibited 7-BSA-immunized sera reactivity to rPDC-E2, rBCOADC-E2, and rOGDC-E2. These data suggest that the induction of AMA reactivity in 7-BSA-immunized rabbits is primarily due to the hapten.

The 7-BSA-immunized rabbits produce self-reactive anti-PDC-E2

In addition to our demonstration that 7-BSA-immunized rabbits recognized rabbit mitochondrial proteins by immunoblotting, we also tested the ability of 7-BSA-immunized rabbit sera to produce mitochondrial immunofluorescence using rabbit epithelial cells. As shown in Fig. 4, anti-PDC-E2 Abs from 7-BSA-immunized rabbits produced a typical mitochondrial pattern by immunofluorescence using rabbit epithelial cells. Sera from preimmunized rabbits and affinity-purified anti-BSA Abs from BSA-immunized rabbits did not produce any such reactivity.

AMA from 7-BSA-immunized rabbit sera and PBC sera recognize similar PDC-E2 epitopes

A competitive inhibition assay was performed to determine whether rabbit and human AMAs recognize overlapping epitopes of PDC-E2. AMA from human PBC sera inhibited the anti-PDC-E2 reactivity from 7-BSA-immunized rabbits to a level of $40 \pm 3.8\%$. We note that control human sera produced a negligible inhibition of anti-PDC-E2 activity of 7-BSA-immunized rabbit sera, with a value of $81.3 \pm 4.1\%$.

Liver enzyme levels of 6-bromohexanoate-immunized rabbits

In addition to the Ab profile, we also analyzed the serum chemistry of 7-BSA-immunized animals and controls for their liver enzyme function. At 4 mo after the initial immunization, levels of alkaline phosphatase were significantly higher in rabbits immunized with 7-BSA (49 ± 3.3 IU/ml) as compared with control BSA-alone-immunized rabbits (23.8 ± 1.9 IU/ml) ($p = 0.0296$). Similar differences in the levels of alkaline phosphatase were noted at 7 and 10 mo postimmunization (data not shown). There was also an elevation of cholesterol at the three time points examined, but no significant differences in serum levels of α-glutamyl transpeptidase, alanine aminotransferase, aspartate aminotransferase, total bilirubin, and total protein were noted. No changes in histopathology of the liver have been noted to date in select animals; additional animals remain under observation.

Discussion

The studies reported in this work demonstrate that sera from rabbits immunized with the 6-bromohexanoate conjugated to BSA develop AMA, with specificity for PDC-E2, BCOADC-E2, and OGDC-E2, the principal autoantigens of PBC. We have focused this study only on female animals, as PBC is predominantly a female autoimmune disease. The rabbit AMA are true autoantibodies, as they recognize rabbit mitochondrial proteins by immunoblotting (Fig. 3) and produce typical antimitochondrial staining pattern on rabbit epithelial cells (Fig. 4). Furthermore, the frequency of reactivity of the rabbit sera against each of the autoantigens follows a hierarchy similar to that seen in patients. The fact that the autoantibodies inhibit PDC-E2 enzyme activity, and rabbit anti-PDC-E2 reactivity competes with human PBC sera support our thesis that the response in these experimentally immunized animals recapitulates the autoimmune response characteristic of human PBC patients. It is also interesting to note that AMA reactivity of 7-BSA-immunized rabbit sera was inhibited by 7-BSA as well as a number of other carrier proteins with little or no significant sequence homology to BSA or PDC-E2. This suggests that the induction of AMA by 6-bromohexanoate is not dependent on any specific carrier protein. Further work is underway to see whether immunization of rabbits with other protein conjugates of 6-bromohexanoate can elicit an AMA response. These data provide evidence for the first time that AMA may be induced by the exposure of a genetically susceptible host to a xenobiotic, leading to the breakdown of tolerance to a self protein. Moreover, we note that AMA are also induced by 8-BSA. Thus, the breakdown of immune tolerance to the OADC enzymes is elicited not only by 6-bromohexanoate, but most likely other xenobiotics sharing similar chemical characteristics.

The mechanisms involved in the breakdown of self tolerance is one of the most important issues in defining the basis of PBC. In this regard, a number of hypotheses about PBC etiology have been tested and discounted: 1) PBC is a form of spontaneous chronic graft-vs-host disease (14). 2) PBC may be a cryptic infection that induces cross-reactive responses (15, 16). 3) Retroviruses are present at high frequency in PBC patients. Although Abs against retroviral elements can be detected in the sera of patients with PBC (17), this reactivity is clearly not disease specific. 4) Finally, unlike scleroderma (18), there is no evidence that fetal microchimerism alone can lead to PBC (19).

Immunization of a wide variety of animals with native (lipoated and/or recombinant lipoated and/or nonlipoated) human PDC-E2 results in Abs against the heterologous PDC-E2 used for immunization, but not any detectable Abs to self PDC-E2 (20). Reports of immunization of SJL mice with PDC-E2 leading to biliary pathology have been demonstrated in only a small number of mice, but the absence of control immunogens in this study, and subsequent failure of the finding to be replicated, makes this work difficult to interpret (21, 22). Moreover, the uniqueness of the model in this study is emphasized by the failure of five different strains of mice to produce AMA when injected with 7-BSA (data not shown), in contrast to the rabbits described in this work. This may be explained by studies that describe numerous species-related differences in susceptibilities regarding actions and toxicities of drugs

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and xenobiotic chemicals. For example, monkey liver P450 proteins are now known to have rates similar to humans for the catalytic activity toward the oxidation of several drugs, including phenacetin, coumarin, erythromycin, and nifedipine. In contrast, oxidation of these drugs by various animals, such as dogs, guinea pigs, and rats, varies a great deal by species (23). In addition, there are numerous differences among the laboratory animal species, particularly between mice and rabbits (24, 25). For instance, mice have only one isoform of CYP2C, while rabbits possess eight.

