Is there a Relation between *Chlamydia* Infection and Primary Biliary Cirrhosis?

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Over the past two decades, a number of studies have failed to provide direct evidence of specific microbial chronic infection in primary biliary cirrhosis (PBC). However, a recent report suggests that there is a specific association of *Chlamydia pneumoniae* in patients with PBC and that *C. pneumoniae* or similar antigens might play a role in the pathogenesis of disease. To determine if *Chlamydia* infection is associated with PBC, we applied a combination of immunological and molecular approaches to investigate (a) the serological reactivity against two common *Chlamydia* human pathogens, *C. pneumoniae* and *C. trachomatis*, by immunoblotting, (b) the presence of *Chlamydia* in liver samples of patients with PBC and controls by PCR amplification of *Chlamydia* specific 16S rRNA and (c) the presence of *Chlamydia* proteins in liver samples of patients with PBC and controls by immunohistochemical staining. By immunoblotting, *C. trachomatis* and *C. pneumoniae* specific serological antibodies were found in 52/57 (91.2%) AMA positive PBC, 7/33 (21.2%) of AMA negative PBC, 1/25 (4%) PSC, 0/15 (0%) Sjorgen’s syndrome and 0/20 (0%) systemic lupus erythematosus patients and 0/20 (0%) healthy volunteers at 1:200 sera dilution. PBC sera reacted to *Chlamydia* and *E. coli* lysates in western blots up to a maximum of $10^{-2}$ dilution. However, PCR amplification of the *Chlamydia* specific 16S rRNA gene was negative in 25/25 PBC livers but positive in 1/4 PSC liver, 3/6 in other liver disease controls and 1/4 normal liver samples. While two commercially available specific monoclonal antibodies stained positive controls (*Chlamydia* infected HEp-2 cells) they failed to detect *Chlamydia* antigens in PBC livers. The detection of *Chlamydia* specific antibodies but not *Chlamydia* rRNA gene and *Chlamydia* antigens in PBC suggests that *Chlamydia* infection is not involved in PBC.

**Keywords**: *Chlamydia*; Cross reactivity; Infection; Primary biliary cirrhosis

**INTRODUCTION**

The breaking of tolerance by molecular mimicry of self-antigens by microbial proteins is an attractive hypothesis in the etiology of primary biliary cirrhosis (PBC) (Bjorkland and Totterman, 1994; Baum, 1995; Sutton and Neuberger, 2002; Datta, 2003; Hayakawa *et al.*, 2003; Plotz, 2003). PBC is serologically characterized by high titer anti-mitochondrial antibodies (AMA) in 95% of patients (Gershwin *et al.*, 2000). AMA are directed against E2 subunits of the 2-oxo-acid dehydrogenase complexes (2-OADC), and also against subunits E1 alpha, E1 beta and E3 binding protein of the pyruvate dehydrogenase complex (Leung *et al.*, 1997; Dubel *et al.*, 1999). AMA of PBC patients also react with bacterial E2s (Gershwin *et al.*, 2000). Reactivities are primarily peptide-specific but cross-reactivity between mitochondrial and microbial E2 antigens of respective complexes have been reported (Fussey *et al.*, 1990). The AMA immunodominant epitopes include the conserved sequence flanking the site of lipoyl attachment site of the 2-OADC.

It has been postulated that the initial stimulus for antibody production is chronic urinary tract infection (Baum, 1995). A number of studies have demonstrated significant bacteriuria in patients with PBC, with *E. coli* as the most common urinary isolate (Burroughs *et al.*, 1984; Rabinovitz *et al.*, 1992; Butler *et al.*, 1993; O’Donohue *et al.*, 1997). For example, *E. coli* rough forms were found in the urine of patients with significant bacteriuria including 39% in patients with PBC, 5.3% in patients with chronic liver disease and 41% in patients from the recurrent urinary tract infection group. Cross-reactivity between AMA and corresponding antigenic bands of *E. coli* has been demonstrated in sera from patients with PBC suggesting that AMA arise in “normal” women with recurrent bacteriuria and in females with PBC (Butler *et al.*, 1993).
Other studies have sought to associate microbial agents responsible for breaking the immune tolerance with mitochondrial proteins. For example, Xu et al. reported the evidence of human betaretrovirus infection in lymph nodes of patients with PBC by RT-PCR and immunohistochemistry (Xu et al., 2003). Screening of random peptide libraries with a PDC-E2 specific monoclonal antibody yielded eight different peptide sequences with little consensus (Chacko et al., 1997). Tanaka et al. concluded that no consensus 16S rRNA microbial sequence could be found in PBC liver specimens by PCR amplification from liver tissue specimens from patients with PBC and non-PBC controls (Tanaka et al., 1999). Altogether, these approaches, although rigorous, could not identify any infectious agent specific associated with PBC.

