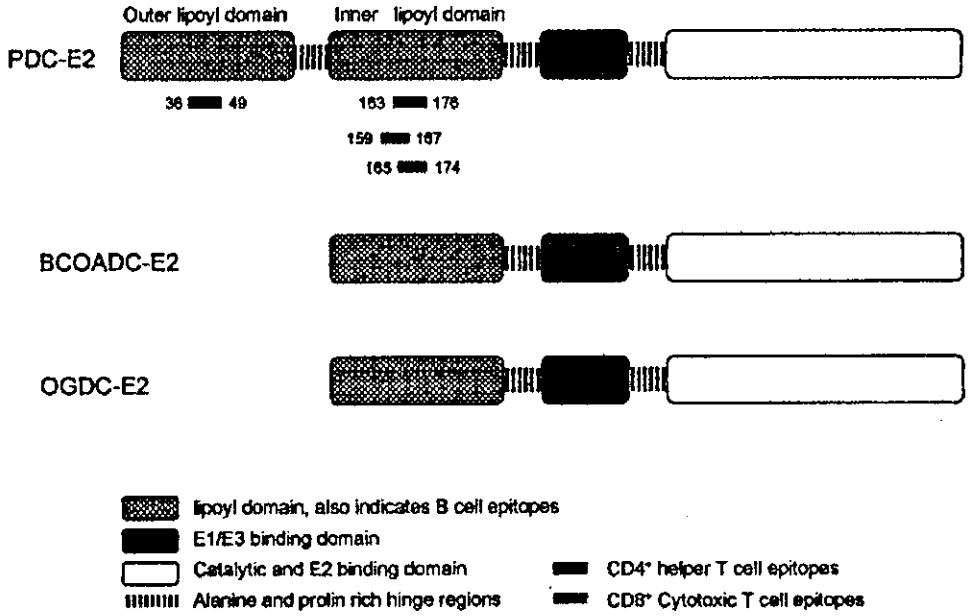

Fig. 1. The structural and functional domains of each E2 subunit of 2-OADC E2 and their corresponding B cell and T cell epitopes.

oligopeptides or recombinant fusion proteins, have shown that the epitope of PDC-E2 is located in the lipoyl domain, whereas the other domains are non-reactive. By expressing various recombinant peptides spanning the entire human PDC-E2 cDNA, PBC sera recognize recombinant peptides corresponding to the outer lipoyl domain (residues 1–96) and inner lipoyl domain (residues 128–227) of PDC-E2 (Surh et al., 1990). Furthermore, the residues 167–186 of PDC-E2 are thought to be the minimal epitope by inhibition assay (Van de Water et al., 1988b). For BCOADC-E2, overlapping expression clones spanning the entire bovine BCOADC-E2 were analyzed for their immunoreactivity against PBC sera, and immunoreactivity was primarily localized in the lipoic acid binding domain (Leung et al., 1995). The epitope of OGDC-E2 was identified within residues 67–147; which again correspond to the lipoyl domain of OGDC-E2 (Moteki et al., 1996). Likewise, the E3BP B cell epitope was mapped within the lipoic acid binding domain (Dubel et al., 1999). Taken together, the B cell epitopes of 2-OADC are located in the lipoyl domains, and antibody binding occurs when the antigen complexes with lipoic acid (Migliaccio et al., 2001). In fact, there are only five proteins in mammals that contain lipoic acid, and four of the five are autoantigens in PBC (Gershwin and Van de Water, 2001).

2.3. AMA and lipoic acid

It is well known that PDC-E2 has a lipoate molecule bonded to a ε-lysine in the domain to which autoantibodies from patients with PBC are directed (Leung et al., 2001). However, the AMA recognition of lipoic acid moiety itself has only been clearly defined recently (Bruggraber et al., 2003). Specifically, sera from 105 patients with PBC, 70 patients with primary sclerosing cholangitis (PSC), 28 patients with rheumatoid arthritis, and 43 healthy volunteers were examined for reactivity against lipoic acid hapten following incubation of aliquots of the sera with human serum albumin (HSA), lipoylated HSA, PDC-E2, lipoylated PDC-E2, polyethylene glycol (PEG), lipoylated PEG, free lipoic acid and synthetic molecular mimics of lipoic acid. Anti-lipoic acid specific antibodies were detected in the sera of 79/97 (81%) of AMA-positive PBC patients but not in the sera of controls. Two previously unreported specificities in AMA-positive sera that recognize (a) free lipoic acid, and (b) a carrier-conjugated form of lipoic acid, were

also identified. Of particular interest, antibodies to the lipoyl-peptide conjugate has a unique specificity for the lipoyl moiety and appear to bind to lipoic acid and a conjugated form of lipoic acid irrespective of the protein carrier but with low to undetectable reactivity against non-lipoylated carrier proteins, providing support for the view that the reactivity is specific for a conjugated form of lipoic acid moiety. These data clearly demonstrate that anti-lipoic acid antibodies are present uniquely in sera of patients with PBC; such antibodies were not seen in control sera. Although previous studies have suggested the presence of cross-reactive antibodies between the inner lipoyl domain of PDC-E2 and lipoic acid, the results were not definitive (Quinn et al., 1993; Flannery et al., 1989). Indeed, it is important to note that the lipoic acid is attached to the ε-amino group of the lysine residue which is part of the signature DKA motif within the inner lipoyl domain of PDC-E2 (Howard et al., 1998). In this study, the lipoic acid was attached to a number of the ε-amino groups of non-PDC-E2 carrier proteins, via the lysine residue of human albumin and rabbit albumin. The number of lysine residues per mole of human albumin and rabbit albumin is 61 and 58, respectively, and the only DKA motif identified was present in human albumin. Thus, the following populations of antibodies to lipoic acid are present in sera from patients with PBC: (a) antibodies to free lipoic acid, (b) antibodies to lipoylated PDC-E2 and (c) antibodies to PDC-E2 unrelated carrier bound lipoate. Subpopulations of AMA appear to recognize the conjugated lipoyl moiety in at least two distinct contexts. The first, and perhaps the major population, recognizes the PDC-E2 peptide both in its lipoylated and non-lipoylated form. The second population specifically recognizes a conjugated form of lipoic acid, but not the inner lipoyl domain of PDC-E2. These two populations of antibodies have different affinities for their antigens, with the antibodies directed against conjugated lipoic acid having a much lower concentration for lipoic acid than the anti-PDC-E2/lipoic acid antibodies for PDC-E2 (Bruggraber et al., 2003). The origin and initiation of anti-lipoic acid antibodies remain a mystery. Further analysis of antibody response against 2-OADC E2 subunits and lipoic acid may reveal how a specific autoimmune response was elicited against a prosthetic group (hapten) of a mitochondrial enzyme complex.

