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Primary Biliary Cirrhosis in Monozygotic and Dizygotic Twins: Genetics, Epigenetics, and Environment

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Background & Aims: There is growing evidence that the interplay of genetic susceptibility and environmental factors leads to primary biliary cirrhosis (PBC). In particular, family members of an infected individual have up to a 100-fold higher risk of developing PBC. Although concordant rates for identical twins in other autoimmune diseases range between 25% and 50%, there are no such data on PBC. Accordingly, we evaluated the concordance of PBC in a genetically defined population of twin sets and evaluated the clinical characteristics between concordant subjects. *Methods:* We identified 16 pairs of twins within a 1400-family cohort followed up by several centers worldwide, evaluated the diagnosis of PBC in all individuals, and determined the zygosity of sets reported as identical by the analysis of 2 highly variable HLA class II regions and 5 short tandem repeats. *Results:* Eight of 16 sets of twins were monozygotic. In 5 of 8 monozygotic twin sets, both individuals had PBC (pairwise concordance rate, 0.63). Among the dizygotic twins (n = 8), no set was found to be concordant for PBC. Interestingly, the age at onset of disease was similar in 4 of 5 concordant sets of monozygotic pairs; however, there were differences in natural history and disease severity. *Conclusions:* The concordance rate of PBC in identical twins is among the highest reported in autoimmunity. However, discordant pairs were identified. The data show not only the role of genetics but also emphasize that either epigenetic factors and/or environment play a critical role.

Primary biliary cirrhosis (PBC) is an autoimmune liver disease of unknown origin characterized by chronic inflammation and destruction of intrahepatic bile ducts, eventually leading to liver cirrhosis.[1] The prevalence of PBC varies in different geographic areas.[2,3] Xenobiotics have been suggested as environmental factors in loss of tolerance, leading to autoimmunity.[4] Although a number of genetic factors have been suggested in population and family studies, no definitive genetic association with the development of the disease or its outcome has been found.[5]

Disease concordance rates (CRs) (roughly defined as the proportion of affected pairs concordant for the disease) in monozygotic (MZ) (who are genetically identical but also share the environmental background) and dizygotic (DZ) (who share the environmental factors but not the genetic background) twin pairs are powerful tools to estimate the weight of genetic and environmental factors in the susceptibility to multifactorial diseases. In autoimmune diseases other than PBC, CRs in MZ twins vary widely (Table 1) and, with the exception of celiac disease, have been reported to be <0.50 in large cross-sectional studies. Data concerning PBC concordance in MZ twins are limited to 2 reports. In 1973, the case of twin sisters both with PBC was reported without ascertaining zygosity.[6] A more recent report described a pair of identical twins discordant for the disease,[7] although the monozygosity of the set has been debated[8] and the healthy twin was found to have low-titer antimitochondrial antibodies (AMA). In this study, we studied the CRs among 8 genetically proven MZ and 8 DZ twin sets.

Abbreviations used in this paper: AMA, antimitochondrial antibodies; CR, concordance rate; DZ, dizygotic; MZ, monozygotic; PBC, primary biliary cirrhosis; PCR, polymerase chain reaction; STR, short tandem repeat.

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Table 1. Evidence of Concordant Occurrence of Autoimmune Diseases in MZ and DZ Twins

	MZ concordance	DZ concordance
PBC[7]	Discordant pair reported	—
Primary sclerosing cholangitis[47]	Concordant pair reported	—
Systemic lupus erythematosis[48]	0.24	0.02
Sjögren's syndrome[49]	Concordant pair reported	—
Type 1 diabetes mellitus[40,50-52]	0.21–0.70[a]	0.00–0.13
Rheumatoid arthritis[53-55]	0.12–0.15	0.035–0.036
Graves' disease[56,57]	0.17–0.29	0.00–0.02
Multiple sclerosis[58,59]	0.25–0.31[a]	0.03–4.7
Celiac disease[43,60]	0.75–0.83	0.11

NOTE. Pairwise concordance rates (ranges) are reported when available.
[a]Pairwise concordance rate over 7.5 years or longer.

Patients and Methods

Subjects

As part of a long-term effort, our laboratory has collaborated with tertiary referral centers as well as the PBCers, an Internet group of patients. This database includes information on the presence of twins within families that have at least one index case of PBC. At the time this study was initiated, we estimate that there were approximately 1400 families in the database; within this group, we requested information as to whether any sets of twins were present. The investigators involved contacted the twins directly to determine whether they were interested in participating in this study. Through this effort, we identified 16 twin sets in which a diagnosis of PBC was encountered. There were no twins who declined participation in the study. To confirm the diagnosis, a protocol explaining the nature of the study and internationally accepted criteria for the diagnosis of PBC[1] was required. There were 8 of 16 twin sets that were believed to be identical. There were 8 additional sets referred to us as being nonidentical, based on either different sex or previously obtained data. To verify the zygosity of those twins referred to us as identical, blood samples were obtained and studied as described below. Clinical data were also obtained. A definite or probable diagnosis of PBC was made when at least 2 of the 3 following criteria were fulfilled: serum AMA positivity at a titer greater than 1:40, elevated serum alkaline phosphatase level for longer than 6 months, and diagnostic liver histology.[9] AMA status was determined by indirect immunofluorescence on Hep-2 cell lines, and a titer greater than 1:40 was considered AMA positive. Liver biopsy was not performed in asymptomatic subjects not presenting other diagnostic criteria. This study protocol respected the ethical guidelines of the 1975 Declaration of Helsinki and subsequent modifications; all patients gave their written consent at the referring center.

DNA and Sera Isolation

Peripheral blood samples were obtained for DNA isolation in 10-mL tubes containing EDTA. Genomic DNA was obtained from peripheral blood lymphocytes using the Qiagen BloodAmp Maxi Kit (Qiagen, Valencia, CA) and stored at −20°C before use.