Recently, it has been suggested Chlamydia pneumoniae or similar antigens play an important role in the pathogenesis of PBC (Abdulkarim et al., 2002a,b). Briefly, C. pneumoniae antigens were detected in explant liver specimens from 25/25 patients with PBC (2 of whom were antimitochondrial antibody-negative) by immunohistochemistry. C. pneumoniae antigens were detected in periportal and lobular hepatocytes of PBC livers, whereas only 9/105 (8.5%) of patients in other categories (primary sclerosing cholangitis (PSC), alcoholic liver disease and chronic hepatitis C combined) were positive (p < 0.01, Fisher’s exact T test). To verify that Chlamydia infection is associated with PBC, we have probed the antibody reactivities of PBC and control sera samples against two common Chlamydia species; C. pneumoniae and C. trachomatis, pursued PCR based molecular detection in liver samples of control and PBC patients of Chlamydia specific 16S rRNA gene and sought to demonstrate the presence of the two Chlamydia types by immunohistochemistry in liver samples from PBC patients and controls.

MATERIALS AND METHODS

Source of Antibodies

A total of 170 sera samples were used in this study. These include sera from patients with PBC (n = 90), PSC (n = 25), systemic lupus erythematosus (n = 20), Sjogren’s syndrome (n = 15) and healthy volunteers (n = 20). All patients were diagnosed according to established criteria (Kaplan, 1996; Venables, 1998; Lee and Kaplan, 1999; Lee and Kaplan, 2002). In addition the sera from patients with PBC have been tested for the presence of AMA as described (Tanaka et al., 2002), which revealed 57 AMA positive and 33 AMA negative sera. Anti-liopoic acid antibody was generated as described (Humphries and Szwedda, 1998). Anti-Chlamydia antibodies were obtained from Virostat (Portland, ME). Monoclonal antibodies against 2-OADC-E2 were generated by immunization of Balb c mice with a recombinant triple hybrid containing the AMA epitopes of PDC-E2, BCOADC-E2 and OGDC-E2 (Migliaccio et al., 1998).

Tissue Samples

Explant liver specimens from 25 patients with PBC and 36 controls were studied. The PBC livers included 5 patients classified as stage I and II and 20 patients classified as stage III and IV. Controls included livers from patients with PSC (n = 10), other liver diseases including hepatitis (n = 6), cryptic cirrhosis (n = 8) and alcoholic liver disease (n = 8) as well as normal liver samples (n = 4).

Preparation of Recombinant Proteins

Recombinant peptides of mammalian PDC-E2, BCOADC-E2, OGDC-E2 were purified from the corresponding expression clone in pGEX (Moteki et al., 1996). Briefly, the E.coli clone was grown in LB containing 50 μg/ml of ampicillin and induced with 1 mM isopropyl beta-D-thiogalactoside (Alexus, San Diego, CA) for 6h. Cells were harvested and recombinant proteins were purified using glutathione agarose (Sigma) as described (Moteki et al., 1996). An irrelevant expression protein of the shrimp tropomyosin in pGEX (Leung et al., 1994) was purified similarly.