2.4. T cells responses against mitochondrial antigens

It has been hypothesized that the destruction of BEC in PBC is mediated by autoreactive T cells infiltrating in the liver (Gershwin et al., 2000; Ishibashi et al., 2003). Proliferation assays demonstrated that the $CD4^+$ helper T cells derived from the patients with PBC responded positively to amino acid residues 163–176 within the inner lipoyl domain and amino acid residues 36–49 within the outer lipoyl domain of PDC-E2 ($CD4^+$ T cell epitopes) in the context of HLA-DR53, and the frequencies of PDC-specific autoreactive $CD4^+$ helper T cells were higher in the liver than in the peripheral blood in patients with PBC (Shimoda et al., 1995, 1998). $CD8^+$ cytotoxic T cells (CTL) epitopes were identified as amino acid residues 159–167 and 165–174 of PDC-E2 in the context of HLA-A2.1, and the frequencies of these autoreactive CTL were also significantly higher in the liver than peripheral blood (Kita et al., 2002a,b; Matsumura et al., 2002a). Interestingly, these T cell epitopes are overlapping not only each other, but also with the B cell (anti-PDC-E2 antibody) epitopes (Fig. 1). These findings suggest that the lipoyl domain of PDC-E2 constitutes the most significant immunodominant regions in PBC. The immunospecificity of the lipoyl domains may be partly attributed to their spatial orientation since the lipoyl domain resides on the outer surface of the mitochondrial protein (Migliaccio et al., 2001).

3. Study of mitochondrial autoantigens in BEC

The intrahepatic biliary system is divided into large ducts, intermediate ducts, small ducts, and ductules. Small intrahepatic bile ducts include septal and interlobular ducts. BEC are heterogeneous not only in their size, but also in the expression of cell adhesion molecules, response to cytokines, growth factors and so on. This heterogeneity may explain why only the small bile duct epithelial cells are the targets of immune-mediated damage in PBC. In addition, only small bile duct BEC may express the target molecules. PDC-E2 and E3BP are upregulated in BEC (Joplin et al., 1994). Furthermore, this upregulation is present early in the natural history of PBC (Tsuneyama et al., 1995). Immunohistochemically,

PDC-E2 appears to be localized to the apical region of the BEC.

The significance of the increased focal expression of PDC-E2 in the surface and apical regions of the BEC is a possible explanation for the observations. It is unclear, however, whether the molecule detected at this special location is the whole PDC-E2 molecule, or a part of PDC-E2 or a mimic molecule that cross-react to PDC-E2. However, PDC-E2 messenger RNA is undetectable in BECs, arguing against simple enhanced synthesis with overspill to the cytoplasm and/or the surface (Harada et al., 1999). An alternative possibility is a trafficking defect, in which PDC-E2 is aberrantly transported to the cytoplasmic membrane. Such an alteration may occur as a result of mutations in the mitochondrial presequence, in a manner analogous to the mistargeting of alanine (Danpure, 1998).

Studies in the rat suggest that 10–15% of basal bile flow is produced by BEC and it has been estimated that the corresponding contribution in humans is 40% (Nathanson and Boyer, 1991). Therefore, bile ducts are one of the routes by which toxic materials or metabolites are removed from the body, and it is possible that select toxic substances may accumulate within BEC and potentially modify the PDC-E2 molecule locally, leading to the production of tissue-specific variants of PDC-E2.

4. Mechanism of BEC destruction in PBC: the role of IgA AMA

4.1. Apoptosis in BEC

Apoptosis is thought to play an important role in a number of liver conditions, including autoimmune hepatitis, chronic viral hepatitis, alcoholic liver disease, and PBC (Bai and Odin, 2003). There are several reports that BEC expressing Fas undergo apoptosis in PBC using in situ nick-end labeling methods for the detection of DNA fragmentation (Harada et al., 1997). In addition, cultured human BEC are sensitive to Fas-mediated apoptosis and this can be initiated by activation of cell surface CD40 (Afford et al., 2001). PDC-E2 is cleaved and loses immunogenicity by granzyme B (Matsumura et al., 2002b). Various stimuli, especially oxidative stress, can lead to damage of the mitochondrial inner membrane, resulting in mitochondrial permeability transition and subsequently the release of cytochrome *c* and apoptosis-inducing factor from the mitochondria.

4.2. Co-localization of IgA AMA with PDC-E2

Although a number of studies were directed in investigating the immunobiology of AMA, the pathological significance of AMA in PBC remains enigmatic. For example, is AMA involved in the pathogenesis of PBC or just epiphenomena? (Davis et al., 1992). We have found evidence that indicates the involvement of AMA in the pathogenesis of PBC.

PBC is a mucosal disease with generalized epithelitis (Epstein et al., 1980). In fact, IgA class AMA is detected not only in the sera, but also in the bile, saliva, and urine of the patients with PBC at the frequency of 47.4, 80 and 68.7%, respectively (Nishio et al., 1997; Reynoso-Paz et al., 2000; Tanaka et al., 2000). In contrast, no AMA is detected in the bile, saliva and urine of control subjects. Also patients with PBC often have concurrent Sjögren's syndrome, or autoimmune destructed salivary glands.

IgA is the major immunoglobulin in mucosal immune system, which acts as a first line of specific immunological defense against harmful external antigens. It is estimated that the total amount of IgA produced daily exceeds that of all other classes combined (Brandtzaeg et al., 1999; Kerr, 1990). IgA in secretions is found as polymers (pIgA), mainly dimmers (dIgA). After synthesis by plasma cells in the lamina propria, pIgA is transported across the epithelium into secretions by transcytosis. Transcytosis of pIgA is mediated by the transmembrane secretory component (SC), also named polymeric immunoglobulin receptor (pIgR), expressed by most epithelial cells lining the secretory epithelial surfaces and the exocrine glands (Brandtzaeg et al., 1994; Giffroy et al., 2001). Human BEC also express IgA-specific surface receptors for the transcytosis of IgA into bile (Brown and Kloppel, 1989). Furthermore, pIgA can interact with its antigens within epithelial cells (Bomsel et al., 1998), and several reports indicate that penetration of autoantibodies into living cells may be linked to the functional effects of

intracellular antibodies on cytopathology (Koscec et al., 1997; Yanase et al., 1997).

