REVIEW

Primary Biliary Cholangitis : Its Pathological Characteristics and Immunopathological Mechanisms

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Abstract : Primary biliary cholangitis (PBC), formerly known as primary biliary cirrhosis, is an organ-specific autoimmune disease that predominantly affects middle-aged women and is characterized by the chronic progressive destruction of small intrahepatic bile ducts with portal inflammation and, ultimately, fibrosis. The serological hallmark of PBC is the presence of anti-mitochondrial autoantibodies (AMA). Several mechanisms have been proposed for immune-mediated bile duct damage in PBC, including the roles of T cells, B cells, other cell phenotypes, and AMA. A sign of fragility of biliary epithelial cells caused by apoptosis, senescence, and autophagy has also been noted. Several complex steps and mechanisms appear to be involved in the induction and progression of cholangitis and biliary degeneration in patients with PBC. J. Med. Invest. 64 : 7-13, February, 2017

INTRODUCTION

Primary biliary cholangitis (PBC) was formerly known as primary biliary cirrhosis. The name change reflects the fact that cirrhosis occurs only in the late stage and therefore does not correctly identify patients with early-stage disease. PBC is an autoimmune liver disease that is characterized by chronic non-suppurative destructive cholangitis (CNSDC) associated with the selective destruction of intrahepatic small bile ducts (interlobular bile ducts and septal bile ducts) by inflammatory cells, mainly lymphocytes and plasma cells. The appearance of anti-mitochondrial autoantibodies (AMA) (90-95%) and a high serum level of IgM are characteristics of the sera of PBC patients (1, 2). Progressive biliary damage eventually results in bile duct loss and fibrosis. The mechanisms by which these antibodies produce liver tissue injury currently remain unknown ; however, exposure to infectious microbes and/or xenobiotics has been suggested to initiate an immune reaction stemming from an individual's genetic predisposition and the participation of innate immunity at the primary stage of PBC.

The immunological interaction between biliary epithelial cells and surrounding inflammatory cells is an important mechanism underlying biliary damage. Biliary epithelial cells in PBC express various cytokines and chemokines in order to generate and sustain the specific surrounding inflammatory conditions. Damaged biliary epithelial cells have an antigen-presenting ability, with the aberrant expression of human leukocyte antigen (HLA) class II and other co-stimulatory molecules (3, 4). Various types of migratory inflammatory cells become effector cells, which attack biliary epithelial cells and also generate additional cytokines and chemokines to produce an inflammatory status and progressive fibrosis. A sign of fragility of biliary epithelium caused by cellular senescence and the disturbance of autophagy accelerates biliary destruction. The immunopathological characteristics of injured bile ducts and infiltrating effector cells were reviewed herein.

1. IMMUNOPATHOLOGICAL CHARACTERISTICS OF DAMAGED BILIARY EPITHELIAL CELLS AND SURROUNDING INFLAMMATORY CELLS IN PBC

The small-sized bile ducts (i.e. interlobular bile ducts), but not the large-sized bile ducts (i.e. septal and intrahepatic large bile ducts) are the specific targets of PBC (5). CNSDC is one of the typical features of the small portal tracts in PBC patients (Fig. 1A). Infiltrating inflammatory cells invade the epithelium and cause epithelial interruption and ductal luminal irregularities. Biliary epithelial cells become fragmented and finally disappear (ductopenia, bile duct loss). Lymphocytes, plasma cells, and often eosinophils infiltrate the areas surrounding damaged bile ducts (Fig. 1B). Epithelioid granulomas of various sizes constructed by aggregated macrophages frequently appear in the portal area and hepatic parenchyma (Fig. 1A).

The biliary epithelial cells of PBC aberrantly express various types of co-stimulatory factors and adhesion molecules as well as major histocompatibility complex (MHC) class II molecules, and may express target molecules (3, 4, 6). Biliary epithelial cells themselves express various types of cytokines/chemokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), regulated on activation, normal T cell expressed and secreted (RANTES), and fractalkine, and also generate the specific surrounding immunological microenvironment (7-12). Biliary epithelial cells also possess several cytokine receptors against IL-4, IL-6, interferon-gamma (IFN-γ), and TNF-α ; therefore, these cytokines exert autocrine and paracrine effects (13).