Clinical Characteristics of Subjects

Clinical data were obtained from the referring centers; further, all physicians were asked to reevaluate the diagnosis of PBC and complete a comprehensive form describing the history and clinical data of each subject. Briefly, 2 of 3 conditions (elevated serum alkaline phosphatase level for longer than 6 months, positivy for serum AMA, or diagnosis by histology) had to be fulfilled to confirm the diagnosis of PBC. Biochemical, serologic, histologic, and ultrasonographic characteristics of patients at the time of blood sampling were also required. Other autoimmune conditions were rigorously evaluated by physicians in each case.

Determination of Zygosity

The zygosity of 8 twin pairs referred as identical was verified by DNA typing of 2 highly variable HLA class II regions and 5 short tandem repeats (STRs) localized on different chromosomes (listed in Table 2). HLA typing was performed at the University of Texas Southwestern in Dallas using a sequence-specific primer molecular method to identify DRB1 alleles present at an intermediate resolution level.[10] The presence of DRB3 (DR52), DRB4 (DR53), and DRB5 (DR51) was also determined to be positive or negative. The analysis of STR polymorphisms was performed at the University of California at Davis. In both cases, researchers were blind with

Table 2. Primer Sequences, Size Ranges, and Annealing Temperature Used for the Study of Microsatellite STR Polymorphisms

Locus	Size range (*base pairs*)	Annealing temperature (°C)	Forward primer	Reverse primer
D4S1647	132–156	45	TATTTCCAACACCCCTGCTA	AAGCAAAGAGGATTGAAAGTG
D5S815	252–296	45	TGGTATACTTGTGTAGCAAATTACA	TGCCATGATTGTTAAGTTTCC
D22S683	160–196	45	AACAAAACAAAACAAAACAAACA	GGTGGAAATGCCTCATGTAG
D7S796	162–198	55	TTTTGGTATTGGCCATCCTA	GAAAGGAACAGAGAGACAGGG
D21S1910	194–266	55	TTCTCTGGAATAAACGTGG	CACGGCAAAGTAGTATTTAATG

respect to the identity of subjects. Genotypes for STR polymorphisms were determined using polymerase chain reaction (PCR) amplification of 10 ng of genomic DNA in 25-μL total volume reactions. The PCR solution included 10 mmol/L deoxynucleoside triphosphate mix, 25 mmol/L $MgCl_2$ solution, 10× PCR Gold Buffer, 1 U AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA), 20 μmol/L Human MapPair Primers (Invitrogen, Carlsbad, CA), and deoxyribonuclease/ribonuclease-free water up to a 25-μL volume. Table 2 shows the sequences of the primer sets, the expected size range of the amplified sequence, and the appropriate annealing temperature. Forward primers were labeled with adenosine triphosphate (γ-33P) (Perkin-Elmer, Boston, MA) using a T4 Polynucleotide Kinase system (Invitrogen). Amplification was performed using a Programmable Thermal Controller (MJ Research Inc., Waltham, MA) under the following conditions: 10-minute denaturation at 94°C, 9 cycles of 45 seconds at 94°C, 45 seconds at 60°C to 52°C (−1°C per cycle), 60 seconds at 72°C, 35 cycles of 45 seconds at 94°C, 45 seconds at annealing temperature appropriate for each primer (Table 2), and 60 seconds at 72°C, followed by 7 minutes at 72°C. On completion of PCR, 1 μL of product was taken and added to 15 μL of dye and water and heated for 5 minutes at 95°C. Finally, 3 μL of product/dye solution was run onto a 7% polyacrylamide Bio-Rad Sequencing Gel (Bio-Rad Laboratories, Inc., Hercules, CA) for 3 hours at 60 W and then transferred to a Molecular Dynamics Phosphor Screen (Amersham Biosciences, Piscataway, NJ). Gel results were analyzed using ImageQuaNT by Molecular Dynamics (Amersham Biosciences). Figure 1 shows the visual results obtained with one set of primers (STR D22S683).

CR in MZ and DZ Twins

Two types of CR for PBC were assessed for MZ and DZ twins. Pairwise CRs were estimated as described by Emery[11] and MacGregor[12] (Pairwise CR = Number of Concordant Pairs/Number of Total Pairs). This CR simply provides the proportion of affected pairs concordant for the disease. To estimate the risk for a twin to be affected given that his or her cotwin has been diagnosed with PBC, we also calculated the proband-wise CR as follows: Proband-wise CR = 2× the Number of Concordant Pairs/(2× the Number of Concordant Pairs + the Number of Discordant Pairs).

Results

Diagnosis of PBC and Determination of Zygosity

Based on internationally accepted criteria,[1,9] we confirmed the diagnosis in 21 of 21 patients previously diagnosed with PBC by their referring physicians. In the remaining 11 of 11 individuals, we confirmed the absence of PBC, once again as noted by their referring physicians. No doubtful diagnosis of PBC (i.e., only one criterion fulfilled) was encountered. Results obtained by genetic analysis (combining HLA and STR independent typings) confirmed the monozygosity of the 8 twin pairs referred as identical. In our series, therefore, 8 MZ (all female; age range, 38–77 years) and 8 DZ (4 female and 4 male-female pairs; overall age range, 31–60 years) twin sets were found.

PBC in MZ and DZ Twin Sets

Among MZ twins (Table 3), PBC was diagnosed in both individuals within 5 pairs of twins; the other 3 sets were discordant for the disease, thus leading to a pairwise CR of 0.63 and a proband-wise CR of 0.77. When the 8 pairs of DZ twins were analyzed, none of them presented concordance for occurrence of PBC, leading to null pairwise CR and proband-wise CR.

In 3 of the 5 concordant MZ twin pairs (Table 4, sets 3–5), there were no significant differences in serology, disease stage, or accompanying symptomatology. The age at onset of PBC was similar in 4 of 5 sets of twins; in only one case (set 5), the 2 sisters were diagnosed with PBC within 5 years. In one case (set 2), the twins had significantly different clinical progressions, with one individual (A) presenting signs of stable disease for more than 13 years of follow-up and the other (B) requiring liver transplantation 8 years after the diagnosis of end-stage PBC. In another set (set 5), 2 different stages of PBC were observed at liver histology. We also note that the duration of follow-up in the 3 discordant DZ twins affected by PBC was between 5 and 12 years; the characteristics of the affected twins are presented in Table 5. No clear environmental differences could be identified between affected and nonaffected MZ twins.

