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The autoimmunity of primary biliary cirrhosis and the clonal selection theory

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Abstract

Primary biliary cirrhosis (PBC) is a chronic cholestatic liver disease in which an immune-mediated injury targets the small intrahepatic bile ducts. PBC is further characterized by highly specific serum antimitochondrial autoantibodies (AMA) and autoreactive T cells, a striking female predominance, a strong genetic susceptibility, and a plethora of candidate environmental factors to trigger the disease onset. For these reasons PBC appears ideal to represent the developments of the clonal selection theory over the past decades. First, a sufficiently potent autoimmunogenic stimulus in PBC would require the coexistence of numerous pre-existing conditions (mostly genetic, as recently illustrated by genome-wide association studies and animal models) to perpetuate the destruction of the biliary epithelium by the immune system via the persistence of forbidden clones. Second, the proposed modifications of mitochondrial autoantigens caused by infectious agents and/or xenobiotics well illustrate the possibility that peculiar changes in the antigen structure and flexibility may contribute to tolerance breakdown. Third, the unique apoptotic features demonstrated for cholangiocytes are the ideal setting for the development of mitochondrial autoantigen presentation to the immune system through macrophages and AMA thus turning the non traditional mitochondrial antigen into a traditional one. This article will review the current knowledge on PBC etiology and pathogenesis in light of the clonal selection theory developments.

Keywords

anti-mitochondrial antibodies; autoimmunity; environmental factors; thymic selection; tolerance

The clonal selection theory and the history of biliary autoimmunity

Two remarkable insights in 1948 and 1957 by Frank Macfarlane Burnet were to set the course for modern immunology [1, 2]. In 1948 he deduced the nature of immunological inertness to self, named this as “tolerance” and proposed it to be a characteristic acquired in developmental life rather than innate as earlier believed [3]. His intuition led to seminal consequences in our understanding of autoimmunity. First, in 1957 (while the hypothesis

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was elaborated in1959[4]) he proposed that specificity of antigen recognition existed in cells of the immune system before any exposure to antigen for which the role was to select cells for clonal proliferation rather than acting in any "instructional" way to do so. Second, these concepts coincided closely in time with the renaissance of the notion of autoimmunity, in the late 1940s, so leading to the idea that an event such as a somatic mutation among cells of an antigen-stimulated clone could result in an aberrant proliferative response to a self antigen. This, together with a resistance to an ill-defined immunological homeostasis, could result in a '"forbidden clone" as the basis for an autoimmune disease [4]. The 'forbidden clone' hypothesis was elaborated in our monograph of 1963 [2], and still retained by Burnet in 1972, but developing knowledge in the 1960s including the B and T cell dichotomy and other advances left the concept increasingly harder to sustain. In particular, T lymphocytes do not undergo post-diversification mutations of their antigen receptor (TCR) whereas hypermutagenesis of genes for the B cell receptor (BCR) in germinal centers of lymph nodes is normally required for affinity maturation, and presumably gives rise constantly to self-reactive BCRs, but such cells for various reasons, including, lack of T-cell help, fail to thrive.

Also many advances were made in the 1960s on the expressions of autoimmunity among which was recognition of two separate albeit overlapping groups of diseases, "organ-specific" (prototypically lymphocytic thyroiditis) in which the identifiable autoantigens (named traditional) were constituents of the affected tissue, and "multisystemic" (prototypically systemic lupus erythematosus -SLE) in which the identifiable autoantigen(s) (non-traditional) were distributed in all cells throughout the body. This latter group includes the clinically, histologically and immunologically well-defined liver disease primary biliary cirrhosis (PBC) wherein there is consistent reactivity with a mitochondrially located autoantigen recognized over some 50 years as eliciting antimitochondrial antibody (AMA) reactivity, and researched from many angles over the past 25 years in our laboratory, as described in this encompassing review. In general terms, the 2010 products of the seminal evidence proposed by Frank Macfarlane Burnet of mutagenesis as the major feature of immune degeneration leading to autoimmunity is the objective of this Special Feature issue to celebrate the 50-year anniversary since the Nobel Prize was awarded for the enunciation of immune tolerance.

The first description of possible biliary cirrhosis, probably obstructive, was by the celebrated Italian pathologist Giovanni Battista Morgagni in 1761 and the earliest report of non-obstructive biliary cirrhosis is attributed to Addison and Gull in 1851 [5]. The disease was put on the map clinically by Ahrens et al in 1950 [6]. Serum "anti-tissue" autoantibodies were described in 1959 and PBC was nominated as an autoimmune disease in 1963 [4]. The anti-tissue autoantibodies were identified as antimitochondrial in 1965 [7]. After a considerable pause, a cDNA encoding the AMA autoantigen was cloned in 1986 and the antigen was identified as subunits (E2) of the pyruvate dehydrogenase complex (PDC) [8, 9] is nuclear encoded and expressed but is imported to become located on the inner mitochondrial membrane.