Previous studies described the profiles of inflammatory cells surrounding bile ducts in PBC (9, 14-17). Cellular profiles may change depending on the process of biliary inflammation. Different immune cell profiles are frequently observed at different portal areas in the same hepatic tissue section. However, T-cell-related cellular immunity is generally considered to be involved in the pathogenesis of CNSDC. Previous studies demonstrated that CD8+
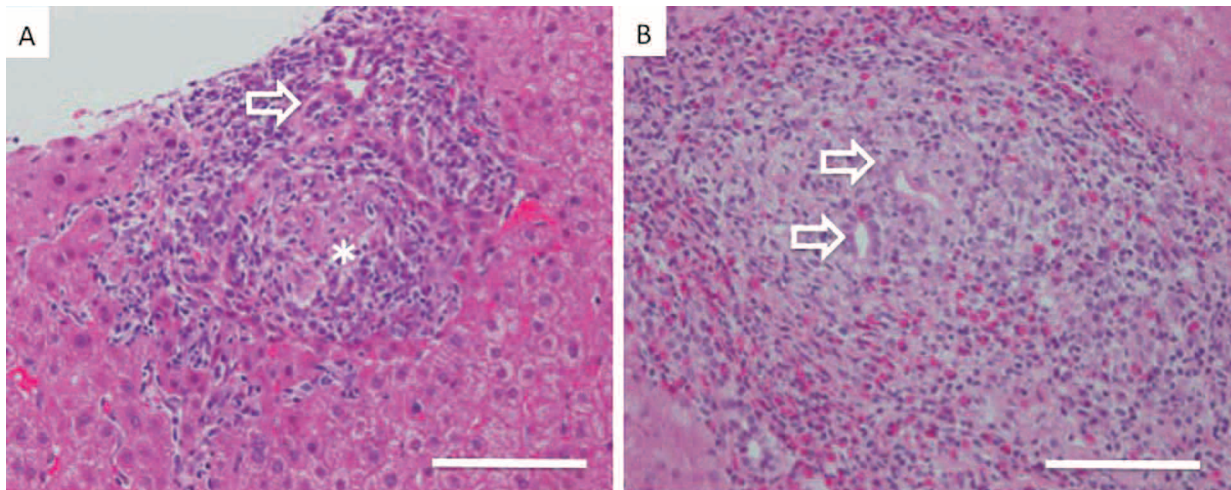
Figure 1
A : Typical images of chronic non-suppurative destructive cholangitis (CNSDC) and epithelioid granuloma (*) near a damaged bile duct (⇨) in a patient with primary biliary cholangitis (PBC). Scale bar : 100 μm.
B : Large numbers of eosinophils as well as lymphocytes and plasma cells infiltrated damaged bile ducts (⇨) in a patient with PBC. Scale bar : 100 μm.

and CD4+ lymphocytes were the predominant cell types among inflammatory cells within the portal area in PBC (18, 19). CD8+ lymphocytes are mostly cytotoxic T cells and affect targets via the perforin/granzyme exocytosis pathway (20, 21). CD4+ lymphocytes, particularly pathogenic autoreactive T cells, regulate autoimmunity around bile ducts in PBC. In the early stage, a Th1-predominant cytokine milieu is characteristic in CNSDC (22) ; however, as the disease progresses, IL-12/Th1 and IL-23/Th17 cells both appear to varying extents. In the late stage of PBC, the ratio of cell formation shifts from Th1 cells to Th17 cells (16). A decreased number of portal-infiltrating regulatory T cells and an imbalanced ratio with cytotoxic T cells may be associated with disease progression (15, 23).

While T cells comprise 55% of the cellular infiltrate, macrophages account for approximately 30% (24) and B cells/plasma cells for approximately 10% (19, 25) in early stage showing CNSDC. Eosinophils may also constitute some proportion of inflammatory cells (9, 26, 27). Natural killer (NK) cells and NK T cells account for approximately 5% of the cellular infiltrate and play important roles in initiating the breakdown of tolerance (28). Interdigitating dendritic cells have been detected between biliary epithelial cells, often near breaks in the basement membrane and in the periductal granulomatous response (4, 29, 30).