Discussion

A large number of genetic and epidemiologic studies in PBC suggest a variable prevalence of disease in different geographic areas as well as a significant risk for development of disease in first-degree relatives of an affected subject.[2,3,13] In addition, the relatives of affected diseased individuals often develop PBC within a short time of the first case, suggesting environmental influences.[13] A large number of discrete genetic alleles have been studied in PBC, but there has not been definitive evidence of a specific association. Importantly, the strong association of HLA observed in other autoimmune diseases has not been found in PBC, and often the data are either conflicting or restricted to a specific geographic area.[14–26] Other polymorphisms have been suggested to confer susceptibility or influence progression of PBC, but again these data are either debated, conflicting, or nonconclusive.[27–30]

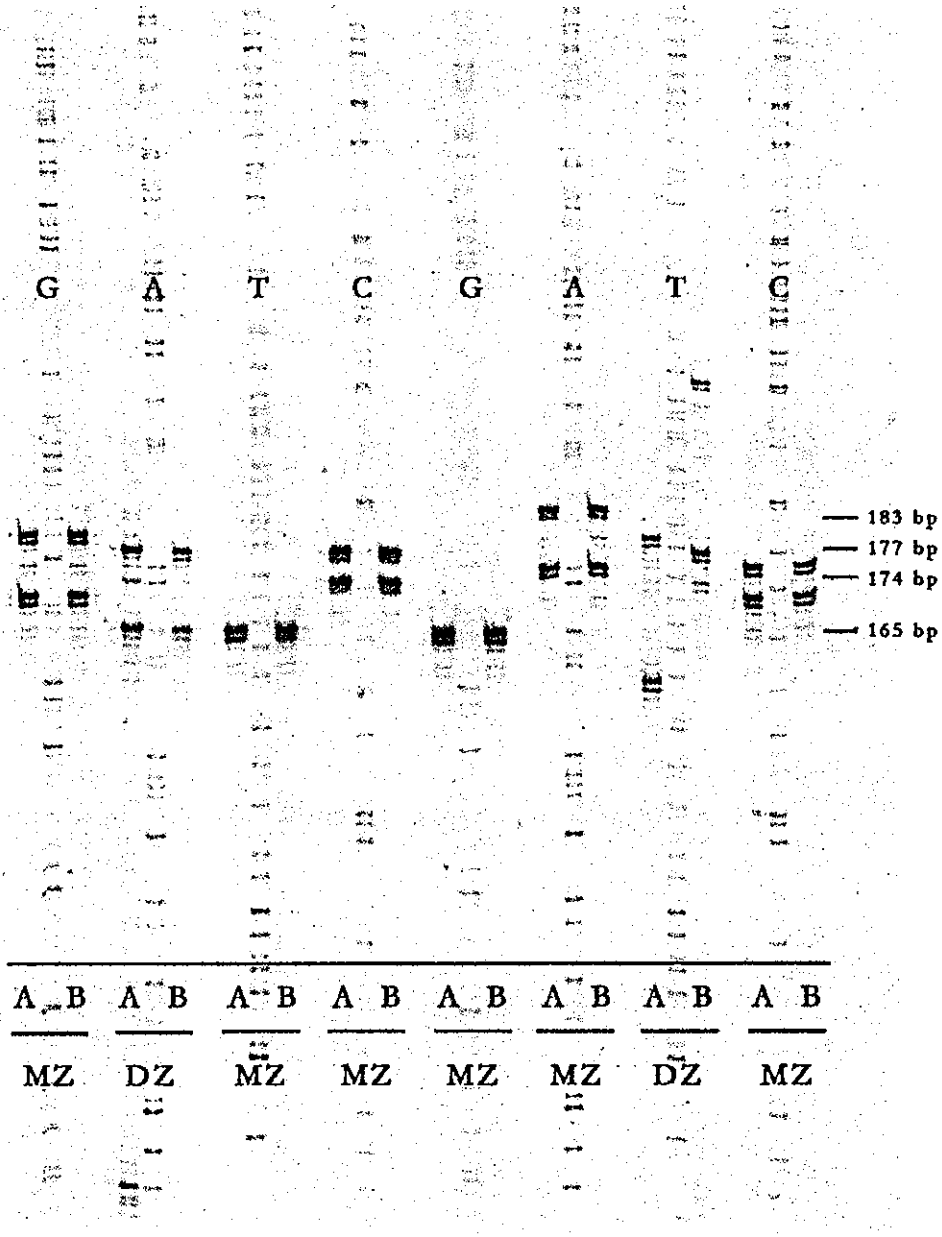

Figure 1. PCR products for the STR polymorphism (range, 160–196 base pairs) at D22S683 are shown for a group of representative MZ and DZ twins.

The frequency of MZ twinning has been relatively constant over the past 5 decades and is estimated to be 4 in 1000 births, whereas DZ rates change over time and are influenced by several factors.[31] We may therefore infer that the 8 MZ twin sets described herein reflect approximately 2000 patients with PBC, an estimate compatible with the 1400 families we identified. Studies comparing the CR between MZ and DZ twins are pow-

Table 3. Pairwise and Proband-wise CRs for PBC Observed Among MZ and DZ Twins

	Sex	Concordant for PBC	Proband-wise CR	Pairwise CR
MZ twin pairs (n = 8)	8/8 F/F	5/8	0.63	0.77
DZ twin pairs (n = 8)	4/8 F/F	0/4	0	0
	4/8 F/M	0/4	0	0