Therefore, we have hypothesized that mucosally derived IgA AMA may be pathogenic for BEC in PBC. We have investigated that the localization and the ability to penetrate cells of IgA from the patients with PBC using Madine-Darby canine kidney cells transfected with the human pIgR (MDCK-pIgR cells), which are thought to be ideal for transcytosis studies since they are established polarity similar to BEC (Song et al., 1995). Interestingly, IgA from all PBC patients co-localized with PDC-E2 in the cytoplasm using dual staining with anti-human IgA and monoclonal antibody directed to PDC-E2 and confocal microscopy (Malmborg et al., 1998; Fukushima et al., 2002). In contrast, co-localization was not observed using control IgA. Moreover, such co-localization pattern was also seen in the BEC of dual-stained liver sections from PBC patients. We also generated recombinant PDC-E2-specific dimeric human IgA monoclonal antibody by using a baculovirus expression vector system. This recombinant dIgA monoclonal antibody also showed a high degree of co-localization with PDC-E2 in MDCK-pIgR cells (Fukushima et al., 2002).

4.3. Induction of apoptosis by IgA AMA

The phenomenon of IgA AMA co-localization with PDC-E2 in the cytoplasm of MDCK-pIgR cells suggests that IgA AMA may play affect mitochondrial function. To investigate the mechanisms of IgA AMA in the pathology of BEC in PBC, we began to study whether IgA AMA affects apoptosis. We took advantage of a novel and unique methodology for isolating intact dimeric IgA from sera of the patients with PBC using a specific Streptococcal IgA-binding peptide (Sap). Sap is composed of 50 amino acids derived from M protein of *Streptococcus Pyogenes* with addition of a C-terminal cystein residue and is known to bind Fc portion of IgA very efficiently in a specific manner, retaining its full biological features (Sandin et al., 2002; Johnsson et al., 1994). Purified IgA was confirmed to contain both monomeric and dimeric forms with similar ratio as serum IgA by SDS-PAGE and immunoblotting. Thereafter, the effects of purified IgA on MDCK-pIgR cells were analyzed using a transwell membrane system

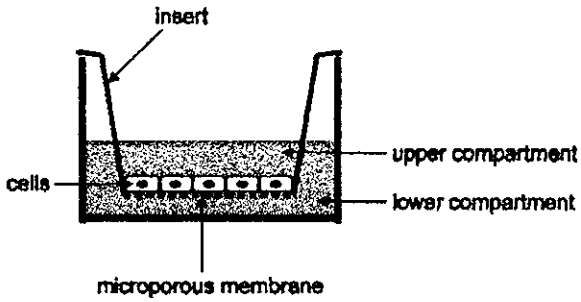

Fig. 2. Scheme of transwell membrane system. MDCK-pIgR cells are cultured on the microporous membrane of the insert and IgA is added in the medium of the lower compartment. MDCK-pIgR cells are established their polarization and IgA is transcytosed from the lower compartment to the upper compartment.

(Fig. 2; Malmborg et al., 1998). Briefly, MDCK-pIgR cells were cultured in the bottom of the insert and IgA was added in the medium of the lower compartment. The cells were analyzed for the binding of carboxyfluorescein-conjugated VAD-fmk peptide against activated caspases by flow cytometry. Surprisingly, we found increased caspases activation in MDCK-pIgR cells treated with IgA from the AMA-positive patients with PBC. In contrast, IgAs purified from AMA-negative patients with PBC or liver disease controls showed no caspases activity (Fig. 3). Moreover, we found that these caspases activating effect of IgA are only seen in IgA class anti-PDC-E2, not in IgG nor IgM class, nor in anti-BCOADC-E2 nor anti-OGDC-E2 (data not shown, manuscript in preparation). We also confirmed that caspase-3 and caspase-9, but not caspase-8 among overall caspases were activated in MDCK-pIgR cells treated with IgA anti-PDC-E2 antibody. This result indicates that the apoptosis is mediated through the mitochondrial pathway.

These caspases activating effects of IgA anti-PDC-E2 were observed in MDCK-pIgR cells and have not yet been demonstrated using human BEC. However, we believe that this MDCK-pIgR system is a good model of BEC, because MDCK-pIgR cells have a polarity similar to BEC and are common models for transcytosis studies (Song et al., 1995). pIgA binding to pIgR involves an intracellular signal, starting by the activation of a protein-tyrosin kinase that rapidly phosphorylates phosphatidylinositol-specific phospholipase Cγ1 (PLCγ1). This enzyme catalyzes the hydrolysis of phosphatidylinositol-4,5-bis-phosphate

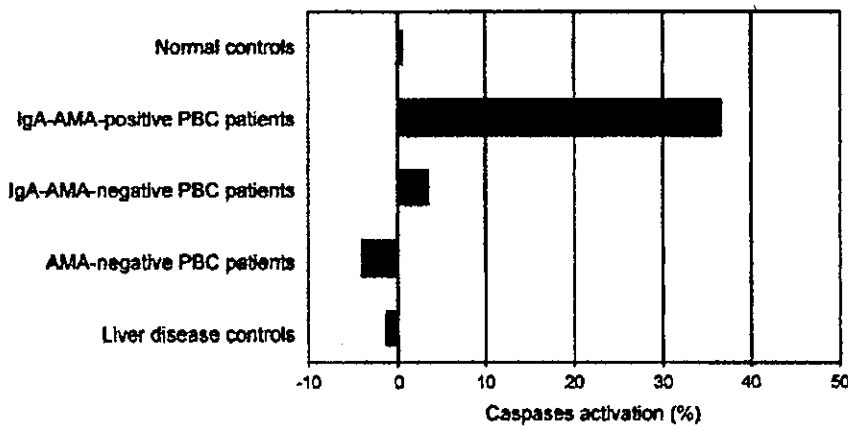

Fig. 3. Overall caspases activation in MDCK-pIgR cells treated with purified IgA. Serum IgAs were purified using Sap column that immobilized Sap peptide. MDCK-pIgR cells were cultured in cell culture inserts. Purified IgAs were inoculated in the lower chambers at the final concentration of 1 mg/ml. After 48-h-incubation at 37 °C, carboxyfluorescein labeled VAD-fmk (FAM-VAD-fmk, Cell Technology, Inc., Minneapolis, MN) was inoculated to the upper chamber at the final concentration of 10 μM. FAM-VAD-fmk was used as a probe which binds overall activated caspases. One hour later, the cells were harvested and analyzed by flow cytometry. Caspases activation index was calculated as; (geometric mean value of IgA-treated cells − geometric mean value of non-treated cells)/geometric mean value of non-treated cells × 100 (%).