Serological Immunoreactivity against Chlamydia by Immunoblotting

Chlamydia trachomatis elementary bodies were obtained from Advanced Biotechnologies (Columbia, Maryland). C. pneumoniae was prepared from HEp-2 cells culture as described (Tjhe et al., 1997). A measured quantity of 100 μg of Chlamydia lysate was resolved on 10% SDS-polycrylamide gel electrophoresis (SDA-PAGE) and transferred to nitrocellulose membrane. The membrane was then cut into 3 mm strips, blocked with 3% non-fat dry milk in phosphate buffered saline (PBS) for 1 h and then incubated with human sera (1:200 dilution) for 1 h. Membranes are then washed with PBS containing 0.05% tween 20 for 4 times, 10 min each before incubating with horseradish peroxidase conjugated anti-human Ig (Zymed, South San Francisco, CA) for 1 h at room temperature. Membranes were then washed with PBS containing 0.05% tween 20. Antibody binding was visualized by incubating with a 0.05% solution of 3, 3’ diaminobenzidine (Sigma) and 0.01% hydrogen peroxide. To determine the identity of the Chlamydia immunoreactive bands, PBC sera (1:200 dilutions) were preincubated with 100 μg of recombinant proteins of PDC-E2, BCOADC-E2 and OGDC-E2 and the irrelevant antigen noted above at 4°C for overnight prior to probing against Chlamydia by immunoblotting. In addition, 100 μg of Chlamydia lysate and the same amount of E.coli lysate were resolved by SDS-PAGE, and transferred onto nitrocellulose membrane.
Reactivity of PBC at $10^3$–$10^6$ of sera dilutions were analyzed by immunoblotting against equal concentrations of Chlamydia and E. coli lysate followed by chemiluminescent detection (Pierce, Rockford, IL).

**PCR Amplification of Chlamydia 16S rRNA Gene**

Total DNA was isolated from explant liver samples using the QI Amp DNA mini-kit (Qiagen, Valencia, CA) according to manufacturer’s instruction. Touch down polymerase chain reaction (PCR) was conducted to amplify the 16S rRNA gene of C. pneumoniae from liver DNA samples using specific primers (CpnA): 5’-TGAC-AACGTGAGAAATACGC-3’ and (CpnB): 5’-CGCCT-CTCTCCTATAAT-3’ as described (Gaydos et al., 1992; Gaydos et al., 1994a,b; Boman et al., 1999). Similarly, the C. trachomatis 16S rRNA gene was amplified using specific primers (CtnA): 5’-TGACCCGCGCAGAAATG-TGCT-3’ and (CtnB) 5’-CGCCTCCTCCTCCTTGCAGG-3’ as described (Gaydos et al., 1992; Gaydos et al., 1994a,b; Boman et al., 1999). C. pneumoniae and C. trachomatis DNA were included as controls. Briefly, the reaction mixture contained liver DNA samples, 0.5 μM primers, 0.2 mM dNTPs, 1 × PCR buffer and 1 unit of Taq polymerase. Samples were subjected to 6 cycles of denaturation (94°C, 1 min), annealing (60–54°C, 1°C step down each cycle, 1 min) and extension (72°C, 1 min), followed by 30 cycles of denaturation (94°C, 1 min), annealing (55°C, 1 min) and extension (72°C, 1 min). Negative (DNAse, RNAse free water and DNA extracted from E. coli) and positive controls (DNA extracted from C. pneumoniae and C. trachomatis) were included. PCR products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining (0.5 μg/ml). Positive and negative controls are included throughout (Boman et al., 1999).

**Immunohistochemical Staining**

Immunohistochemical staining with monoclonal antibodies against C. pneumoniae were performed on liver sections from patients with PBC (n = 10) and other disease controls (six PSC, one alcoholic liver cirrhosis, five cryptic cirrhosis and six Non-A, Non-B hepatic cirrhosis). Briefly, paraffin sections were incubated at 65°C for overnight and deparaffinized in four changes of xylene. The slides were then washed in two 100% alcohol and two 95% alcohol rinses and then finally rinsed in tap water. The slides were washed in phosphate buffered saline (PBS, pH 7.4) and incubated with blocking solution (1.5% of normal horse serum in PBS). Following blocking, slides were incubated with 1:20 diluted monoclonal antibody against C. pneumoniae (Virostat, Portland, ME) for 1 h in a humidified chamber and thereafter washed three times in PBS. After that, the slides were incubated with biotin labelled anti-mouse-IgG 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).