Lymph follicles are frequently observed in the portal tracts of PBC patients in the early and late stages (31) (Fig. 2A). In an immunohistochemical analysis, the lymph follicles of PBC are regarded as tertiary lymphoid organs (TLOs) (Fig. 2B). The MECA-79 monoclonal antibody recognizes a sulfated carbohydrate epitope present on human high endothelial venules (HEVs) called peripheral lymph node addressin (PNAd). Several HEVs expressing MECA-79 have been observed in the portal lymph follicles of PBC (Fig. 2C, D). A follicular dendritic cell (FDC) network expressing CD21 was detected around the HEV (Fig. 2E). B cells regularly accumulate along the FDC network and are located in the central part of lymph follicles. A layer of T cells has been observed in the outer part of the B cell layer (Fig. 2F). Approximately 80% of PBC cases with inflammatory cell infiltration showed MECA-79-positive HEV formation. We hypothesized that the inflammatory process may initially and primarily induce MECA-79-positive HEV followed by the formation of TLOs in the portal area. These TLOs often include injured bile ducts ; however, TLOs formed at a distant location

from injured bile ducts have also been detected. A direct correlation between TLOs and injured bile ducts has not yet been demonstrated. Takahashi *et al.* reported that, rather than the follicle-like aggregation of CD20-positive Bcells, the periductal infiltration of CD38-positive plasma cells was strongly associated with bile duct injury (32). TLOs may indirectly correlate with bile duct injury via the maturation of effector cells.

2. MECHANISMS UNDERLYING BILE DUCT INJURY IN PBC

There are several pathogenetic possibilities for the mechanisms underlying bile duct injury in PBC. One hypothesis for the selective destruction of biliary epithelial cells is that the pyruvate dehydrogenase complex (PDC)-E2 subunit, which is typically located in the mitochondrial inner membrane, is aberrantly expressed on the surface of biliary epithelial cells (3). Not only PDC-E2 itself, but also its mutant form and tissue-specific variants may cause the same phenomenon. Lleo *et al.* reported the existence of intact immunoreactive PDC-E2 within the apoptotic blebs of cholangiocytes during the process of apoptosis in PBC (33, 34). An autoimmune response may accelerate the process against these modified intrinsic PDC-E2 or related molecules.

Another hypothesis is the occurrence of an immune reaction against extrinsic antigens located in the biliary epithelium. The microbial mechanism termed "molecular mimicry" is a strong hypothesis that is being advanced to account for the break in tolerance against mitochondrial antigens.

Epidemiological studies have also suggested that infectious agents trigger or even exacerbate the disease (35). Gram-positive and Gram-negative bacteria have both been suspected, particularly *Escherichia coli* and *Novosphingobium aromaticivorans*, which are the most commonly associated agents that have been reported to date (36-38). Another candidate for the role of an extrinsic antigen is xenobiotics (chemicals). Many chemicals, including pharmaceuticals and household detergents, have the potential to form metabolites that show molecular mimicry to PDC-E2 (39, 40). Amano *et al.* reported that 2-octynoic acid was unique in both its quantitative structure-activity relationship analysis and reactivity. Sera from PBC patients exhibited high Ig reactivity against the
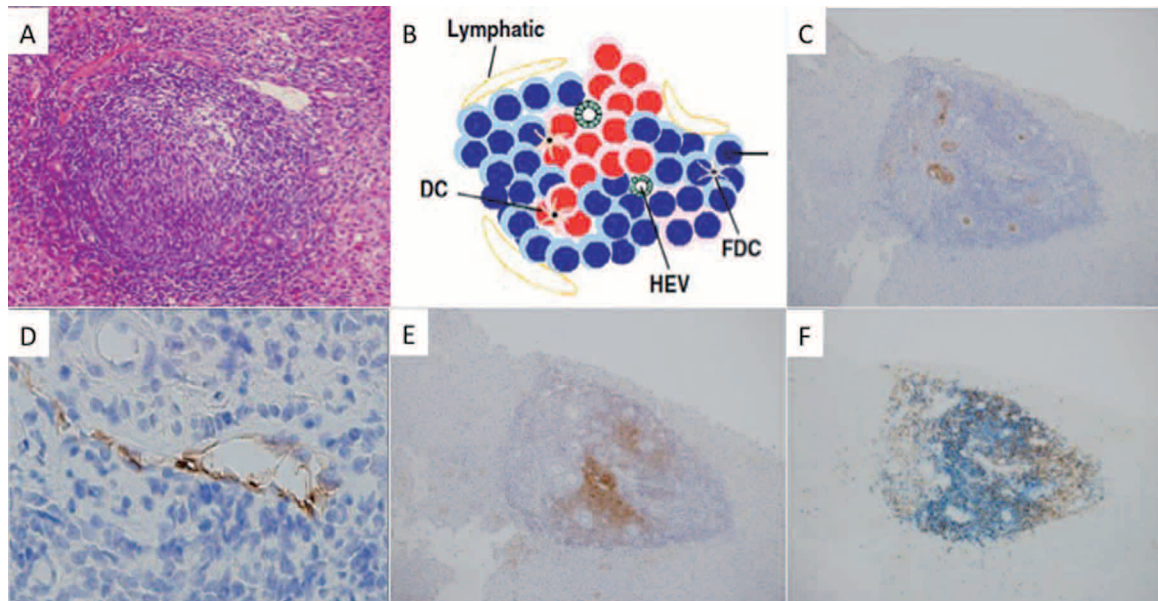
Figure 2
A : Lymph follicle formation with germinal centers in the portal tract of a patient with PBC. Scale bar : 100 μm.
B : Schema of tertiary lymphoid organs (TLOs) in the portal tract of a patient with PBC. DC : Interdigitating dendritic cells, HEV : high endothelial venules, FDC : follicular dendritic cells.
C : Immunostaining for MECA-79, which is a marker of high endothelial venules (HEV) (brown color : positive, counterstaining was performed by hematoxylin).
D : Higher magnification of MECA-79-positive HEVs (brown color : positive, counterstaining was performed by hematoxylin).
E : Immunostaining for CD21, which is a marker of follicular dendritic cells
(brown color : positive, counterstaining was performed by hematoxylin).
F : Double immunostaining for CD3, a marker of T cells, and CD20, a marker of B cells (brown color : CD3, blue color : CD20).