Table 4. Clinical Characteristics of MZ Twin Pairs Concordant for PBC

	Set 1		Set 2		Set 3		Set 4		Set 5	
	A	B	A	B[a]	A	B	A	B	A	B
Ethnicity	Asian		White		White		White		White	
Country of residence	United States		United States		United States		United States		Ireland	
Birth year	1925		1952		1964		1946		1953	
Occupation	Housewife	Housewife	Clerical	Consultant	Housewife	Housewife	Nurse	Publisher	Housewife	Housewife
Year of diagnosis (age [yr])	1990/65	1990/65	1990/38	1988/36	1997/33	1997/33	1995/49	1995/49	1998/55	2003/60
Status	Alive	Alive	Alive	Orthotopic liver transplantation (1996)	Alive	Alive	Alive	Alive	Alive	Alive
Follow-up (yr)	13	12	13	8	6	6	8	8	6	1
Symptoms	Pruritus	Fatigue	None	Liver failure	Fatigue	Fatigue	Fatigue	Fatigue	Pruritus, fatigue	Pruritus, fatigue
Latest histologic stage (yr)	III (1997)	II (1993)	I (1990)	IV (1996)	III (1999)	II-III (1997)	I-II (2000)	I-II (1995)	I (1998)	II-III (2003)
Endoscopic signs of portal hypertension	Mild portal hypertensive gastropathy	None	None	Esophageal varices	—	—	None	None	None	Not done
Associated autoimmune conditions	Autoimmune hepatitis	Sicca	Sicca	Sicca	None	Sicca	Hypothyroidism	Hypothyroidism	Raynaud's syndrome	None

NOTE. All subjects were female and AMA positive.
[a]All clinical data refer to the condition before orthotopic liver transplantation (1996).

erful tools to evaluate the role of genetic and environmental factors in determining the susceptibility to diseases that appear as multifactorial. However, it should be emphasized that the immune response depends on somatic mutation and such events are stochastic. In other words, there is evidence that the immune response of identical twins may not be identical, as recently described in multiple sclerosis.[32]

Increasing evidence, however, has been provided to challenge the traditional view of MZ twins as being "identical." Such evidence derives from studies on the effects of intrauterine characteristics as well as on epigenetics. Briefly, in the former case, it has been shown that there may be, during an antenatal infection, a differential involvement of twin fetuses.[33] Additionally, twins may be different based on the timing of the division of the fertilized egg into 2 separate embryos and ultimately the sharing of the amniotic sac, or chorionic and placental formation.[33] These specific environmental factors have been shown to be involved in the determination of the phenotype of the fetuses, albeit preserving their identical genome background. Furthermore, epigenetic alterations, indicating differences in the layer of protein and chemicals surrounding the DNA and particularly the

Table 5. Clinical Characteristics of Affected Subjects From MZ Sets Discordant for PBC

	Affected twin from discordant MZ sets[a]		
	5A	6A	7A
Sex	Female	Female	Female
Ethnicity	White	White	Asian
Country of residence	United States	United States	Japan
Birth year	1943	1946	1942
Occupation	Teacher	Teacher	Housewife
Year of diagnosis (age [yr])	1996 (53)	1989 (43)	1997 (55)
Status	Alive	Alive	Alive
Follow-up (yr)	8	12	5
AMA	Positive	Positive	Negative
Symptoms	None	None	None
Latest histologic stage (I–IV)	II (2001)	II-III (1999)	II (1997)
Endoscopic signs of portal hypertension	None	Not done	None
Associated autoimmune conditions	None	Psoriasis	Type 2 diabetes mellitus

[a]The unaffected twins from these sets remain asymptomatic.

sites or levels of DNA methylation, can also interfere with the direct genotype-phenotype correlation. Indeed, epigenetics play a role in cell aging[34] and neoplasia[35] and could lead to the discordance observed between MZ twins. For example, based on the surprising data on the variable expression of the sheep *callipyge* gene,[36] efforts are now being dedicated to the study of imprinted human genes. Additional factors that might also potentially interfere with the classical model of zygosity in twins are chimerism and mosaicism; these pockets of genetically mismatched cells appear to be relatively frequent in both single and twin births, possibly due to the antenatal sharing of blood supply.[37] A role for chimerism and mosaicism has been suggested in a number of complex diseases, including autoimmune disorders.[38] Similarly, recently suggested acquired haplotype deficiencies of specific chromosomes[39] could also be involved in explaining the discordant cases encountered.

In autoimmune conditions other than PBC (such as systemic lupus erythematosus, type 1 diabetes mellitus, Graves' disease, systemic sclerosis, and rheumatoid arthritis), CRs among MZ twins have been found in cross-sectional studies to be generally <0.50 (Table 1). Some of these studies, however, showed that CR could often be increased by longer periods of follow-up of unaffected individuals.[40]

Our population is the largest group of twins thus far described in PBC. There are, however, several limitations in our data. Firstly, of course, there is potential bias derived from enrollment. We note that this type of recruitment may be responsible for a higher prevalence of females, and that could lead to an overestimation of CR through an overascertainment of concordant pairs.[41,42] Such biases could be reduced by twin studies based on national or local registries. However, the lack of a reliable register of patients with PBC (as the one recently used for celiac disease[43]) and of a sensitive 100% reliable noninvasive marker of the disease (5%–15% of patients with PBC lack detectable AMA when tested by indirect immunofluorescence technique[1]) militate against this approach. Second, we note that longer observation periods will influence CRs; however, we note the long duration of follow-up of the discordant MZ sets described herein and, therefore, the consistent long-standing status of the unaffected twin. Finally, the data described herein show lower CR among MZ twins compared with our earlier abstract,[44] thus emphasizing the necessity of a comprehensive determination of zygosity. For example, one set of twins that was previously believed to be MZ and concordant for PBC turned out to be not twins at all but rather nontwin siblings.