In the wider field of autoimmunity numerous self antigens became recognized as functional structures of the cell, particularly nuclear components, the autoepitopes were mapped, but adverse effector/inhibitory properties of autoantibodies *in vivo* remained incompletely defined [10]. Among the characterized autoantigens, functional sites were found within cell nuclei as chromatin, nucleoli and ribonucleoproteins additional to the mitochondrial proteins. DNA molecules and the associated histones were among the most common of the reactive nuclear autoantigens, being recognized by almost all sera from patients with SLE giving reactivity for ANA. Other ANA included anti-Scl70 antibodies directed against topoisomerase I (Scl-70), a nuclear non-histone protein that uncoils condensed chromatin

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during mitosis [11], the anti anti-Sm [12] antibodies, and SS-B (or La) antibodies directed at eukaryotic RNA polymerase III in Sjögren's syndrome and SLE. The genesis of all of these "non-traditional" autoantibodies seemed harder to explain than that of the "traditional" autoantibodies of organ-specific autoimmunity such as thyroid peroxidase (TPO) and thyrotropin receptor (TSHR) recognized by autoantibodies in autoimmune thyroid diseases. The discovery of PBC-specific antinuclear antibodies (ANA) came after AMA description and led to further possible implications in the pathogenesis of the disease, although our knowledge on the ANA onset and role in PBC remains largely incomplete. We will herein provide a conspectus of mitochondrial autoimmunity before returning to the question of how clonal selection theory might relate to the non-traditional, if not paradoxical, autoimmunity of PBC by invoking our recent discoveries on patterns and pathways of apoptosis in the target cell, the cholangiocyte.

Biochemical properties of the PBC autoantigens

The 2-OADC autoantigens are multi-enzyme complexes essential in energy metabolism [13].Since this enzyme family has been repeatedly reviewed in the context of PBC, the data are presented in summary form in Table 1 and Figure 2 for the constituent pyruvate dehydrogenase complex (PDC), the 2-oxo glutarate dehydrogenase complex (OGDC), and the branched chain 2-oxoacid dehydrogenase complex (BCOADC). Each of the three complexes consists of three subunits, i.e. E1, E2 and E3. The E2 components consist of several functional domains. There is the inner catalytic domain containing the active site, one or more lipoyl domains containing the lysine residue to which the essential cofactor lipoic acid is attached, and an E3-binding domain. PDC-E2 and E3BP are the major autoantigens for serum AMA. Both PDC-E2 and E3BP fold into distinct domains linked by flexible regions rich in alanine and proline residues; interestingly, such flexibility is important for the enzyme catalytic function [14]. Moreover, both polypeptides have a central core region, responsible for binding to other polypeptides. The E2 core, moreover, contains residues essential for its catalytic activity and is linked to a binding domain, which accounts for the binding to E1 (and possibly E3). On the other hand, the corresponding E3BP region binds E3 only. Both polypeptides include at their amino terminals compact domains containing the covalently attached lipoic acid co-factor [13]. PDC-E2 has two and E3BP a single lipoylated domain [15]. These lipoyl domains are exposed on the surface of the E2 core, a necessity for the function of the molecules. In all three instances, the domain is composed by a single lipoic acid residue covalently attached to a lysine residue in a constant DKA sequence motif. There are no crystallographic structural data but there is available for the inner lipoyl domain of human PDC-E2 a three dimensional model derived by nuclear magnetic resonance (Figure 1)[16].

Antigen-specific adaptive response in PBC

AMA specifically recognizes lipoylated domains of the 2-oxoacid dehydrogenase (2-OADC) family of enzymes (Table 1) of the mitochondrial respiratory chain. All immunodominant epitopes contain a ExDKA (glutamic acid –E-, x, aspartic acid, -D-, lysine –K-, and alanine –A-) motif, with lipoic acid attached to K at position 173, which is necessary and/or sufficient for antigen recognition [17]. Amongst the 2-OADC constituents, the major autoantigen is the E2 subunit of pyruvate dehydrogenase complex (PDC-E2). Less frequent autoantigenic are the E2 components of 2-oxo glutarate dehydrogenase (OADC-E2) and branched-chain 2-oxo acid dehydrogenase (BCOADC-E2) complexes and the E3 binding protein (E3BP) [8, 18]. In addition, nucleotide sequence analysis of genes encoding specific human monoclonal antibodies to PDC-E2 and combinatorial Fabs strongly suggest that these autoantibodies have been selected from a restricted set of somatically mutated immunoglobulin germline genes.

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T helper (CD4+) TCR αβ+ and CD8+ T cells are present in portal tracts, around damaged bile ducts, strongly suggesting the participation of cellular immune mechanisms in the biliary damage [19–26]. Autoreactive CD4 T cells that specifically target PDC-E2-self-antigen are present in peripheral blood and liver. There is a specific 100–150 fold increase in number of PDC-E2-specific CD4 T cells in the hilar lymph nodes and liver versus peripheral blood in patients with PBC [21], while it is of interest that their presence is independent of the serum AMA status [22]. Autoreactive CD8 T cells likewise have been characterized in PBC, and are considered the major effectors of tissue injury in PBC. The HLA class I restricted epitope for CD8 T cells, namely the 159–167 aa sequence, maps in close vicinity to the epitopes recognized by CD4 T cells as well as by AMA (Figure 2). Notably the autoepitope for T cells, both CD4 and CD8 T cells, overlaps with the B cell (AMA) counterpart and includes the lipoylated amino acid K173 of the inner lipoyl domain. Similar to CD4 autoreactive T cells, there is a 10-fold higher frequency of PDC-E2 159–167 specific CD8 T cells within the liver versus peripheral blood Moreover, the precursor frequency of PDC-E2-specific autoreactive CD8 T cells is significantly higher in early rather than late stage of the disease. The autoreactive CD8 T cells in PBC have specific cytotoxicity against PDC-E2 antigen and, as well, produce IFN-g rather than IL-4/IL-10 cytokines [27].