2-octynoic acid-PDC-E2 peptide. 2-Octynoic acid has the potential to modify PDC-E2 *in vivo*, and, importantly, is widely used in daily life such as in perfumes, lipsticks, and many common food flavorings (41). Mice immunized with 2-octynoic acid serve as a unique PBC animal model showing autoimmune cholangitis, typical anti-mitochondrial autoantibodies, and elevated numbers of liver lymphoid cells with an increase in the number of CD8 (+) cells in the liver (Fig. 3 A, B) (42). However, difficulties are associated with demonstrating the localization of 2-octynoic acid in the liver because of its small molecular size. We recently developed a new technique to highlight the expression of low-molecular-weight molecules without any labeling of frozen liver sections using nano-particle-assisted laser desorption/ionization (nano-PALDI) imaging

mass spectrometry (IMS) (43). We examined the localization of 2-octynoic acid in the liver using 2-octynoic acid-induced PBC model mice. 2-Octynoic acid was mainly located in the portal area with marked inflammatory cell infiltration (Fig. 3C) (44). Since 2-octynoic acid was also detected in bile juice, we speculated that it may be deposited in biliary epithelial cells. These findings imply that aberrantly deposited extrinsic xenobiotics (chemicals) or their metabolites function as pathogens.

The activation of the innate immune response appears to be another key event in early PBC that leads to autoimmune injury of the small intrahepatic bile ducts. Biliary epithelial cells possess an innate immune system consisting of the Toll-like receptor (TLR) family, which recognizes pathogen-associated molecular patterns
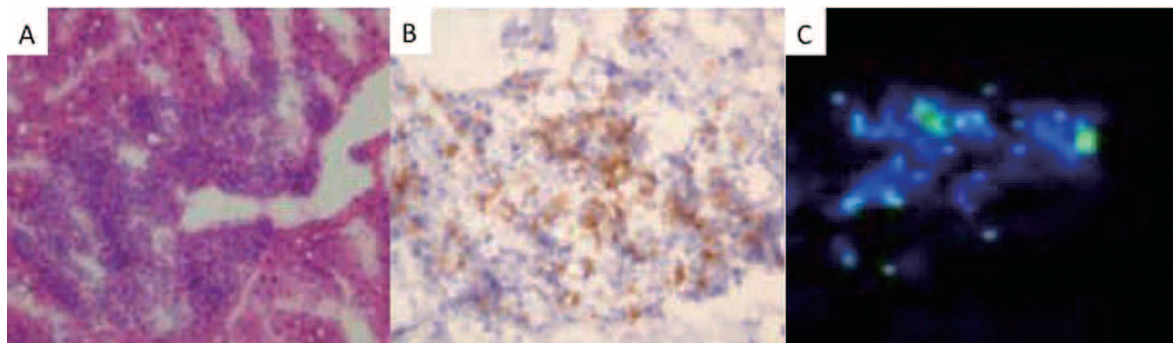

Figure 3
Mice immunized with 2-octynoic repeatedly showed PBC-like biliary damage with marked inflammatory cell infiltration. Serial images of portal tracts in the liver tissues of 2-octynoic acid-induced PBC model mice. A : HE staining, B : CD8 immunostaining, C : MS spectra reconstructed as ion images of 2-octynoic acid by high-spatial resolution nano-PALDI imaging mass spectrometry.