into diacylglycerol and inositol 1,4,5-triphosphate (IP3). Diacylglycerol is known to activate protein kinase C (PKC), more specially its α and ε isoforms, and direct activation of PKC by phorbol esters indeed stimulates transcytosis. IP3 production causes the release into the cytosol of Ca^{2+} from intracellular stores, and an artificial increase in cytosolic $[Ca^{2+}]_i$ by thapsigargin also stimulates tanscytosis (Giffroy et al., 2001). There are no apoptosis-inducing signals in these intracellular signal transductions via pIgR. Hence, our data on specific caspases activation by anti-PDC-E2 IgA may explain IgA AMA induced destruction of BEC in PBC. Briefly, dIgA is transcytosed through pIgR into BEC. J-chain of dIgA binds to pIgR. While non-specific IgAs are being transferred into bile duct lumen, anti-PDC-E2 IgA may bind to newly synthesized PDC-E2 protein during protein translocation from ribosome to mitochondria. Alternatively, anti-PDC-E2 IgA may bind to PDC-E2 on the mitochondrial membrane. The binding of PDC-E2 by anti-PDC-E2 in the cytoplasm might effectively reduce the available PDC-E2 subunits for protein turnover and thereby inhibits mitochondrial function. These may cause mitochondrial damage, and several apoptogenic proteins, including cytochrome *c*, are released from the mitochondria, then caspase-9 followed by caspase-3 is activated, finally apoptosis may be induced (Fig. 4). In contrast, PDC may be released from apoptotic mitochondria to the cytoplasm, then autoreactive epitopes may be present on the still-intact cell surface. BEC may be particularly susceptible to this process, as other cell types efficiently delete cytoplasmic PDC by glutathiolation, which eliminates the autoreactive epitope (Odin et al., 2001).

5. Diagnostic value of AMA in PBC

Since AMA is present in the sera of 90–95% of patients with PBC, they constitute the most important diagnostic criteria of PBC. Currently, several methods are commonly used in the detection of AMA. Indirect immunofluorescence (IIF) microscopy is routinely utilized in clinical laboratories. However, IIF lacks specificity and sensitivity. Other methods such as ELISA or SDS-PAGE followed by immunoblotting on mitochondrial samples/recombinant mitochondrial autoantigens provide alternative methods for the specific determination of the AMA profile. Genetically engineered recombinant molecules (pML-MIT3) containing the AMA epitopes of PDC-E2, BCOADC-E2 and OGDC-E2 have been used in the refinement of ELISA and provide a highly sensitive diagnosis of PBC (Moteki et al., 1996). Recently, AMA has been detected in the saliva and urine of PBC patients with

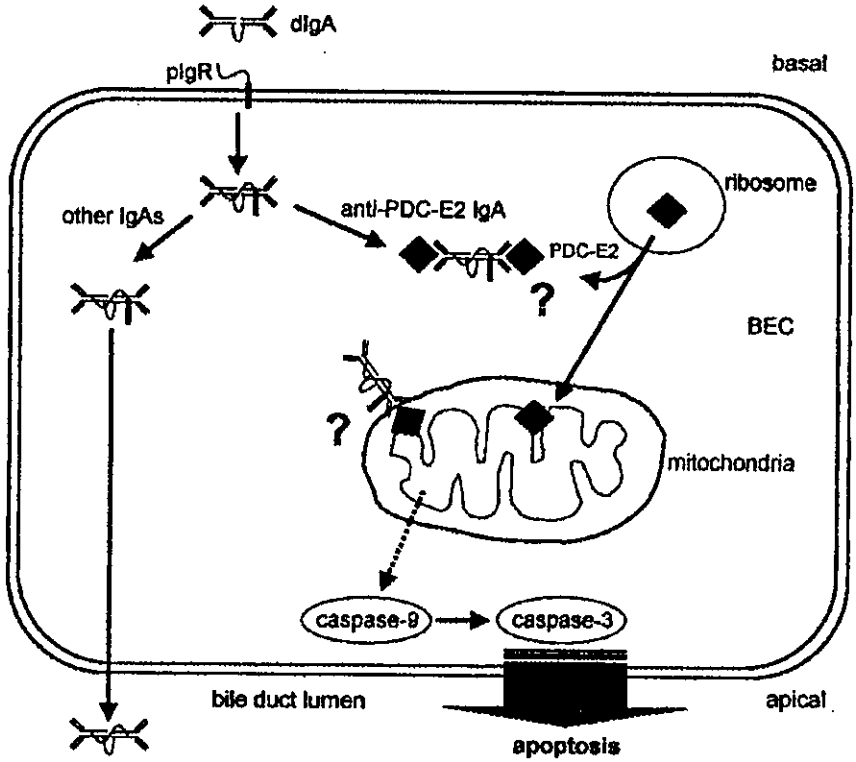

Fig. 4. Hypothetical role of IgA anti-PDC-E2 on the destruction of biliary epithelial cells. dIgA is transcytosed through pIgR into biliary epithelial cells (BECs). The J-chain of dIgA is thought to bind to pIgR. Non-specific IgAs should be transferred into bile duct lumen. However, anti-PDC-E2 IgA may bind to PDC-E2 protein is moving from ribosome to mitochondria in the cytoplasm. Alternatively, anti-PDC-E2 IgA may bind to PDC-E2 on the mitochondrial membrane. These may cause the mitochondrial damage, and caspase-9 followed by caspase-3 is activated; BECs then undergo apoptosis.

specificity against one or more of the OADC subunits (Reynoso-Paz et al., 2000; Tanaka et al., 2000). The use of the genetically engineered autoantigenic epitope combined with the ability to utilize excretory fluid such as saliva clearly provides a much less invasive and sensitive method for the detection of AMA. Although there are approximately 5% of patients with PBC who are seronegative for AMA, AMA detection is a powerful tool for diagnosis when in conjunction with combination of serological, biochemical and histological data (Tanaka et al., 2002).

6. Discussion

An orchestrated immune response against the intrahepatic BEC through 2-OADC specific $CD4^+$ helper T cells and $CD8^+$ CTL are the major players in the immunological destruction of BEC in PBC. We believe that a prior/primary event of specific intrahepatic BEC malfunction (possibly caused by environmental insults such as xenobiotics, microorganisms with xenobiotic modified 2-OADC) leads to BEC destruction and the subsequent release of mitochondrial autoantigens, both in their native and modified form. This is responsible for the breaking of tolerance to 2-OADC in PBC. The generation of AMA and 2-OADC specific T cells further accelerates the BEC pathology. In addition to studies on BEC apoptosis, the data presented in this review indicate that IgA AMA may participate in the destruction of BEC by damaging mitochondria. Further studies of the biochemical mechanisms of mitochondrial protein synthesis, turnover and their modification in BEC will be necessary to reveal the molecular basis of the specific immune response to the 2-OADC.