The pairwise and proband-wise CRs for PBC in MZ twins described herein (Table 3) were found to be among the highest observed in cross-sectional twin studies on other autoimmune conditions except celiac disease (Table 1). This is a consistent finding with the relative risk factor for development of PBC within a family, which was also found to be the highest among autoimmune disease.[13] Moreover, we found that both twins within concordant sets were diagnosed with PBC within a short period of time in all but one set (Table 4), although this could be due to the increased awareness related to the diagnosis of PBC in the twin presenting earlier signs of disease. It is also interesting to note that the clinical and biochemical characteristics were nearly identical in only 2 of 4 concordant MZ pairs. In one set (Table 4; set 2), 2 very different clinical history patterns were encountered. One individual has had a nonprogressing early-stage disease for more than a decade of follow-up, whereas her twin sister underwent orthotopic liver transplantation 8 years after diagnosis of end-stage PBC. No apparent difference in any environmental factor could be identified between the 2 sisters. In another set (set 5), different disease stages were encountered at liver histology, although we emphasize that the liver biopsy specimen showing stage I in patient A was performed 5 years before the histology showing stage II–III was available in patient B. Moreover, 2 slightly different patterns of autoimmunity were noted in another set (Table 4; set 1) in which both sisters presented positive AMA as well as antinuclear antibodies. In one case (set 1, A), histologic analysis showed a PBC/autoimmune hepatitis overlap, whereas only PBC was found in the other (set 1, B). We also note that 2 of 5 MZ twin pairs (sets 2 and 4) also presented concordant occurrence of sicca syndrome and hypothyroidism as accompanying conditions. We submit that the relatively small number of observations in this study might account for a type II error, possibly responsible for the lack of clinical difference observed in 2 of 5 concordant sets. In diseases as uncommon as PBC, these data still represent a significant number of patients.

The finding of this high degree of concordance seems to suggest that genetics play a major role in the induction of PBC. The possible confounding role for antenatal factors,[33] epigenetics,[15] or chimerism and mosaicism,[37] as previously discussed, could strengthen this assumption. Our data do not rule out the likelihood that environmental factors serve as a trigger, as observed in celiac disease, that presents a high CR in MZ twins and that shares, like PBC, both genetic and environmental etiologic factors.[46] We suggest that a vigorous study using, for example, inheritance by descent or linkage analysis, is

critical to determine the genetic basis of PBC. Nonetheless, the size of the PBC cohort represented by the 8 MZ sets described herein is representative, based on the variable reported prevalence rates of PBC, of approximately 5–100 million general population individuals. We encourage an international effort to develop a database on a larger number of twin sets or representative families with more than one member affected to define the genetic basis of PBC.

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Biliary Epithelial Cells Regulate Autoreactive T Cells: Implications for Biliary-Specific Diseases

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The biliary epithelial cell (BEC) is the target for several human immune mediated liver diseases, including primary biliary cirrhosis, but it is not always clear whether the BEC functions as an accessory cell or an antigen presenting cell, although it is well documented that BECs express high levels of human leukocyte antigen Class II, intercellular adhesion molecule-1, and lymphocyte function–associated antigen-3. To examine this issue, we established autoreactive T-cell clones from human leukocyte antigen–DR53 patients with primary biliary cirrhosis and characterized BEC function as a function of the ability of BECs to regulate T-cell activation. We report herein that BEC-mediated T-cell activation occurs partially via programmed death 1 ligands in a cell-contact–dependent manner. Further, such activation occurs via prostaglandin E2 production in a cell-contact–independent fashion. Moreover, the production of prostaglandin E2 was partially controlled by interleukin-1β and tumor necrosis factor α. In conclusion, the regulatory activities of BECs are important for the maintenance of peripheral immune tolerance. Further, modulation of BEC function may be used for therapeutic modulation. (HEPATOLOGY 2005;41:151–159.)

The biliary epithelial cell (BEC) is the target lesion of several enigmatic human immunologically mediated liver diseases, including primary biliary cirrhosis (PBC), but it is unclear whether the biliary epithelium is the innocent victim of an immune attack or whether it participates actively as either an accessory cell (AC) or as an antigen-presenting cell (APC). One explanation for the vulnerability of BECs in PBC and other cholestatic pathology to immune attack is their relatively high expression of human leukocyte antigen (HLA) class I, HLA class II, intercellular adhesion molecule-1, and lymphocyte function–associated antigen-3.[1-7] In PBC, BECs also express B7 molecules.[8,9] Further, following *in vitro* stimulation with the proinflammatory cytokines (*i.e.*, interferon γ [IFNγ]), BECs express HLA class I, HLA class II, intercellular adhesion molecule-1, lymphocyte function–associated antigen-3, and CD40.[10,11] In addition, BECs produce chemokines, including interleukin (IL)-8, monocyte chemotactic protein-1, and stromal cell–derived factor-1.[12-14] Thus, while BECs are the targets of immune attack, they may also be actively involved in the regulation of peripheral immunity.

We have previously reported the characteristics of different classes of autoreactive T-cell clone (TCC). One type of clone-recognized antigen (Ag) in a costimulation–independent manner while the other, in contrast, was costimulation dependent. Further, we demonstrated that BECs from patients with PBC do not function as APCs to either of these autoreactive TCCs. In fact, our data illustrated that BECs regulate the proliferation of costimulation–dependent TCCs after rendering them anergic.[15] This latter finding was unique and led us to investigate the mechanism of this regulatory effect of BECs in detail. We report herein that BECs regulate autoreactive T cells even in the presence of professional APCs through the direct action of several inhibitory molecules, including ligands of programmed death 1 (PD-1)

Abbreviations: BEC, biliary epithelial cell; PBC, primary biliary cirrhosis; AC, accessory cell; APC, antigen-presenting cell; HLA, human leukocyte antigen; IFNγ, interferon γ; IL, interleukin; TCC, T-cell clone; Ag, antigen; PD-1, programmed death 1; PD-L1, programmed death 1 ligand 1; PD-L2, programmed death 1 ligand 2; PG-E2, prostaglandin E2; PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction; TNFα, tumor necrosis factor α; LPS, lipopolysaccharide; Ab, antibody; TGFβ, transforming growth factor β.

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(PD-1 ligands 1 and 2 [PD-L1 and PD-L2]) and through the indirect action of prostaglandin E2 (PG-E2). We postulate that the regulatory role of BECs on autoreactive T cells is important for the maintenance of peripheral immune tolerance in the portal area.