The role of lipoic acid in PDC-E2 recognition

The three-dimensional model of PDC-E2, and its unique oxidative mechanisms, raise the idea that foreign compounds that mimic or alter lipoic acid could bind AMA when the lipoic acid molecule becomes conjugated to any carrier molecule, such that immunization of rabbits with a bovine serum albumin (BSA) conjugate of a lipoic acid mimic (6-bromohexanoic acid) would induce AMA[28], and it was later demonstrated that lipoic acid can indeed be mounted on a protein background [29]. Thus PBC could be due to chemical exposure, and lipoic acid or a lipoic acid mimic could be important in failure of tolerance to mitochondrial antigens [28, 30, 31]. Several lipoic acid (IA) mimotopes have been identified with the use of mimotope conjugated carrier molecules and affinity purified anti-PDC-E2 antibodies; specifically, 79/97 (81%) of AMA-positive PBC sera reacted to lipoylated human albumin (HSA-LA), and a high reactivity to HSA-LA correlated with the level of reactivity to PDC-E2. Also, PDC-E2 affinity purified sera reacted with HSA-LA, suggesting that some of the antibodies to HSA-LA are a subset of anti-PDC-E2 specificities. The antibody reactivity to lipoylated PDC-E2 and PDC-E2 is predominantly IgG and IgM whereas that to HSA-LA is predominantly IgM. Bruggraber and colleagues [17] have demonstrated the presence in sera of patients with PBC of (a) antibodies with specificity against PDC-E2 that are capable of recognizing both PDC-E2 and lipoic acid and (b) antibodies to lipoic acid that recognize a conjugated form of lipoic acid but not the PDC-E2 backbone. Notably, antibodies to PDC-E2 LA and antibodies to lipoylated peptide conjugates are not merely cross-reactive antibodies: they differ in their epitope specificity and also in their Ig subclass and affinity for antigens. Previous studies had failed to detect anti-lipoic acid antibodies when free lipoic acid was used as an antigen [32], yet our findings otherwise on the presence in PBC of autoantibodies to lipoic acid [17] are of particular interest in resembling the immune response to iodine in autoimmune thyroiditis observed in chickens, rats and NOD mice [33–37].

Cholangiocyte apoptosis and “traditional” mitochondrial autoantigens

The mitochondrial antigens recognized by both B and T cell autoimmune responses in PBC are ubiquitously expressed in all nucleated cells, and are highly conserved in phylogenesis [38]. Mitochondrial 2-OADC antigens are not “cryptic” to the immune system, and normally there is tolerance to these, even if there are responses to bacterial homologs which are phylogenetically distant from human proteins. During spontaneous or induced apoptosis,

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numerous---perhaps all--- cell types express mitochondrial antigens on the intact plasma membrane and within apoptotic blebs [39, 40] which then acquire the capability to initiate an autoimmune response by presentation of 2-OADC-derived autoantigens [41]. Notably this latter process is specific to cholangiocytes, explaining in part why PBC recurs after liver transplantation [42, 43]. Indeed, AMA may react, though weakly, against biliary epithelial cells of normal subjects [44] and specifically reactive T cells[45, 46], and B cells, and serum AMA [47], have been found (at low levels) in the serum of non-PBC subjects. Nevertheless, the immune system starts a progressive autoimmune attack against cholangiocytes only in patients destined to develop PBC, and this occurs irrespective of whether the biliary epithelium is derived from a patient with PBC or a control subject [48]. Accordingly, liver infiltrating autoreactive T cells to 2-OADC were found only in patients with PBC[49], irrespective of their serum AMA status [22]. The mitochondrial autoantigens undergo a particular cell-specific processing that may well contribute to, if not entirely explain, the organ specificity of PBC [50, 51]. The lack of putative post-translational modifications alters protein degradation leading to the accumulation and exposure of large amounts of autoantigens, as postulated for the “traditional” autoantigens of the organ-specific autoimmune diseases [52]. In most cell types, lysine-lipoylated sequences when released from mitochondria during apoptosis [50] are oxidized by glutathiones; the oxidated forms are not immunogenic and are not recognized by serum AMA because glutathionylation masks the autoantibody recognition site [50 53]. On the other hand, cholangiocytes and cells from certain other epithelia fail to covalently link glutathione to lysine-lipoyl groups during apoptosis [50]. In cholangiocytes cleavage of the immunodominant PDC-E2 epitope has not been detected in vivo during either apoptosis [50, 54] or phagocytosis [55]. Moreover, the enhanced expression of 2-OADC proteins, with a particular luminal concentration, is seen early in cholangiocytes in PBC versus other chronic inflammatory biliary disease e.g. primary sclerosing cholangitis [44]. This abnormal expression of PDC-E2 may depend on self-antigens being presented by cholangiocytes after binding to HLA molecules, although HLA class II on cholangiocytes have more an intrahepatic basolateral than luminal surface expression [56–60] and anyway, are expressed only weakly and in the early stages of disease [60]. This observation could be also secondary to immune complexes deposition rather than membrane protein expression. There are different opinions on exposure of self-antigens on the cell membrane during cholangiocyte apoptosis [39, 50], or during the rearrangement of lipid rafts as seen after TLR activation by microbial infection [61] or after ingestion of apoptotic cholangiocytes by other cholangiocytes [55]. Whilst cholangiocyte phagocytosis of neighboring apoptotic cholangiocytes is not specific to PBC, this effect could be involved in the presentation process of mitochondrial antigen observed in PBC indicating that mitochondrial autoantigens are similar to the ‘traditional’ group.