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T Cell Immunity in Primary Biliary Cirrhosis.

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Primary biliary cirrhosis (PBC) is a chronic inflammatory cholestatic liver disease that predominantly affects middle-aged women (1). Histologically, PBC is characterized by infiltration of mononuclear cells in portal tracts and destruction of intrahepatic small bile ducts. Portal inflammation is followed by fibrosis and then cirrhosis, eventually, liver failure (2). The serological hallmark of PBC is the presence of antimitochondrial antibodies (AMA), which are almost specific to PBC. It has been clarified that the major autoantigens members are of the 2-oxo-acid dehydrogenase complexes (2-OADC) (3) including the E2 subunits of the pyruvate dehydrogenase complex (PDC-E2), the branched chain 2-oxo-acid dehydrogenase complex (BCOADC-E2) and the 2-oxo-glutarate dehydrogenase complex (OGDC-E2) as well as the E1 subunits of the PDC and the E3-binding protein (E3BP). Interestingly, the immunodominant epitope of each of the 2-OADC enzymes locates around the lypoic acid bound to lysine in the lipoyl domain (4).

While the association between AMA and PBC has been clearly established, there has been no evidence that AMA is causally involved in the damage of biliary epithelial cells (BEC) and the pathological significance of AMA in PBC remains enigmatic. However, the presence of autoantibodies, such as AMA and antinuclear antibodies (ANA) (5), together with the presence of autoreactive lymphocytes (6) and the frequent association with other autoimmune diseases such as Sjögren's syndrome, Hashimoto's thyroiditis, scleroderma and rheumatoid arthritis (1) collectively suggest that PBC is mediated by autoimmunity.

Interestingly, PBC is often considered to be a dry gland disease caused by frequent involvement of salivary and lacrimal glands, and the importance of mucosal immunity attract the attention. However, little is known about the presence of mitochondrial autoantigens in mucosal compartments such as saliva or urine, although high titers of AMA have long been recognized in PBC. Gershwin et al. investigated saliva and sera in PBC patients for the presence of AMA and mitochondrial antigens. They detected AMA in saliva in over 90% of PBC patients, 80% of the isotype of which was immunoglobulin A (IgA)(7). Similar to serum and bile anti-PDC-E2 IgA antibodies, the autoantibodies in saliva reacted to the inner lipoyl domain of PDC-E2. Furthermore, saliva from patients with PBC but not controls inhibited pyruvate dehydrogenase enzyme activity *in vitro*. In addition, a molecule with a molecular weight corresponding to PDC-E2 (74 kDa) was detected in PBC but *not in control saliva*. These findings suggest the presence of localized mucosal immunity in the secretory system of PBC.

Recent findings suggest a possible role for IgA AMA-mediated biliary injury in PBC (8) in addition to cytotoxic T cells and natural killer T (NKT) cells which have been found to play a major role in the destruction of bile ducts (9, 10).

Immunohistology of PBC

Characteristic immunohistochemical findings of the PBC liver include extensive infiltration of T cells, predominantly activated $CD4^+$ T cells in the portal tracts. However, a predominance of $CD8^+$ T cells has been revealed during early stages of PBC (11-13). Within the CD4+ population, the percentage of CD45RO+ helper-type T cells is increased. Activated T cells have also been shown to be present in areas of piecemeal necrosis, and these are predominantly of the CD8+CD11b- phenotype (11).

$CD4^+$ helper T cells

A subpopulation of liver-infiltrating T cells in PBC were demonstrated to be specifically directed against PDC-E2, a highly conserved enzyme which plays a critical role in intermediate metabolism, by using recombinant truncated fragments of PDC-E2 (6). CD4+T-cells specific for PDC-E2 are present in the peripheral repertoire of the majority of patients with PBC. The observations that peripheral blood PDC-E2-specific T cells are most commonly seen in early stage disease, when active bile duct damage is occurring, and that PDC-E2-specific T cells can be found in the portal tract infiltrate at times when this damage is occurring, suggest that these autoreactive T cells may play an important role in the etiology of primary biliary cirrhosis.

Using a panel of 33 overlapping peptides spanning the entire PDC-E2 sequence, Shimoda et al (14) identified the amino acid residues 163–176 (GDLLAEIETDKATI) within the inner lipoyl domain of PDC-E2 as the minimal T cell epitope. The $PDC\text{-}E2_{163\text{-}176}$-specific T cell clones were shown to be positive for CD4, CD45RO and TCR αβ. Further studies have shown that the HLA-restriction molecules for such PDC-E2 epitope is HLA-DR53 (B4*0101) (14) and that the amino acid residues E, D and K (at positions 170, 172 and 173, respectively) are essential for the recognition by the T cell clones. Interestingly, the ExDK motif can also be found within the outer lipoyl domain of PDC-E2, OGDC-E2, BCOADC-E2 and E3BP, and is recognized by T cell clones specific for $PDC\text{-}E2_{163\text{-}176}$ (14). More specifically, T cell clones

continued on page 11

continued from page 10

cross-reacted with the amino acid residues 100–113 within OGDC-E2, 90–103 within BCOADC-E2, and 34–47 within E3BP, all located in the lipoyl domain of the respective subunit and possess the ExDK motif (15, 16). A quantitative analysis of autoreactive T cells in different tissues from patients with PBC showed that the frequency of precursor PDC-$E2_{163-176}$–reactive T cells is 100–150-fold higher in the hilar lymph node and liver than in the peripheral blood (15).

T cell receptor (TCR) usages have been investigated by several researchers in autoreactive T cells. The TCR Vβ usage of liver-infiltrating T cells was shown to be more heterogeneous, without denying its oligoclonality (17). The TCR Vβ- or Jβ-gene usage of PDC-$E2_{163-176}$–reactive T cell clones is highly heterogeneous despite a GxG or GxS motif found in the complementarity determining region 3 (CDR3) of all T cell clones examined (18). Other experimental data, indicated that other amino acids (G as well as A and L) were conserved residues in the junctional N-D-N regions of the CDR3 (17).