(PAMPs). In PBC, deregulated biliary innate immunity, namely, hyper-responsiveness to PAMPs, is associated with the pathogenesis of cholangiopathy. Moreover, the targeted biliary epithelial cells may play an active role in the perpetuation of autoimmunity by attracting immune cells via chemokine secretion. Biliary innate immune responses induce the production of fractalkine and several Th1 shift chemokines, causing the migration of inflammatory cells including NK cells. TLR4 ligand-stimulated NK cells destroy autologous biliary epithelial cells in the presence of IFN-α, which is synthesized by TLR3 ligand-stimulated monocytes. These findings provide new insights into the pathogenesis of PBC (45, 46).

The injured bile ducts and bile ductules of PBC indicate cellular senescence. Senescent biliary epithelial cells may modulate the microenvironment surrounding bile ducts by expressing senescence-associated secretory phenotypes (SASP) and contribute to maintaining inflammation and fibrosis around bile duct lesions in PBC. Deregulated autophagy followed by cellular senescence in biliary epithelial cells may be closely related to the abnormal expression of mitochondrial antigens and subsequent autoimmune pathogenesis in PBC (47, 48). The biliary epithelial cells of PBC are exposed to excessive oxidative stress because of a decrease in their anti-oxidative abilities (49). Oxidative stress may accelerate cellular senescence and the disturbance of the autophagy system, thereby causing complex biliary damage.

3. RELATIONSHIP BETWEEN THE MANIFESTATION OF AMA AND BILE DUCT INJURY

Various hypotheses have been proposed for the participation of AMA in bile duct injury. We recently suggested the protective contribution of AMA against biliary damage (31). The degree of bile duct damage around the portal areas was significantly milder in AMA(+) PBC than in AMA(-) PBC in liver biopsy examinations of Chinese PBC patients (31). Although detailed comparison of clinical information between AMA(+) and (-) PBC patients did not clearly figure out in this report, portal areas from AMA(-) patients had a significant increase of CD5+ T cells infiltrating the ductal regions and the levels of B cell infiltrates were worse in the early phase of bile duct damage. In contrast, Lleo *et al.* suggested that AMA promotes the inflammatory process by demonstrating the prominent production of inflammatory cytokines in the presence of biliary epithelial cell apoptopes, macrophages from patients with PBC, and AMA. The secretion of cytokines was inhibited by anti-CD16 and this was not due to differences in apoptope uptake. Moreover, mature monocyte-derived macrophages from PBC patients cultured with biliary epithelial cell apoptotic bodies in the presence of AMA markedly increased TNF-related apoptosis-inducing ligand expression (33).

Several studies have been conducted on AMA in animal models (23). A unique murine PBC model expressing a dominant negative form of transforming growth factor beta receptor II (dnTGFbetaRII) under the control of the CD4 promoter developed colitis and autoimmune cholangitis with elevated serum levels of IL-6. Based on these findings, IL-6-deficient mice with a dnTGF-betaRII background (dnTGFbetaRII IL-6(-/-)) were produced and examined for the presence of AMA, cytokine levels, histopathology, and hepatic immunohistochemistry. Serum AMA levels were decreased in dnTGFbetaRII IL-6(-/-) mice ; however, autoimmune cholangitis was significantly exacerbated, including elevated levels of inflammatory cytokines, increased numbers of activated T cells, and worsening hepatic pathology. These findings suggest the inhibitory effects of AMA on inflammation.