The data described in both the current and previous studies (11) are consistent with two possible mechanisms for breaking tolerance to the mitochondrial enzymes. These are illustrated in Fig. 5. The unifying theme in these mechanisms is that the autoimmune response is directed against the inner lipoyl domain of PDC-E2. They differ in how the initial immune response is mounted. Our rabbit immunization data suggest how AMA can be produced. In this mechanism, we postulate that exposure of carrier protein (A1) lysine residues with xenobiotics leads to lysine adducts (A2) that elicit AMA production perhaps through sequence or structural analogy to AMA epitope. Another possibility invokes formation of an initial adduct (A3) that undergoes subsequent biological modification to give the immunogen A2. The Abs are thus produced by breaking tolerance with the inner lipoyl domain of PDC-E2 leading to AMA.

Our recent study (11) probed a postulated mechanism in which we viewed lipoic acid-modified PDC-E2 (B1) as the immunogen for AMA production (Fig. 5). Indeed, as our previous study indicated, several size and shape xenobiotic modified lipoic acid analogs cross-react with PBC patient sera. This led to the hypothesis that an in vivo reaction of PDC-E2-LA with a xenobiotic forms immunogen B1, which breaks tolerance. Once again, the exact nature of B1, specifically $\otimes$, is unknown.

Our rabbit study embraces the possibility outlined in Fig. 5, in which AMA was induced by 7-BSA and 8-BSA, but another straight chain-halogenated hydrocarbon 9-BSA did not lead to AMA production. Why this difference between 7-BSA/8-BSA and 9-BSA? As illustrated in Fig. 5, the target of PBC AMAs is the inner lipoyl domain of PDC-E2, e.g., PDC-E2-LA. We postulate that a xenobiotic modified peptide cross-reacts with PDC-E2-LA when the lysine substituent of the xenobiotic modified peptide bears meaningful similarity to lipoic acid. Although $\otimes$ in 7-BSA, 8-BSA, and 9-BSA is unknown at present (note: we presume that $\otimes$, although structurally different, stereoelectronically approximates the $C_3H_5S_2$ moiety of lipoic acid), n in 7-BSA and 8-BSA equals 4 or 5 (note: n in PDC-E2-LA $= 4$), but the $n = 1$ of 9-BSA is too dissimilar from PDC-E2-LA.

Therefore, one possible chain of events that could lead to the generation of AMA is the introduction of a xenobiotic, either alone or conjugated to a carrier, with the capacity to conjugate to the OADC-E2 enzymes, either during synthesis or at some later stage, within the bile duct epithelial cells (BECs). This could result in an altered form of lipoic acid, or the replacement of lipoic acid with the xenobiotic hapten. The precise mechanism by which this would occur is unknown. However, once a xenobiotic adduct is conjugated to PDC-E2, this form would be more immunogenic, generating a cross-reactive response resulting in the breakdown of tolerance. One possibility is that the xenobiotic-conjugated PDC-E2 is toxic, thus generating apoptosis of the BECs, leading to fragmentation of the BECs and eventual uptake by APCs. The uptake and processing of this xenobiotic PDC-E2 molecule by APCs singly, or during the chronic phase of the disease in the form of immune complexes with anti-PDC-E2, could facilitate cross-presentation, followed by determinant spreading to BCOADC-E2 and OGDC-E2. A second possibility is that the carrier protein-xenobiotic conjugate is a mimic for PDC-E2 and as such is able to break tolerance similar to our rabbit model. Serum albumin binds a variety of hydrophobic compounds, including nonesterified long chain fatty acids, for which it serves as the principal transport protein in the plasma compartment (26). Thus, serum albumin may in fact be a prime candidate for the role of carrier/mimic when bound to the correct hapten. Of particular interest to the present communication is the recent data that posttranslational modifications of Ag may lead to altered processing and a breakdown in tolerance (27). This has been shown in both human and murine systems for collagen-induced arthritis, experimental autoimmune encephalomyelitis/multiple sclerosis, systemic lupus erythematosus, and celiac disease (28–33).

Xenobiotics have been suspected to contribute to the induction of autoimmunity (34). Many environmental chemicals or drugs are toxic to hosts, and their detoxification is achieved primarily in the liver. During their metabolism, they may form reactive metabolites, which can then modify cellular proteins to form neoantigens. The precise mechanisms that lead to modification of self proteins and the molecular requirements for this modified self to induce tolerance breakdown remain to be established. However, it is important to note that the direct toxic effect of xenobiotics is usually dose dependent and may be evident in the majority of individuals shortly after drug intake; hence, they are relatively easy to identify. In contrast, the immune-mediated effects that follow the intake of drugs or xenobiotics may take a prolonged period of time to be clinically manifest, making the identification of the causative agents a formidable task. Future studies are focusing on these issues, including the possibility that induction of disease will require not only a long exposure (chronicity), but also the presence of an inappropriate cytokine (i.e., Th1) liver environment.

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