The intact PDC-E2 in apoptotic fragments could be uptaken by local antigen presenting cells and transferred to regional lymph nodes for priming of cognate T cells thus initiating PBC. This is indeed an attractive possibility, however solid data of such antigen presentation are awaited and it could not be excluded that the reported mechanisms are not PBC specific. A major contribution came from Lleo and colleagues who first demonstrated that PDC-E2 is found in the blebs of human intrahepatic bile duct cells undergoing apoptosis [40] and subsequently that macrophages are capable to uptake the autoantigen found in apoptotic blebs (coined apotopes) [41]. The addition of serum AMA to the coculture of macrophages and apotopes led to a significant increase in proinflammatory cytokine secretion. These phenomena were not observed in other epithelial cell lines and appears to confirm the importance of apoptosis in the perpetuation of the autoimmune injury [62] as well as the view that PBC bile duct cells are not unique [48].

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Antinuclear antibodies (ANA) in PBC

Serum ANA are detected in nearly 50% of patients with PBC with some reports suggesting that the prevalence may be higher in AMA-negative sera [63] PBC-specific indirect immunofluorescence patterns include 'nuclear rim', based on the recognition by the autoantibodies of gp210 and nucleoporin 62 (within the nuclear pore complex which also includes LBR) and 'multiple nuclear dots', based on the reactivity with Sp100, PML, and most recently, Sp140 (nuclear body proteins) [64, 65]. In addition, the cross-reactivity with small ubiquitin-like modifiers (SUMO) bound to both Sp100 and PML have been suggested as independent antigens also specific for PBC [66]. The prevalence of 'nuclear rim' ANA in PBC is similar with the different techniques utilized for the test, particularly when recombinant or isolated antigens are included. Gp210 consists of three main domains: a large glycosylated lumenal domain, a single hydrophobic transmembrane segment and a short cytoplasmic tail. The antigenic epitopes recognized by anti-gp210 ANA are located within the glycosylated lumenal domain (a 64 kD fragment) and the cytoplasmic tail (15 amino acids) [67]. In general terms, anti-gp210 are detected in 26% of cases using the gp210-C terminal peptide a.a.1863–1887 [68] and 27% when using isolated nuclear pore complexes [69]. The major nuclear body protein is Sp100 which consists of at least three non-overlapping major autoantigenic domains in sp100 recognized by Sp100 positive PBC sera and two stretches of 16–20 amino acids are the predominant autoepitopes [67]. One domain, which contains the sequence similarity with HIV nef proteins, was recognized by all anti-sp100 sera. The prevalence of anti-Sp100 ANA in PBC is estimated to range between 9% [68] and 30% [70] when different methods are used. It was first supposed that ANA-positive patients with PBC are more frequently AMA-negative, possibly because of the lack of a masking effect of these latter antibodies in such sera, yet this remains to be determined and current data do not support this view.

There is a third type of ANA associated with PBC and are directed against centromeric proteins (ACA) that occur in PBC mostly together with the usual "clinical partner" of ACA, limited CREST-type scleroderma, at a prevalence formerly cited as 10% [71] but now seemingly higher [68], although with specificity limited by the rheumatological comorbidity.

No studies have been able to discern any link between PBC-specific ANA or the antigens they recognize and the immunopathology of PBC, nor it is clear how or why these ANA are generated in individuals with PBC. Whether ANA-positivity should be regarded as the result of the unique cholangiocyte apoptotic features (discussed below) or of the T regulatory defect [72] is a fascinating hypothesis that awaits experimental confirmation and provides additional evidence that 'nontraditional' autoantigens in PBC could in fact be 'traditional'. Nevertheless, significant associations between the presence of ANA and a worse prognosis have been independently reported [73, 74], different from AMA [75].

Innate immunity in PBC

Innate immunity as an activator of autoimmune responses is receiving much attention [76]. The liver is a major organ of innate immunity in containing the largest resident population of innate immune system cells, including NK and NKT cells. As with other autoimmune diseases innate immune mechanisms likely contribute to the initiation and progression of liver damage [77], and in PBC in particular as judged by features such as epithelioid granulomas, elevated levels of polyclonal IgM, hyper-responsiveness of the immune system to CpG, increased levels in blood and liver NK cells [78] and an indicative cytokines response [77]. There is a consistent elevation of serum polyclonal IgM in PBC, regardless of the AMA or ANA status [79], and reduction is usually observed during treatment [80]. The hyper-IgM appears secondary to a chronic innate immune response of memory B cells to specific

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bacterial molecular motifs such as unmethylated CpG motifs [81]. Furthermore patient peripheral B cells exposed to CpG motifs express increased amounts of TRL9 and CD86 so enhancing their production of AMA. This evidence strongly suggests a profound disease-specific dysregulation of B cells and supports the proposed link between bacterial infection and PBC i.e. B cells become hyper-responsive to innate stimuli, such as microbial CpG motifs, favoring perpetuation of the autoimmune process [82]. Other innate immune cells such as monocytes have also been implicated in the pathogenesis of PBC since their pro-inflammatory activity is greatly enhanced in PBC. Functionally, monocytes become activated by pathogen-associated patterns through TLR to release pro-inflammatory cytokines, including IL-1, IL-6, IL-18, IL-12, and TNF-α that can amplify adaptive T cell mediated immune responses against pathogens [83]. Thus peripheral blood monocytes in PBC are overly sensitive to infectious stimuli resulting in hyper-secretion of pro-inflammatory cytokines; the relevant mechanisms are unknown but might relate to the higher frequency of recurrent Gram-negative bacterial infections (*e.g.* urinary tract infections) in PBC. In other words both B cells and monocytes constantly exposed to bacterial products from portal blood could participate in the modulation of adaptive cellular immune response and possibly also in its priming.