$CD4^+CD28^-$ or costimulation-independent T cells

Previous work has suggested that $CD4^+CD28^-$ or costimulation-independent T cells are increased in autoimmune diseases. Kamihira et al. (19) compared the frequency and qualitative characteristics of autoreactive costimulation-independent or $CD4^+CD28^-$ T cells in PBC by taking advantage of the well-defined immunodominant autoepitope of PDC-E2. They studied the precursor frequency of costimulation-independent $CD4^+$ T cells that respond to PDC-E2 163-176 and found that the frequency of $CD4^+CD28^-$ T cells were dramatically elevated in PBC. Furthermore, two types of T cell clones that respond to PDC-$E2_{163-176}$ emerged from their study; one type was costimulation dependent and the other costimulation independent. Both types of clones lyse biliary epithelical cells (BEC) in a similar effector target (E/T) ratio distribution. However, BEC did not help the proliferation of any T cell clones. Furthermore, costimulation-independent T cell clones do not become anergic by BEC. From these observations they concluded that costimulation-independent autoreactive T cells, which do not become anergic, increase and maintain the autoimmune response in PBC. In controls, although autoantigens are expressed on BEC and autoantigen-reactive T cells exist around BEC, autoantigen-reactive T cells are costimulation dependent and will become anergic, thus maintaining peripheral tolerance.

$CD8^+$ cytotoxic T lymphocytes (CTL)

A marked enrichment of $CD8^+$ cytotoxic T cells among liver-infiltrating T cells has been reported (20). Although this observation suggested an important role for $CD8^+$CTL in the immunopathogenesis of PBC, very limited numbers of studies have been reported. Kita et al (21) generated $CD8^+$ CTL lines from peripheral blood cells of patients with PBC, using autologous immature dendritic cells pulsed with a panel of 12 peptides derived from PDC-E2. Such peptides were selected by scanning the amino acid sequence of the autoantigen for the presence of 9-mer peptides containing HLA-A*0201–binding motifs (21). The obtained CTL lines produced interferon (IFN)-γ after restimulation with a peptide containing amino acid residues 159–167 of the inner lipoyl domain of PDC-E2. These data indicate that such amino acid residues constitute an HLA-A*0201-restricted CTL epitope, and that autoreactive PDC-$E2_{159-167}$-specific CD8+CTL can be found in the peripheral blood of patients with PBC. Based on these results and using PDC-$E2_{159-167}$-HLA-A*0201 tetramers, they were able to estimate that the frequency of PDC-$E2_{159-167}$-specific CTL was 10-fold higher in PBC liver than in the peripheral blood as well as in the peripheral blood of patients with early stages of PBC (9). An HLA-A*0201-restricted epitope was further mapped to amino acids 165-174 of PDC-E2, also locating within the inner lipoyl domain of PDC-E2 and therefore partially overlapping with the previously described $CD8^+$ and $CD4^+$ T cell epitopes (22).

Natural killer T (NKT) cells

Using immunohistochemistry, NKT cells were found predominantly within portal tracts and parenchyma in the liver of patients with PBC or other liver diseases, as well as healthy controls (23). Compared to healthy and disease controls, however, PBC liver samples contained a significantly higher frequency of $CD3^+CD57^+$ NKT cells, localized in particular where damaged interlobular bile ducts could be found. NKT cells produce both IFN-γ and interleukin (IL)-4 and have been implicated in several models of autoimmunity. Quantitated studies using a tetramer complexing human CD1d and α-GalCer (10) showed that CD1d-α-GalCer-restricted NKT cells were significantly more represented in the liver compared to the peripheral blood of patients with PBC, and were also more frequent in the liver and blood of patients compared to healthy individuals.

Cytokines in the recruitment of autoreactive cells

Several studies (24-26) suggest that type 1 cytokines (mainly IL-2 and IFN-γ) are produced by activating CTL and macrophages, and play a dominant role in the pathogenesis of PBC. On the other hand, type 2 cytokines such as IL-5, IL-6, and IL-10 (25, 27) have also been found in PBC livers. These observations together indicate that both type 1 and type 2 cytokines might be involved in the pathogenesis of PBC possibly during different stages of the disease.

Summary

The presence of AMA, highly specific autoantibodies for PBC, and autoreactive T cells (both helper and cytotoxic) point toward an autoimmune pathogenesis for PBC. Interestingly, epitopes recognized by AMA and T cell clones are all located within the overlapping areas of the antigens, and present significant overlapping in their amino acid sequence. Autoreactive lymphocytes, with their diverse effector functions and their

continued on page 12

continued from page 11

regulatory effects on other immune cells, play a central role in PBC. An imbalance in cytokine pattern and natural killer lymphocytes also plays an important role in the pathogenesis of PBC. However, despite several experimental reports, no clear evidence on the interaction of these factors leading to bile duct destruction has been available.

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Editorial

Hen and egg, you mitochondria! PBC revisits mitochondria again

Primary biliary cirrhosis (PBC) is a hallmarked autoimmune liver disease, in the point of which anti-mitochondrial antibodies are positive in most of the patients [1] and autoimmune responses against mitochondrial proteins, such as the E2 component of pyruvate dehydrogenase complex (PDC-E2), are well documented [2]. Nevertheless, its etiology remain obscure, even though environmental [3], microbial [4,5], and genetic factors [6] are each believed to play pivotal, and not mutually exclusive roles for initiating and sustaining the pathophysiology in PBC. Moreover, the cause for the organ specific autoimmune reaction needs plausible explanations: pathological targets are biliary epithelial cells (BECs), while molecular targets are ubiquitously expressed in mitochondria throughout the body. Are mitochondria themselves in the PBC liver pathogenetic? Or, may autoimmune destruction of BECs via mitochondrial antigens in autoimmune reactions cause further pathological changes in mitochondria in inflamed liver? Those questions have hardly been solved so far.