Materials and Methods

Generation of Autoreactive TCCs That Respond to Human PDC-E2 163-176. Using methods previously described,[15] we isolated and characterized 2 human autoreactive TCCs, coined HT7 and HK15, from 2 HLA-DR53 patients with early-stage PBC. Peripheral blood mononuclear cells (PBMCs) from these 2 patients were placed in RPMI medium containing 10% fetal calf serum and repeatedly stimulated by PDC-E2 163-176–pulsed irradiated autologous PBMCs. The 2 PDC-E2 163-176–specific TCCs were established by limiting dilution. These 2 TCCs were $CD3^+$, $CD4^+$, $CD8^-$, $CD45RO^+$, and $TCR\alpha\beta^+$ and produced a Th1-type cytokine profile, expressing high levels of IFNγ and low levels of IL-4, and recognized PDC-E2 163-176 in an HLA-DR53–restricted manner.[15] Both clones recognize amino acids 163-176 (GDLLAEIETDKATI) in PDC-E2 within the context of HLA-DR53. The antigenic peptide used in this study, PDC-E2 163-176, was synthesized on a solid phase simultaneous multiple peptide synthesizer (PSSM-8, Shimadzu, Kyoto, Japan), based on previously described Fmoc strategy.[16]

Generation of Human Intrahepatic BEC Lines. We simultaneously established BEC lines from human liver obtained at the time of orthotopic liver transplantation, using tissue derived from 3 different HLA DR53–positive patients with either end-stage PBC or other liver disease.[11,17] Briefly, liver specimens were digested with collagenase type IV (Sigma, St. Louis, MO); the digested cells were then harvested. BECs were immunomagnetically separated using Dynabeads-conjugated BerEp4 (Dynal Biotech, Brown Dear, WI), an epithelium-specific antibody. Cells were resuspended in complete BEC medium (a 1:1 mixture of Ham's F12 and DMEM, supplemented with 5% fetal calf serum, epithelial growth factor [10 ng/mL], cholera toxin [10 ng/mL], hydrocortisone [0.4 μg/mL], tri-iodo-thyronine [1.3 μg/L], transferrin [5 μg/mL], insulin [5 μg/mL], adenine [24.3 μg/mL] [Sigma], and hepatocyte growth factor [10 ng/mL] [R&D Systems, Minneapolis, MN), was then seeded in 12-well plates. The BECs were passaged before confluence. Importantly, the isolated cells were positive by immunohistochemistry for cytokeratin 7 and cytokeratin 19 (using specific antibodies obtained from Dako [Carpinteria, CA]) and immunoperoxidase detection. These criteria defined these cells as the BEC phenotype. Purified cells were greater than 99% pure, and the cultures used in the studies herein were between 4 to 6 passages.

Flow Cytometry. To analyze surface expression of costimulatory molecules, BECs were stained by indirect immunofluorescence using anti–HLA-DR (Becton Dickinson, San Jose, CA), anti–PD-L1 (R&D Systems) and anti–PD-L2 (Genzyme, Cambridge, MA). TCCs were stained by indirect immunofluorescence using anti–PD-1 (Becton Dickinson, Franklin Lakes, NJ). Dilutions of each antibodies were 10 μg/mL. Known positive and negative cells were used in parallel, and stained cells were analyzed on a FACScan (Becton Dickinson).

Isolation of Messenger RNA and Reverse-Transcriptase Polymerase Chain Reaction (PCR). BECs were cultured in 6-well plates for 0, 2, 4, 8, or 24 hours in the presence of IFNγ (1,000 U/mL), tumor necrosis factor α (TNFα) (1,000 U/mL), or lipopolysaccharide (LPS) (1 μg/mL). Total RNA was extracted from cultured BECs using an RNeasy kit (QIAGEN, Hilden, Germany) and the isolated RNA stored at −80°C. First-strand complementary DNA synthesis was performed with 5 μg of total RNA as a template using SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Quantitative PCR of first-strand complementary DNAs were performed using a LightCycler with gene-specific primer pairs of GADPH (Roche, Indianapolis, IN) as a control. After quantitation of GADPHcontents, each sample was diluted to equalize the concentration of GADPH. PCR for PD-L1 and PD-L2 were thence performed with gene-specific custom primer pairs (Sigma Genosys, St. Louis, MO); the conditions for amplification were 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 45 seconds, and extension at 72°C for 45 seconds. PCR products were resolved on a 1.5% agarose gel containing ethidium bromide. Gels were visualized under ultraviolet light and photographed. To reconfirm that BECs had a biliary phenotype and had lost a hepatocyte phenotype, PCR for albumin and alpha-fetoprotein were thence performed with gene-specific custom primer pairs (Sigma Genosys). The HepG2 cell line was used as positive control for albumin and alpha-fetoprotein.

Antigen Presenting Activity of s, HLA-DR53 Transfected Mouse L Fibroblasts, or BECs. T-cell proliferation was assayed by culturing the TCCs (5×10^4/well) with APCs in a 96-well plate for 72 hours. During the final 16 hours of culture, 0.5 μCi/well ^{3}H-thymidine was added. IFNγ-treated BECs (2×10^4), irradiated PBMC (2×10^5), or mitomycin C–treated HLA-DR53 transfected mouse L fibroblasts (2×10^4) were used as APC after pulsing with PDC-E2 163-176 peptide. Following cell harvest, ^{3}H-thymidine uptake was measured in a beta

Table 1. Thymidine Incorporation and PG-E2 Production of Coculture Experiments

		^{3}H-TdR* (cpm)	PG-E2† (pg/mL)
#1	BEC	13,879 ± 350	87 ± 23
#2	TCC	12,928 ± 264	79 ± 31
#3	TCC, Ag-nonpulsed PBMC	15,462 ± 122	203 ± 37
#4	TCC, Ag-pulsed PBMC	16,803 ± 43	407 ± 9
#5	BEC and TCC, Ag-nonpulsed PBMC	12,239 ± 92	213 ± 115
#6	BEC and TCC, Ag-pulsed PBMC	5,219 ± 1,628	44,333 ± 8,956
#7	BEC cocultured with supernatant of #3	12,330 ± 1,628	293 ± 34
#8	BEC cocultured with supernatant of #4	5,415 ± 428	46,666 ± 4,988

NOTE. Data points represent mean ± SD of triplicate cultures. BECs were preincubated with IFNγ.