NKT cells are now implicated in autoimmunity[84] as innate effector cells which are regulated by self and non-self glycolipid antigens presented by the antigen-presenting molecule CD1d, allowing for a rapid NKT cells production of effector cytokines and chemokines, thus modulating both innate and adaptive immune responses. The involvement of NKT cells in the pathogenesis of PBC was suggested by our study reporting a higher than normal frequency of CD1d-restricted NKT cells in PBC patients and, as for autoreactive T cells, CD1d-restricted NKT cells were more frequent in liver than peripheral blood [26]. Chuang *et al.* found likewise an increased number of CD1d-restricted NKT cells in the liver of a mouse model for PBC transgenic for directed expression of a dominant-negative form of TGF-beta receptor type II (dnTGFβRII). Such CD1d-restricted NKT cells in the liver had an increased IFN-γ production after exposure to α-galactosylceramide (α GalCer) and there was a decreased hepatic lymphoid cell infiltration and milder cholangitis than in controls [84] Innate immune system hyper-responsiveness is probably insufficient *per se* for disruption of immune tolerance, might well participate in the initiation and/or perpetuation of autoimmune injury. Thus Mattner *et al* demonstrated that, in a murine model of PBC (discussed below), *N. aromaticivorans* induced autoreactive AMAs and T cell-mediated autoimmunity against small bile ducts via NKT-dependent mechanisms [85].

Environmental agents and PBC autoantigens

Environmental agents, chemical compounds or infecting microbes, are suspected to be involved into the breakdown of tolerance, through molecular mimicry and cross reactivity mechanisms [86]. This idea is supported by the less than complete genetic evidence including ~60% concordance rate for PBC in monozygotic twins [87] and insufficient associations in PBC patients from genome wide association studies [88, 89]. Thus a mimotope carried by a microbe or a neo-antigen generated by xenobiotic-modified self-antigen that mimics mitochondrial proteins may activate autoreactive lymphocytes that have “leaked out” into the peripheral repertoire.. The process becomes self-perpetuating because of the presence of cross reactive unmodified self-antigens on cholangiocytes surface.

Similar to the majority of autoimmune diseases and their specific autoantibodies, the pathogenetic role of serum AMA remains debated and data questioned the sequence of the immunodominant B cell epitope and the role of the lysine-lipoylated motif in the PBC B cell response [90]. Ultimately, only the recapitulation of an AMA-mediated injury in an animal model will provide definitive evidence of the autoantibody role in PBC pathology [91].

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However, the study of the immunodominant T cell epitope, PDC-E2 163–176, has provided important evidence on pathogenesis of PBC, based on the reactivity of cloned PDC-E2 163–176-specific T cell lines. Thus, for this peptide, the contact residues with T cell receptors (TCRs) are ^{168} EIExDK^{173} so that microbial proteins, whether or not related to PDC-E2, that have an ExDK sequence are potentially capable of recognition by autoreactive T cells. Interestingly, this activating PDC-E2 peptide was not lipoylated at K^{173} and conservative substitutions at position 173 did not abrogate T cell response, indicating that the lysine-lipoylated motif is a minor role participant in T cell responsiveness. However glutamic acid (E)^{170} is crucial to T cell recognition as its substitution abrogates reactivities. Considering the proximity of E^{170} to K^{173} (Figure 2), we hypothesize that the customary glutathionylation of the lysine-lipoyl residue at position 173 can mask or alter the exposure of E^{170} so as to abrogate contact between this residue and CDR3 of the TCR. Of note, this mechanism can be considered as an “immunologic defense” in most cell types with the exception of cholangiocytes [17]. The experimental evidence illustrated thus far (along with the demonstration of autoantibody reactivity with lipoic acid) has obvious fascinating implications in terms of autoantigen selection. Indeed, one of the major issues in classical immunology is what makes an antigen an autoantigen. Indeed, this commonly takes place for intracellular enzymes (as in the case of PBC) and cell surface receptors. While molecular mimicry and epitope spreading (discussed below) cannot be overlooked, additional mechanisms have been sought and currently imply the structural features of antigens, particularly flexibility, as well illustrated by the three major type 1 diabetes autoantigens [92, 93]. Other factors may also include dysfunctions of vitamin D [94] based on genetic polymorphisms [95] or other modulators [96].