In this issue of the Journal, Chen et al. [7] reported the enhancement of the mitochondrial gene expression in the PBC liver. They performed suppression subtractive hybridization (SSH) to analyze the differential mRNA expression in the liver of a patient with PBC, and found over-expressed genes encoded by mitochondrial DNA, compared to normal liver. They further investigated the mechanism of increased mitochondrial transcription, and revealed that up-regulation of mitochondrial transcriptional factor A (mtTFA) and its transactivator mitochondrial nuclear respiratory factor 1 (NRF-1) was associated with the phenomena. They first took advantage of SSH for screening of overexpressed genes in the needle biopsied specimen that was obtained from a PBC patient of Scheuer's stage II. Sequence analysis of clones from subtracted PBC cDNA gave them substantial fragments of cDNAs derived from mitochondrial DNA(mtDNA)-encoded genes, namely mitochondrial 12S rRNA, cytochrome *b*, mitochondrial tRNA-Val, and cytochrome *c* oxidase. After the confirmation of overexpression of these genes by RT-PCR, they evaluated the expression of three nuclear encoded genes that control the transcription and replication of these mitochondrial DNA *in trans*: mtTFA, its transcriptional activator NRF-1, and the NRF-1 coactivator, PPAR gamma coactivator 1(PGC-1) [8]. Quantitative competitive RT-PCR analysis with liver biopsy samples from patients in the chronic stage of cholangitis or hepatitis revealed the significantly higher NRF-1 and mtTFA expression levels in PBC liver than in other liver diseases, while the PGC-1 was suppressed. mtTFA is a known single nuclear DNA-derived transcriptional factor, which enhances both mtDNA replication and transcription of mtDNA-encoded genes, resulting in mitochondria biogenesis. Although they did not check the transcription levels of other mitochondrial genes even in the index cases, these data suggested that enhanced-activation of NRF-1–mtTFA axis may be a cause of the increased mitochondrial transcription detected in PBC liver, in accordance with previous literatures observing the increased mitochondria biogenesis in PBC liver [9–11].

There are some apparent limitations within this report: too small number for each chronic hepatitis group and no inclusion of other cholestatic diseases do not give us the information that those phenomena are truly specific in PBC. Moreover, lower mRNA levels of PGC-1 in PBC was not fit well for their conclusion that NRF-1 dependent mtTFA transcription was activated, even if post-transcriptional modification of PGC-1 [8], resulting in its activation and stabilization, may occur in the inflamed liver. In addition, they did not even mention, through which master molecule, PGC-1α or its homologue β, their postulated molecular signature works around in mitochondria [12].

Nevertheless, this study raises interesting questions and provides important informations, regarding the mitochondria-associated pathology in the liver.

First, what is the mechanism of NRF-1 and mtTFA overexpression? May it be the probable enhancement of mitochondria biogenesis in the PBC liver? Reactive oxygen species (ROS), produced in the mitochondria as byproducts of cellular respiration, are the known candidates for the enhanced mitochondria biogenesis [13], as was discussed in the ar-

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ticle. The increase in mitochondrial mass and concomitant NRF-1 overexpression in human fibroblasts under oxidative stress and during replicative senescence was shown in in vitro study [13]. Valgimigli et al. [14] reported the progressively increasing levels of ROS in liver tissues, passing from control values to type B and C chronic hepatitis and PBC. Cellular stress, especially chronic state of inflammation occurred in the liver, should induce ROS, resulting in subsequent activation of NRF-1–mtTFA axis. NRF-1 induced genes are originally shown to be implicated in anti-oxidant pathways, as was confirmed in the recent paper describing that NRF-1 deficient fetal livers exhibited increased oxidative stress [15].

Taken together, even ROS-induced NRF-1 overexpression may work in compensatory way for reducing the ROS abundance in the inflamed liver, a certain epiphenomenon that is the enhancement of mtDNA transcription/biogenesis through mtTFA, might further produce ROS. Accelerated replication of mtDNA with ROS should be the prerequisite for the accumulation of mtDNA mutations, probably influencing the physiology of the cell or causing mitochondriopathy, once it has accumulated to the significant fraction of all mtDNA in the cell [16]. Results by Chen et al. may represent that the inflammation in PBC is generally the most prominent state of the vicious circle through ROS, accompanying possible mitochondriopathy, among various state of chronic liver diseases.

Second and the last question is that the overexpression of mitochondria genes is mainly from hepatocytes, or BECs? The answer is surely from hepatocytes, but as was discussed in the article, the previous paper used SSH with isolated BECs also reported the overexpression of mitochondria genes in BEC [17], suggesting that both BECs and hepatocytes are responsible for the transcriptional changes in mitochondria. In accordance with that, electron microscopic study has already shown that both hepatocytes in PBC liver [9,10] and BECs [11] had increased mass of mitochondria, and the latter called them "intrahepatic bile duct oncocytes". Again, it should be true that any type of chronic inflammation in the liver, albeit in different etiology, has some common characteristics of ROS/mitochondria-related pathology, but PBC might be the extreme case in its nature, possibly related to the sustained presence of autoimmune reaction to mitochondrial proteins.

Irrespective of the precise pathogenetic mechanism(s), PBC revisits mitochondria again through SSH. Future studies including the mutational analysis of mtDNA, coupled with the phenotypical analysis of mitochondria itself, in the PBC specimen should broaden our understanding for the mitochondria-associated pathology in the liver in PBC.

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T cell immunity in hepatitis B and hepatitis C virus infection: implications for autoimmunity

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Abstract

T cells are involved in the pathogenesis of important liver diseases including both autoimmune liver diseases and viral hepatitis. In addition to playing a crucial role in the control of hepatitis viruses, T cell responses are also responsible for the liver injury during acute and chronic phases of viral hepatitis. In this article, we reviewed current literature on T cell immunity to hepatitis B and C viruses. In addition, antigen presenting cells that are critical for T cell immunity against these viruses are also discussed. This will provide insights to the understanding of T cell immunity in autoimmune liver diseases due to the similar role of T cells in autoimmune liver diseases and viral hepatitis.

Contents

Abbreviations: APC, antigen presenting cells; CTL, cytotoxic T lymphocytes; DC, dendritic cells; HBV, hepatitis B virus; HCV, hepatitis C virus; IFN, interferon; IL, interleukin; Th1 cells, type 1 helper T cells; Th2 cells, type 2 helper T cells; TNF-a, tumor necrosis factor-a.

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1. Introduction

T lymphocytes, with their diverse effector functions and their regulatory effects on other immune cells, play a central role in inflammatory diseases such as infectious diseases, autoimmune diseases, graft-versus-host disease and allograft rejection. Of particular interest, T cells are believed to involve in the pathogenesis of important liver diseases, including both autoimmune liver diseases and viral hepatitis. Accumulating evidences indicate that similar to type I diabetes and multiple sclerosis, primary biliary cirrhosis (PBC), an autoimmune liver disease, is also a T cell-mediated autoimmune disease. While PBC and other autoimmune liver diseases are a relatively rare liver diseases, chronic viral hepatitis, especially those caused by hepatitis B virus (HBV) and hepatitis C virus (HCV), are the most common chronic liver diseases, with more than 400 million and more than 170 million people worldwide chronically infected with HBV and HCV, respectively. In addition to playing a crucial role in the control of HBV and HCV, T cell responses are also responsible for the liver injury during acute and chronic phases of viral hepatitis. Studies on T cell immunity in viral hepatitis are much more extensive than that in autoimmune liver diseases. Due to the similar role of T cells in autoimmune liver diseases and viral hepatitis, and a much larger body of literature on viral hepatitis, knowledge on hepatitis virus immunology has provided important insights to the understanding of T cell immunity in autoimmune liver diseases. Therefore, this article will be devoted to the T cell immunity against two major hepatitis viruses.