**RESULTS**

Serological Immunoreactivity against Chlamydia

By immunoblotting, Chlamydia specific serological antibodies were found in 52/57 (91.2%) AMA positive PBC and 7/33 (21.2%) of AMA negative PBC sera. Out of the 90 PBC sera, 59 (65.6%) reacted with C. trachomatis and 51 (56.7%) reacted with C. pneumoniae. 1/25 (4%) of PSC sera reacted to both C. trachomatis and C. pneumoniae, 0/15 (0%) of Sjogren syndrome and 0/20 (0%) of healthy subjects reacted to either of the Chlamydia species (Table I). Moreover, the identities of the major Chlamydia reactive proteins were determined as the E2 subunits of 2-oxo-acid dehydrogenase complex by the antigen inhibition studies and confirmed by their specific reactivities to monoclonal antibodies to mammalian 2-OADCC-E2 (Fig. 1). Irrelevant recombinant protein of the shrimp tropomyosin did not inhibit.

<table>
<thead>
<tr>
<th>Chlamydia species</th>
<th>Group (No. of samples)</th>
<th>No. of positive samples/total (%)</th>
</tr>
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<tbody>
<tr>
<td><strong>Chlamydia trachomatis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary biliary cirrhosis</td>
<td>(n = 90)</td>
<td>59/90 (65.6%)</td>
</tr>
<tr>
<td>Primary sclerosing cholangitis</td>
<td>(n = 25)</td>
<td>1/25 (4%)</td>
</tr>
<tr>
<td>Sjogren’s syndrome</td>
<td>(n = 15)</td>
<td>0/15 (0%)</td>
</tr>
<tr>
<td>Systemic lupus erythematosus</td>
<td>(n = 20)</td>
<td>0/20 (0%)</td>
</tr>
<tr>
<td>Normal</td>
<td>(n = 20)</td>
<td>0/20 (0%)</td>
</tr>
<tr>
<td><strong>Chlamydia pneumoniae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary biliary cirrhosis</td>
<td>(n = 90)</td>
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<td>0/20 (0%)</td>
</tr>
<tr>
<td>Normal</td>
<td>(n = 20)</td>
<td>0/20 (0%)</td>
</tr>
</tbody>
</table>
Detection of C. trachomatis and C. pneumoniae Specific 16S rRNA Gene

PCR amplification of C. trachomatis and C. pneumoniae specific 16S rRNA gene showed that neither Chlamydia 16S rRNA gene was present in livers from patients with PBC, but was present in low percentage of the other controls (Table II).

Immunohistochemical Detection of Chlamydia in Liver Samples

C. pneumoniae were not detected on the bile ducts and hepatocytes of the liver sections from 10 PBC patients as well as 18 livers sections from other disease controls.

Titer of PBC Patient Sera against Chlamydia and E. coli

PBC sera reacted to both Chlamydia and E. coli at $10^{-3}$ and $10^{-4}$ sera dilution but did not react at sera dilution greater than $10^6$ (Fig. 2).

DISCUSSION

The underlying mechanism(s) in the breaking of immune tolerance is one of the most important issues in the etiopathology of autoimmune diseases. One hypothesis is based on molecular mimicry, i.e. mimicry by infectious agents of host antigens may induce cross-reactive autoimmune responses to epitopes within host proteins which, in susceptible individuals, may tip the balance towards mounting an immunological response and subsequently lead to autoimmune disease (Davies, 1997). In fact, a number of reports support this hypothesis (Zhao et al., 1998; Fournel and Muller, 2002). It has been suggested that infection can probably cause T cell activation in multiple sclerosis (MS) susceptible individuals (Hafler, 1999) and several microbial agents including C. pneumoniae have been implicated as the intracellular pathogens causing MS (Hunter and Hafler, 2000). On the other hand, a search for molecular mimicry in PBC has been done using monoclonal antibodies to PDC-E2 and random peptide libraries (Cha et al., 1996; Leung et al., 1996) and found mimeotope peptides that did not show any significant sequence homology to any known microbial peptides and did not generate PBC in animals.