Infectious agents

The question of an environmental factor initiating PBC [97], this is supported by the incomplete concordance among monozygotic twins [87], there are reported instances of non familial clustering [98–100], and a claimed changing risk of PBC in individuals moving from high risk to lower risk locations (i.e. geoepidemiology) [101, 102 103, 104]. Infectious agents are the obvious choice for environmental candidates and support for an infectious hypothesis is garnered from data that lipopolysaccharide (LPS), a specific component of gram negative bacterial cell wall, injected into mice either alone or in combination with PDC-E2 induce the appearance of portal lymphocytic infiltration and cholangiocyte degeneration as seen in the human PBC liver. Further, lipoteichoic acid (LTA), the gram positive cell wall component, has been detected in PBC liver samples around damaged bile ducts, and serum levels of LTA-specific IgA are significantly higher in PBC than in normals [105], and bacterial DNA containing unmethylated CpG motifs triggers a PDC-specific Th1 response in PBMC from mice immunized with PDC [106]. Finally, Th17 cells that are important components of the mucosal host defense system against infections (although also involved in the pathogenesis of various autoimmune diseases [107]), are constituents of the periductular infiltrates in human PBC [108, 109]. However, the identity of the suspected pathogen (if such does exist) and the exact initiating mechanisms remain unrecognized.

Among mechanisms proposed to explain just how an infectious agent could contribute to the onset of an autoimmune disease in genetically susceptible subjects, molecular mimicry remains popular, having been reported for many microbes [110]. Shared sequences between human and microbial proteins can disrupt immune tolerance by inducing cross reactive antibodies or effector T cells and/or by promoting epitope spreading [111], although this has been refuted in PBC [112]. Other non-exclusive mechanisms involve superantigen polyclonal activation of T cells, i.e. staphylococcal enterotoxins [113], mouse mammary tumor virus antigens [114] and viral polyclonal activation of B cells, i.e. Epstein Barr virus [115], IgA production, and Th17 differentiation.

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We may summarize that numerous specific infectious agents, mainly bacteria, but also viruses, parasites, and fungi, have been suspected in PBC [116], but these studies have failed to demonstrate any clear association of a microbial agent with the disease and the evidence at best is only circumstantial, such as linear or conformational mimicry between microbial proteins and human mitochondrial antigens.

Xenobiotics

The other environmental factor seriously proposed for is constituted by foreign chemicals i.e. xenobiotics that can modify a defined self or non-self protein so as to cause a change in its molecular structure that enhances immunogenicity. This has been proposed for numerous autoimmune diseases and is consistent with the observed geoepidemiological gradient [117–119] and is supported by a number of epidemiology studies as previously discussed. One particular example is development of autoantibodies in subjects to halothane, a previously used inhalatory anesthetic that can induce antibodies reactive with lipoylated PDC-E2 [120]. As stated above, lipoic acid attaches to only a very limited number of proteins, yet is a critical component of the PDC-E2 epitope [17]. The PDC-E2 structure exposes lipoic acid at the exterior of the protein complex making it accessible to chemical modification [29]. The role of xenobiotics in PBC is supported by serum reactivity against specific organic compounds with structures similar to lipoic acid [121]; further, two of these compounds (6-bromohexanoate and 2-octynoic acid) are capable of inducing AMA and PBC-like liver lesions in guinea pigs [122] and NOD.1101 [123] or C57BL/6 [124] mice, respectively. The ability of *N. aromaticivorans* to metabolize chemical compounds might link xenobiotics and bacteria in the etiology of PBC, as discussed above.

The 2-OADC antigens undergo several post-translational modifications endogenously, and such changes may alter the epitope regions of the proteins. Nevertheless, external influences can also contribute to protein alterations and neo-antigen formation [51]. Of note, the liver is constantly exposed to chemicals derived from the gut through the portal circulation to be metabolized, activated, or excreted in the bile, and there is evidence that xenobiotics can modify mitochondrial proteins. Thus Long and colleagues in 2001 demonstrated that specific organic structures attached to the mitochondrial antigens were recognized by PBC sera with a higher affinity than the native forms of such antigens [125] suggesting that an organic compound may serve as a mimotope for an autoantigen; one such halogenated compound induced AMA production in rabbits with no requirement for the peptide backbone of PDC-E2 [28] albeit without producing liver lesions (possibly due to latency of disease expression), and antibodies disappeared when the stimulus was discontinued [30]. In another study induction of PBC-like liver lesions did follow during a longer follow-up in guinea pigs [122] while yet a further study reported two new xenobiotic-induced PBC murine models based on the immunization of NOD.1101 [123] or C57BL/6[124] mice with 2-octynoic acid. Both models illustrated breakdown of tolerance in the absence of exposure to PDC-E2 but there was no progression to liver disease. Utilizing a different approach, we next demonstrated that 2-nonynoic acid is capable of being recognized by PBC sera with high affinity [121], of interest since this non-naturally occurring compound is known to be found in several cosmetic products including nail polish and their frequent use among women could contribute to female predominant PBC [126, 127].

Cumulatively, the xenobiotic theory in PBC induction is fascinating and may well fit into the previously illustrated view that conformational changes of self-antigens may provide an efficient way to overcome the numerous checkpoints [128] for ‘forbidden’ clones to survive and expand to ultimately produce the orchestrated autoimmune response based on cellular and humoral responses [129].

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The genetics of PBC

Not mutually exclusive with regard to environmental factors leading to PBC, the challenge to identify susceptibility gene(s) that predispose to the development of the diseases is still open. Of interest, a most recent multi-center study reported of the first genome-wide association study and identified interleukin 12 and its relative receptor as susceptibility genes for PBC [130]. These data were later confirmed by our group in an independent cohort of Italian patients and controls as well as in a meta-analysis with Northern American subjects [131]. Among significantly associated genes we recapitulated the proposed importance of HLA and STAT4 genes.