*The supernatant of combined culture medium was added to the T-cell proliferation assay. $P < .001$ when compared #1 to #6, #3 to #6, #4 to #6; $P < .01$ when compared #2 to #6, #5 to #6, #7 to #6, #7 to #8; $P < .0001$ when compared #1 to #8, #2 to #8, #3 to #8, #4 to #8, #5 to #8; $P = .85$ when compared #6 to #8.

†The supernatant of combined culture medium was measured. $P < .01$ when compared #1 to #6, #2 to #6, #3 to #6, #4 to #6, #5 to #6; $P < .0001$ when compared #7 to #8.

scintillation counter. In specific experiments, anti–PD-L1 antibody (Ab) (10 μg/mL) or anti–PD-L2 Ab (10 μg/mL) (eBioscience, San Diego, CA) was used to block the effect of PD-L1 or PD-L2, respectively. For anti–PD-L1 Ab and anti–PD-L2 Ab, BECs were precultured with these antibodies for 30 minutes before removing excess antibody by washing prior to T-cell addition.

Transwell Assay. To assess the dependence of BEC regulation of T-cell activation on cell contact, transwell assays were performed using cell culture inserts (0.4-μm pore size; Becton Dickinson). Ag-pulsed irradiated PBMCs (3,000 rad) (1×10^6) and TCCs (5×10^5) were cocultured in the lower compartment of a 24-well plate, while BECs (1×10^5) were added to the lower or the upper compartment as accessory cells. Cells were then cultured for 72 hours; 0.5 μCi/well ^{3}H-thymidine was added to the cultures for the final 16 hours. ^{3}H-thymidine uptake was measured in a beta scintillation counter.

The Effect of BECs as ACs and the Effect of Supernatants From BECs. A T-cell proliferation was assayed by culturing TCCs (5×10^4/well) with PDC-E2 163-176 peptide pulsed or control (not pulsed) irradiated PBMCs (2×10^5) in a 96-well plate for 72 hours. During the final 16 hours of culture, 0.5 μCi/well ^{3}H-thymidine was added. Following cell harvest, ^{3}H-thymidine uptake was measured in a beta scintillation counter. To assess the effect of BECs as ACs on the proliferation of T cells, BECs (5×10^3) or BEC-cultured supernatants were added to this assay. In nested experiments, indomethacin (1 μg/mL) (Sigma), anti–transforming growth factor β (TGFβ) Ab (10 μg/mL) (R&D Systems), anti–IL-10 Ab (10 μg/mL) (R&D Systems), or NG-monomethyl-L-arginine (1 mmol/L) (Wako, Richmond, VA) were used to block the effect of PG-E2, TGFβ, IL-10, and nitric oxide, respectively. To assess the effect of supernatant of BECs on the proliferation of T cells, the supernatant of the combination of TCCs (5×10^5), PBMCs (1×10^6) and BECs (1×10^5) was added to the T-cell proliferation assay. Combined cells were cultured in 500 μL RPMI containing 10% fetal calf serum for 72 hours in a 24-well plate, and the supernatant was filtered and mixed 1:2 to the T-cell proliferation assay. The schema and groups used are shown in Table 1: (#1) BECs cultured alone; (#2) TCCs cultured alone; (#3) BECs cultured with nonantigen pulsed PBMCs; (#4) BECs cultured with Ag-pulsed PBMCs; (#5) BECs cultured with nonantigen pulsed PBMCs and TCCs; (#6) BECs cultured with Ag-pulsed PBMCs and TCCs; (#7) BECs cultured with supernatant of 36 hours cultured nonpulsed PBMCs and TCCs; or (#8) BECs cultured with supernatant of 36 hours cultured Ag-pulsed PBMCs and TCCs.

Quantitation of Cytokines, PG-E2,and Nitric Oxide. BECs (1×10^5) were cocultured in 500 μL RPMI containing 10% fetal calf serum in 24-well plates for 72 hours alone, with TCCs (5×10^5) and control Ag nonpulsed PBMCs (1×10^6) or with TCCs (5×10^5) and Ag (10 μg/mL) pulsed PBMCs (1×10^6), as described in Table 1. When specified, BECs were cultured with recombinant human IFNγ (1,000 U/mL) (Sigma), TNFα (1,000 U/mL), IL-1β (10 ng/mL) (Strathmann Biotech GmbH, Hannover, Germany), or LPS (1 μg/mL) (Sigma). BECs were also cultured in the presence of supernatants of TCCs and Ag-pulsed PBMCs with or without neutralizing anti-TNFα, anti–IL-1β, control immunoglobulin G (Genzyme), or indomethacin. TGFβ (R&D Systems), IFNγ, IL-10, TNFα (Japan Immunoresearch Laboratories Co., Gunma, Japan), IL-1β (BioSource Europe, Nivelles, Belgium), and PG-E2 (Amersham Life Science, Piscataway, NJ) from the culture supernatants were measured by enzyme-linked immunosorbent assays. The production of nitric oxide was estimated by measuring the amount of nitrite using the Griess reagent (Wako).[18]

Results

Characterization of BECs. Three BEC lines were used herein from 3 HLA-DR53–positive patients, 1 with PBC and 2 with HCV-positive cirrhosis. The 3 BEC lines gave similar data. As determined by reverse-transcriptase PCR, BECs have a biliary phenotype (Fig. 1A). As determined by flow cytometry, BECs did not express HLA-DR, while IFNγ-treated BECs expressed HLA-DR. BECs expressed the inhibitory molecules, PD-L1 and PD-L2, the ligands of PD-1, and TCCs expressed PD-1 (Fig. 1B). BECs also manifest messenger RNA encoding PD-L1 and PD-L2; the expression of which peaked at 2 to 8 hours after IFNγ treatment, as determined by semiquantitative PCR (Fig. 1C). Expression of PD-L1 and PD-L2 were also upregulated after treatment with either TNFα or LPS (Fig. 1C).