Based on the significant sequence homology between the AMA epitopes and the E. coli lipoil enzymes, it was hypothesized that the initial stimulus for AMA production may result from chronic urinary tract infection (Baum, 1995). Although AMA themselves are not pathogenic, antigen specific CD4+ T cells could be primed, recognizing the lipoyl domain epitope in association with class II HLA and initiate the autoimmune cascade in patients with PBC. The association of urinary tract infections in patients with PBC has been studied and the data are inconclusive (Burroughs et al., 1984; Rabinovitz et al., 1992; Butler et al., 1993; O’Donohue et al., 1997). Similarly, data on association of PBC with other microbial agents remain elusive (Tanaka et al., 1999; Heathcote, 2000; Samonakis et al., 2003). We have used a three prone approach to determine if Chlamydia infection is involved in the pathogenesis of PBC through the use of a combination of immunological and molecular approaches.

Chlamydia are obligate intracellular euobacteria which are phylogenetically distinct from other bacteria. Two well-known Chlamydia human pathogens are

**TABLE II**  PCR amplification of Chlamydia 16S rRNA gene

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of samples tested</th>
<th>No. positive to C. pneumoniae</th>
<th>No. positive to C. trachomatis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary biliary cirrhosis</td>
<td>25</td>
<td>0/25</td>
<td>0/25</td>
</tr>
<tr>
<td>Primary sclerosing cholangitis</td>
<td>10</td>
<td>1/10</td>
<td>1/10</td>
</tr>
<tr>
<td>Hepatitis</td>
<td>6</td>
<td>1/6</td>
<td>0/6</td>
</tr>
<tr>
<td>Cryptic cirrhosis</td>
<td>8</td>
<td>3/8</td>
<td>3/8</td>
</tr>
<tr>
<td>Alcoholic liver disease</td>
<td>8</td>
<td>2/8</td>
<td>1/8</td>
</tr>
<tr>
<td>Normal</td>
<td>4</td>
<td>1/4</td>
<td>1/4</td>
</tr>
</tbody>
</table>
C. pneumoniae and C. trachomatis. C. pneumoniae is a widespread pathogen of humans causing pneumonia and bronchitis and it accounts for approximately 10% of pneumonia and 5% of bronchitis cases in the United States. In addition, there are reports associating C. pneumoniae infection with atherosclerosis and abdominal aortic aneurysm (Danesh et al., 1997; Juvenen et al., 1997a; Juvenen et al., 1997b). C. trachomatis infection causes trachoma, an ocular infection that leads to blindness and sexually transmitted diseases (Tabbara, 2001; Gaynor et al., 2003; Jensen et al., 2003). Analysis of the C. pneumoniae and C. trachomatis genome (Kalman et al., 1999) showed that the two genomes have a low level of DNA homology to the mammalian genome. However, genes for central metabolic pathways, including aerobic respiration are present and electron transport may be supported by pyruvate. Thus, it is not surprising that lipoyl containing proteins, which are necessary in mammalian aerobic respiration, are detectable in Chlamydia when anti-lipoic acid antibody was used as a probe (Fig. 1). Furthermore, the Chlamydia lipoyl acid containing proteins are recognized by AMA and the Ig reactivity can be inhibited by absorption with recombinant E2 proteins of the 2-OADC pathway (Fig. 1), suggesting that AMA in PBC are binding to the lipoyl domains of the Chlamydia homologue of the mammalian respiratory enzymes. 65.6% of the PBC sera sample tested reacted positively to Chlamydia, which is similar to the prevalence of antibodies to C. pneumoniae (40–60%) in the general population (Cook and Honeybourne, 1995; Niki and Kishimoto, 1996). It is interesting to note that a surprisingly low percentage of control sera reacted positively to either C. pneumoniae or C. trachomatis. On the other hand, 70% of the PBC sera, were positive to C. trachomatis. When the titer of PBC sera against Chlamydia vs. against E. coli, PBC sera reacted to both E. coli and Chlamydia at sera dilutions up to 10^4 as detected by chemiluminescence. This modest titer suggests that the serological antibody reactivity to Chlamydia is at best comparable to other common human microbes.