The majority of previous studies not only have been derived solely from case-control designs [132] but were also limited by poor control matching criteria and sample size or selection. A plethora of association studies have been conducted (reviewed elsewhere), mainly focused on immune genes that affect the immune system belonging to both the HLA family and non-HLA immune modulators genes, including CTLA-4, IL-1, IL-10 and vitamin D-receptor [132, 133].

Pertinent to the individual susceptibility to PBC, several sex-related factors appear to increase the risk of developing the disease, mostly by means of reproductive life variables. Among these are the role of pregnancies [134, 135], contraceptives, estrogen replacement treatments [126], and recurrent vaginitis [136] but the mechanisms remain to be elucidated. However, the novel hypothesis of sex chromosome-related effects on PBC appears promising [137], and may possibly operate via gene dosage or epigenetic changes [138] which appear to be common to autoimmunity in general [139–142].

Of interest to the individual genetic susceptibility to PBC is the observation that several animal models have been recently proposed based on a determined genetic background. Indeed, two animal models, i.e. dnTGFβRII and IL-2Rα knockout mouse, point out the possible crucial role of Tregs deficiency in loss of immune tolerance with consequent development of autoimmune response against PDC-E2 in PBC. The selective deficiency of the TGFβR-signaling pathway exclusively in T lymphocytes accounts for major impairments of peripheral tolerance as Treg cells depend on TGFβ for their regulatory activity, triggering the emergence of tissue-specific autoreactive effector T cells [143]. A mouse deficient for IL2 receptor α (IL-2R α), which is highly expressed on Tregs developed 100% AMA positivity against PDC-E2, 80% ANA positivity, and lymphocyte infiltration around the portal tracts associated with cholangiocyte injury [144]. These data illustrate the important relationships between Tregs and the appearance of autoimmune portal tract pathology and serum AMA. An additional animal model is a variant of the non obese diabetic (NOD) mouse model (NOD.c3c4) manifesting autoimmune cholestasis and PBC-specific serology, showing AMA positivity of 50%–60% and ANA positivity of 80%–90%. This mouse is protected from diabetes by B6/B10 regions on chromosomes 3 and 4 that contain B6/B10 insulin-dependent diabetes (*Idd*) loci. Histologically, it presents lymphocyte infiltration around portal tracts with chronic nonsuppurative destructive cholangitis and epithelioid granuloma formations; nevertheless, the morphological features of bile ducts differ somewhat from those in human PBC [145]. Based on these animal models we may surmise that PBC susceptibility should be seen as a tolerance dysregulation secondary to a permissive background (as in the NOD model) or the lack of T regulatory mechanisms (as in the mouse deficient for IL2 receptor α on which an environmental insult could rapidly establish an autoimmune reaction [123, 124].

One cumulative theory has been recently proposed [128] and is based on the frequency of germline gene mutation, multiplied over the large number of known ‘tolerance/

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autoimmunity' genes which are well illustrated in the numerous genome-wide association studies. These would result in a high frequency of deleterious mutations in a heterozygous state on which an autoimmune-prone phenotype could ensue either in the event of homozygosity of one or another such genes, if a somatic mutation occurred, or via a chromosome-specific haploinsufficiency, as well illustrated for the X chromosome [138]. Further, most autoimmune diseases manifest a striking prevalence in older ages and the observed disease latency (in the case of PBC represented by the long latency between AMA appearance and disease manifestations) suggests that successive mutations may need to occur in a stepwise stochastic manner for the autoimmune phenotype to become apparent. Similar stochastic events may take place also at different levels, including the exposure to specific environmental factors or the interaction between different cell types in the maintenance of immune tolerance. This is supported by most recent evidence obtained in inflammatory bowel diseases [146] where genes and infections concur to the development of Crohn's disease in an animal model [147]. There are multiple other environmental agents and factors that have been incriminated in other autoimmune diseases, including the potential role of nucleic acids as adjuvants that have not yet been studied in PBC, but which should be addressed as possible modulators of pathology, including epigenetic influences, commensal microbiota, nanoparticles, ultraviolet light, tobacco smoke, nutrition and stress [148–156].

Linking AMA in the pathogenesis of PBC

In contrast to classic systemic autoimmune diseases, PBC is highly tissue specific with the cells of the small intrahepatic bile ducts as the primary target. However, apparently paradoxically, the autoantigens of PBC, PDC-E2, OGDC-E2, and BCOADC-E2, are ubiquitous mitochondrial proteins in all nucleated cells and hence seen as "non-traditional" at least as compared with the "traditional" autoantigens of thyrogastric organ-specific autoimmunity---this is possibly resolved as discussed below by the unique immunopathological characteristics of cholangiocytes.