In order to inhibit the secretion of AMA, autoreactive B-cell depletion therapy using several PBC model mice was also performed. Moritoki *et al.* examined the therapeutic efficacy of B-cell depletion

using anti-CD20 (50). In mice in which treatment was initiated at 4-6 weeks of age (early treatment group), anti-CD20 therapy resulted in a significantly reduced incidence of liver inflammation associated with decreased numbers of activated hepatic CD8(+) T cells. In contrast, in mice treated at 20-22 weeks of age (late treatment group), anti-CD20 therapy had a negligible effect on the liver. All treated animals had reduced B cell numbers, the absence of AMA, and increased levels of inflammatory cytokines such as TNF-α in sera. AMA may play some roles in the induction state of pathogenesis, but not in disease progression in this model (50). However, B-cell depletion using another murine PBC model (genetic B-cell deficient Igmu(-/-) NOD.c3c4 mice) revealed reduced B cell numbers, the absence of AMA, and decreased non-B cell numbers in the liver accompanied by fewer activated NK cells. Since liver inflammation was significantly attenuated, B cells and AMA may play important roles in pathogenesis in the model (31). Given the disparate nature of these findings, we consider the role of B cells and AMA to depend on the disease phase and a number of other factors.

4. HYPER-IGM PRODUCTION AND IMMUNOPATHOLOGY

Elevated levels of IgM and the presence of AMA are characteristic of the sera of PBC patients. This increase in serum IgM has been attributed to chronic B-cell activation induced via the TLR-signaling pathway. Peripheral blood mononuclear cells (PBMCs) from PBC patients produce significantly higher levels of polyclonal IgM and secrete more AMA than controls after an exposure to CpG, which is a natural ligand for TLR9 (51, 52).

The primary site of IgM production in PBC patients is still unclear. Takahashi *et al.* reported that CD38-positive plasma cells accumulated around the bile duct in PBC patients (32). These periductal plasma cells produced IgM and IgG, but not IgA ; therefore, they may be candidates for the source of serum IgM. We focused on the spleen of PBC patients because B-cell maturation and differentiation occur in the splenic white pulp and produce IgM in response to an innate immunity stimulus with the capsular polysaccharide of a pneumococcus. In an immunohistochemical analysis using surgically resected spleen and autopsied spleen, IgM-producing plasma cells aggregated near the CD21-positive FDC network in the germinal center of the spleen of PBC patients (53). The chemokine, CXCL13, which has a chemotactic function for B cells, localized near IgM in the lymph follicles of PBC spleen. In addition to portal-infiltrated B cells in the liver, splenic B cells also produce IgM in PBC patients. The production of IgM in PBC patients may be systemically regulated rather than being a local event in the liver.

5. ESTABLISHMENT OF A NEW THERAPEUTIC APPROACH AGAINST B CELLS IN PBC

A new therapeutic approach that targets B cells in PBC patients has recently been clinically examined. Tsuda *et al.* reported the safe and potential efficacy of B-cell depletion with the anti-CD20 monoclonal antibody rituximab in patients with PBC who had an incomplete response to ursodeoxycholic acid (UDCA) (54). After the treatment, serum levels of total IgG, IgM, and IgA as well as those of AMA-IgA and AMA-IgM decreased significantly from baseline levels by 16 weeks and subsequently returned to baseline levels by 36 weeks. Transient decreases in memory B cells and T cells and an increase in CD25(high) CD4+ T cells were observed after the treatment. These changes were associated with significant increases in the mRNA levels of Foxp3 and TGF-β and a

decrease in TNF-α in CD4+ T cells. Notably, serum alkaline phosphatase levels were significantly reduced up to 36 weeks after the rituximab treatment. Based on these findings, Tsuda *et al.* concluded that the depletion of B cells provides a potential mechanism for the treatment of patients with PBC who have an incomplete response to UDCA. However, the potential for herpes zoster reactivation and upper respiratory infection was acknowledged in a few PBC patients administered rituximab in this trial. This is regarded as an important issue of infection control in the performance of B-cell removal therapy. We also encountered a case of PBC that rapidly progressed to liver cirrhosis after a treatment including rituximab (55). A 66-year-old Japanese female patient with PBC who presented with gastric lymphoma had been treated with a regimen containing rituximab for incidental malignant lymphoma. She showed biochemical and immunological improvements, and her liver histology before and after the rituximab treatment confirmed the amelioration of liver inflammation. However, she developed liver cirrhosis soon after the rituximab treatment without biochemical or immunological deterioration. Autoimmune B-cell removal therapy has potential as a novel treatment for PBC ; however, caution needs to be exercised and the careful monitoring of patients is required.

CONFLICT OF INTEREST

None of the authors has any conflicts of interest to declare.

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