PCR is a highly sensitive and specific method for detection and quantification of specific nucleic acids and its application to the detection of slow growing microorganisms in autoimmune diseases has been recently reviewed (Cuchacovich et al., 2003). C. pneumoniae and C. trachomatis specific 16S rRNA was not detected in any of the PBC liver samples but present in low percentages in the other liver disease control and normal volunteers. Due to the ubiquitous nature of Chlamydia, special precautions were specifically used to avoid not only false positive but false negative results in the detection of Chlamydia by PCR. Thus, the absence of any Chlamydia specific 16S rRNA gene in PBC livers seems unlikely to be due to experimental or technical issues such as DNA quality, primer specificities and reaction conditions. Of note, C. pneumoniae has been reported to have a strong association with patients with MS, but a recent report using a large population of MS patients and controls was unable to confirm the increase presence of C. pneumoniae genome in the spinal fluid of MS patients (Boman et al., 2000; Krameter et al., 2001). Furthermore, a surprisingly low prevalence of C. trachomatis infection was reported in the three studies using PCR (1.06, 0 and 1.48%, respectively) giving an overall prevalence of 0.98% (Andreu Domingo et al., 2002). Thus the detection of Chlamydia specific antibodies but not Chlamydia rRNA gene and Chlamydia antigens in PBC suggests that Chlamydia infection is not involved in PBC.

Despite multiple and rigorous studies, the linkage between microbial infections and autoimmune diseases remains still elusive. The apparent lack of involvement of Chlamydia infection in PBC as reported here suggests that the molecular mimicry hypothesis in PBC may require modification. Recently, the hypothesis that PBC may be due to an initial insult with an environmental agent, possibly as a xenobiotic agent has been explored (Sasaki et al., 2000; Long et al., 2001). Since optimal reactivity of AMA requires the lipoyl domain as a component of the autoantigen (Migliaccio et al., 2001), its structural configuration has been reasoned to be important for the breakdown of self tolerance in PBC. Hence, exposure of genetically susceptible individuals to a xenobiotic agent, which mimics the lipoylated peptide of PDC-E2, may be important in the etiology of PBC. In a recent study, we synthesized the immunodominant 12 amino acid peptide epitope within the inner lipoyl domain of PDC-E2 and replaced the lipoyl acid moiety with a battery of synthetic structures designed to mimic a xenobiologically-modified lipoyl hapten, and quantified the reactivities of these structures with sera from PBC patients. Particularly noteworthy was our finding that AMA from all patients with PBC, but not controls, reacted against several of the organic modified mimetopes as well as, or significantly better than, to the native lipoyl domain (Long et al., 2001). This is further supported by the recent data that rabbits immunized by one of such xenobiotic agents, 6-bromohexanote induced the production of AMA (Leung et al., 2003). Most recently, a specific association of a unique xenobiotic metabolizing bacteria Novosphingobium aromaticivorans (Takeuchi et al., 2001; Pinyakong et al., 2003) and PBC has been reported (Selmi et al., 2003). N. aromaticivorans is ubiquitous in the environment (Takeuchi et al., 2001; Fujii et al., 2002; Tiirila et al., 2002; Fujii et al., 2003) and has the highest homology in the lipoyl domains between two of its lipoylated proteins and the human PDC-E2 in the known microbial world. Moreover, 100% (77/77) of anti-PDC-E2 positive and 12% (2/17) of AMA negative sera from patients with PBC recognize these two lipoyl containing proteins, while no such reactivity was detected in 200 control sera. Antibody titers to these N. aromaticivorans lipoyl-containing proteins was detectable at dilutions of 1:1,000,000 and were up to 1000-fold stronger than the titers for E. coli lipoylated proteins. In addition to
the reactivity towards these two lipoylated proteins, sera from patients with PBC also recognize other non-lipoylated *N. aromaticivorans* proteins. Moreover, PCR amplification of the *N. aromaticivorans* 16S rRNA gene from fecal samples showed that *N. aromaticivorans* is present in approximately 25% of PBC patients and controls. Altogether, these data suggest that xenobiogenic modification of lipoyl moiety of the *N. aromaticivorans* PDC-E2 homologues as a possible mechanism in the etiology of PBC. The origin of autoimmunity is clearly multifactorial and molecular mimicry of infectious agent itself cannot fully account for the specificities and spectrum of autoimmune diseases. The role of xenobiotics, in conjunction with presence of xenobiogenic metabolizing microorganisms and the hypothesis of molecular mimicry in the induction of autoimmune diseases (Pumford and Hames, 1997) warrant further investigation.

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**References**


**INFECTION AND PBC COINFECTION 233**


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