However, first of all, staining of small bile ducts with a panel of monoclonal antibodies against the mitochondrial autoantigens has demonstrated that some give an intense staining at the apical surface of the cells lining the bile duct lumen, and this is specific for PBC liver[157, 158] versus other liver pathology. This apical staining is seen only with selected PDC-E2 specific monoclonal antibodies and that distinct epitopes could identified with such monoclonal antibodies, leading to the hypothesis that a PDC-E2-mimicking (and thus cross-reactive with AMA protein is recognized by the human autoantibodies [157–159]. Others have suggested that the apical staining is due to immune complexes of IgA AMA and PDC-E2 [160, 161]. While a solid proteomic confirmation of this critical issue is awaited, it does appear that cholangiocytes are not just an innocent bystander in PBC pathology, but rather are active participants. They may be involved in biliary and mucosal secretory transfer including that of dimeric IgA [162–164]. In particular, experimental data [161] suggest that PDC-E2-specific IgA may enter bile duct cells via a poly-Ig receptor (pIgR) and complex with PDC-E2, thereby potentially contributing to pathology. Anti-PDC-E2 IgA antibody titer in PBC sera directly correlates with the level of caspase activation [54] and suggest that during transcytosis through $pIgR^+$ cells, exposure to PDC-E2-specific dimeric IgA can result in the initiation of caspase activation. Based on the presence of dimeric AMA-IgA in biliary and mucosal secretions [165], constant transcytosis may render the exposed cells more susceptible to apoptosis, thus producing the bile duct damage. The apoptosis of biliary epithelial cells in PBC warrants further discussion and may be proved to be crucial for immune tolerance breakdown [40, 62], as illustrated in other experimental settings [166]. It was first reported that PDC-E2 remains intact and retains its immunogenicity during cholangiocyte apoptosis, due to a cell-specific lack of glutathionylation of biliary epithelial cells [50]. The intact PDC-E2 in apoptotic fragments could be uptaken by local antigen presenting cells and transferred to

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regional lymph nodes for priming of cognate T cells thus initiating PBC. A major contribution came from Lleo and colleagues who most recently demonstrated that PDC-E2 is found in the blebs of human intrahepatic bile duct cells undergoing apoptosis [40]. More importantly, this phenomenon was not observed in other epithelial cell lines and appears to confirm the importance of apoptosis in the perpetuation of the autoimmune injury [62] as well as the view that PBC bile duct cells are not unique [48]. This is indeed an attractive possibility, and data supporting such antigen presentation have been recently proposed [41] to ultimately support that PBC autoantigens (both nuclear and mitochondrial) are indeed 'traditional'.

Concluding remarks

Based on the data discussed, PBC can be sustained as a model autoimmune disease in which there is loss of immune tolerance to the mitochondrially located PDC-E2 autoantigen with the emergence of autoreactive populations of T and B lymphocytes (forbidden clones in the old parlance). Among many likely contributors to PBC pathogenesis is a strong genetic predisposition, not yet sufficiently dissected out, and environmental influences among which some could determine the structure and flexibility of the autoantigen, and others (life-style factors) could impact more on immune function itself [126]. Particularly promising are current studies on a cholangiocyte-specific form of apoptotic degradation of the PDC-E2 autoantigen which in our view could blur distinctions between the "traditional" autoantigens of the organ-specific autoimmune diseases and the "non-traditional" autoantigens of PBC, chiefly PDC-E2 [40, 41]. Further, we may expect that additional unsuspected factors will be investigated in the pathogenesis of PBC. These may include the recently suggested immunomodulatory effects of the gut microbiota [167] as intestinal adsorption capacity was already found impaired in patients with PBC [168]. Clearly there are many avenues still wide open to would-be investigators of the pathogenesis of PBC.

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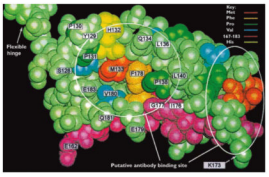

Figure 1.
Three-dimensional structure of the PDC-E2 inner lipoylated domain based on published NMR structure [16]

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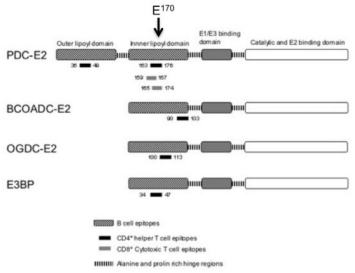

Figure 2.
The mapping of AMA (B cell) and T cell (CD4, CD8) epitopes recognized in PBC. Glutamic acid (E) at position 170 is highlighted for clarity purposes.

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Table 1

Molecular weights and functions of the 2-oxo-acid dehydrogenase complexes

Enzymes	MW (kDa)	Function
Pyruvate dehydrogenase complexes	**(PDC)**	
E1a decarboxylase	41	Decarboxylates pyruvate with thiamine pyrophosphate (TTP) as a co-factor
E1b decarboxylase	36	Decarboxylates pyruvate with TTP as a co-factor
E2 acetyltransferase	74	Transfers acetyl group from E1 to coenzyme A (CoA)
E3 lipoamide dehydrogenase	55	Regenerates disulphide of E2 by oxidation of lipoic acid
E3 binding protein (protein X)	56	Anchoring E2 to the E2 core of pyruvate dehydrogenase complex
2-oxoglutarate dehydrogenase complex	**(OGDC)**	
E1 oxoglutarate dehydrogenase	113	Decarboxylates a-ketoglutarate with TTP as a co-factor
E2 succinyl transferase	48	Transfers succinyl group from E2 to CoA
E3 lipoamide dehydrogenase	55	Regenerates disulphide of E2 by oxidation of lipoic acid
Branched-chain 2-oxo-acid dehydrogenase complex	**(BCOADC)**	
E1a decarboxylase	46	Decarboxylates a-keto acids
E1b decarboxylase	38	Derived from leucine, isoleucine, and valine with TTP as a co-factor
E2 acyltransferase	52	Transfers acyl group from E1 to CoA
E3 lipoamide dehydrogenase	55	Regenerates disulphide of E2 by oxidation of lipoic acid