BECs Do Not Induce Proliferation of TCCs. To assess the ability of HLA-DR–expressing BECs to function as APCs, our costimulation independent or dependent auto Ag specific TCCs were cultured with IFNγ-treated BECs for 72 hours. Both costimulation independent HT7 and dependent HK15 proliferated when cocultured with Ag-pulsed HLA-DR53–positive PBMCs (18,060 ± 1,033 and 14,036 ± 2,220 cpm) as professional APCs. However, HT7, but not HK15, proliferated when cocultured with Ag-pulsed L-DR53 (21,203 ± 1,211 and 166 ± 32 cpm) (Fig. 2A-B). Both costimulation independent HT7 and dependent HK15 did not proliferate in response to Ag-pulsed IFNγ-treated BECs expressing HLA-DR53 (155 ± 32 and 154 ± 29 cpm) (Fig. 2A-B). These results indicate that BECs do not function as professional APCs to induce T-cell activation. To determine the mechanism of BEC-mediated T-cell inhibition, TCCs were cocultured with Ag pulsed BECs in the presence of anti–PD-L1 or anti–PD-L2 blocking antibodies. Costimulation–dependent HK15 did not proliferate in the presence of anti–PD-L1 (422 ± 33 cpm) or anti–PD-L2 (491 ± 9 cpm) blocking antibodies (Fig. 2B). In contrast, costimulation independent HT7 could be partially activated using the combination of both anti–PD-L1 and anti–PD-L2 antibodies (5,765 ± 123 cpm) during coculture with Ag-pulsed BECs ($P = .0002$, compared with anti–PD-L1, $P < .0001$, compared with anti–PD-L2) (Fig. 2A). These data indicate that HLA-DR–expressing BECs regulate T-cell activation, partially due to the expression of PD-L1 and PD-L2, which are the ligands of PD-1 of T cells.

BECs as ACs Regulate T Cells in a Cell-Contact–Independent Manner. To further assess the effect of BECs, TCCs were cultured with Ag-pulsed irradiated PBMCs as APCs in the presence or absence of BECs as ACs

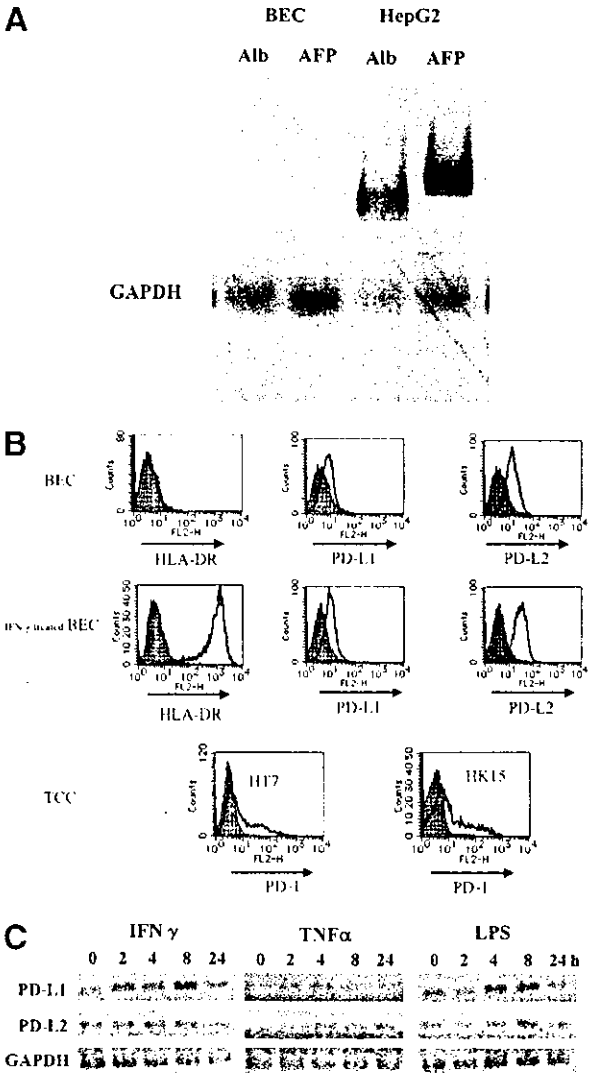

Fig. 1. Demonstration of biliary epithelial cell (BEC) phenotype, including the expression of human leukocyte antigen (HLA)-DR, programmed death 1 ligand 1 (PD-L1), and programmed death 1 ligand 2 (PD-L2) on BECs and the expression of programmed death 1 (PD 1) on T-cell clones (TCCs). (A) The expression of messenger RNA in BECs and the HepG2 cell line encoding albumin (Alb) and alpha-fetoprotein (AFP) was assessed by semiquantitative reverse-transcriptase polymerase chain reaction. The expression of messenger RNA encoding Alb and alpha-fetoprotein was detected in HepG2 but not in BECs. (B) Surface expression of HLA-DR, PD-L1, and PD-L2 on BECs was assessed by flow cytometry. Nontreated BECs (shaded histograms) did not express HLA-DR, while interferon γ (IFNγ) treatment (open histograms) upregulated HLA-DR. PD-L1 and PD-L2 were constantly expressed on IFNγ-treated or control (nontreated) BECs. PD-1 was expressed on TCCs regardless of their costimulation dependency. (C) The expression of messenger RNA in BECs encoding PD-L1 and PD-L2 was assessed by semiquantitative reverse-transcriptase polymerase chain reaction after treatment with IFNγ, tumor necrosis factor α or lipopolysaccharide for 0, 2, 4, 8, and 24 hours. The expression of messenger RNA encoding PD-L1 and PD-L2 was upregulated as early as 2 hours after stimulation, reaching a maximum at 4 to 8 hours after IFNγ, tumor necrosis factor α, or lipopolysaccharide treatment.