Hepatitis B virus (HBV) and hepatitis C virus (HCV) are the major causes of chronic viral hepatitis. HBV is the prototype member of a family of related viruses known as hepadnaviruses (hepatotropic DNA viruses), while HCV is a positive stranded RNA virus belonging to the family of flaviviruses. Replication of both viruses takes place primarily in the liver, making HBV and HCV infections the leading causes of chronic hepatitis worldwide, affecting 350–400 million and 170 million people, respectively [1,2]. HCV persists in the majority of infected individuals (~70%), but the incidence of asymptomatic infection or transient disease followed by spontaneous recovery may be underestimated. In contrast, chronic hepatitis B develops in only ~5% of HBV-infected adults,

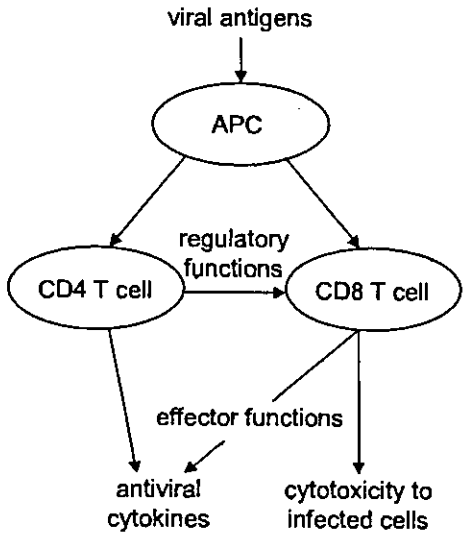

Fig. 1. T cell immunity in HBV and HCV infections. Extensive interactions between different components of the immune system are critical for a successful cellular immune response against HBV and HCV infection.

although the majority of HBV-infected neonates become chronic carriers. There is growing evidence that the nature of the cellular immune responses determines long-term outcomes in both diseases, but there is limited information on what underlies the differences in the individual immune responses. In this article, we review the current literature on T cell immunity to HBV and HCV, including the $CD4^+$, $CD8^+$ and NKT cell response. Further, since antigen presenting cells have a profound effect on the development of T cell immunity, we also discuss current data on dendritic cells. While these are discussed in different sections separately for clarity, a successful immune response against HCV and HBV is believed to involve multiple subsets of immune cells as well as extensive interactions between different components of the immune system (Fig. 1). Some of these interactions will also be discussed in respective parts of this review.

2. $CD4^+$ T cell response

2.1. HCV

There are several lines of evidence suggesting an important role for $CD4^+$ helper T cells in the elimination of both HBV and HCV. One is the significant overrepresentation of certain HLA class II alleles in patients with a self-limiting disease course compared to patients who develop chronic disease. In particular, carriage of DQB1*0301 or the closely linked DRB1*1101 is consistently correlated with spontaneous clearance of HCV [3–6]. Patients with one of these alleles exhibit proliferative $CD4^+$ T cell responses to a significantly higher number of peptides derived from core and nonstructural proteins of HCV [6].

Further evidence comes from direct analyses of T cell immune responses in acutely infected individuals and their comparison to results obtained in chronically infected patients. A study of five healthcare workers who were infected with HCV and followed weekly from the time of accidental needlestick exposure provides invaluable information on the immune response during the incubation and acute phase of HCV infection [7]. In the only subject who cleared the virus, an early, vigorous and sustained $CD4^+$ and $CD8^+$ T cell response was observed. Initially, tetramer$^+CD8^+$ T cells were positive for CD38 and failed to produce IFN-γ and eliminate the virus. Viral clearance was associated with a switch from the $CD38^+IFN\text{-}\gamma^-$ to a $CD38^-IFN\text{-}\gamma^+$ phenotype, which was temporally associated with a rise of the $CD4^+$ T cell proliferative response, whereas $CD4^+$ T cell responses in subjects who became chronically infected were short-lived or weak. This suggests that $CD4^+$ T cells contribute to viral clearance, possibly by inducing the required antiviral functions in $CD8^+$ T cells.

Similar results were reported from other longitudinal studies of acutely HCV^--infected patients using proliferation and/or IFN-γ ELISPOT assays for the assessment of $CD4^+$ T cell function [8–10]. In particular, some analyses confirmed the temporal association between viral elimination and vigorous virus-specific $CD4^+$ T cell responses [8,10]. The observation of a strong correlation between the strength and breadth of the HCV-specific $CD4^+$ T cell response and the specific cytotoxicity, proliferation and IFN-γ production of HCV-specific $CD8^+$ T lymphocytes provides further evidence for the importance of $CD4^+$ T cells in shaping the $CD8^+$ T lymphocyte response [9].

These findings also agree with the results of earlier longitudinal studies reporting a significantly higher frequency and broader specificity of $CD4^+$ T cell proliferative responses in patients who normalized ALT and cleared the virus than in those who did not [11–14]. While it has been suggested that $CD4^+$ T cell responses to specific epitopes provided protection against the development of chronic disease [11], studies examining a wider range of viral antigens generally found that the multispecificity of the response rather than the targeting of a specific epitope was an important determinant of the disease outcome [6,12,14].

Only transient clearance followed by HCV recurrence was observed in a patient in whom the initially strong $CD4^+$ T cell responses were lost [13]. These and similar results [15] suggest that permanent maintenance of the $CD4^+$ T cell response is required for the long-term control of HCV infection. This is further supported by the finding of vigorous and multispecific $CD4^+$ T cell responses in subjects who had spontaneously cleared HCV up to 20 years earlier [2].

Chronic HCV infection is associated with weaker proliferative responses to fewer antigens, and such a pattern was already apparent in patients with evolving chronicity during acute infection [16]. Cross-sectional studies confirmed that vigorous $CD4^+$ T cell responses were detectable in acutely infected patients, while significantly less frequent and narrower responses were found in chronically HCV-infected patients [10,17]. Interestingly, through the assessment of CD25 upregulation in response to stimulation with viral antigens, it was recently demonstrated that some patients with chronic hepatitis C had a significant number of HCV-specific $CD4^+$ T cells [10]. However, antigen-specific proliferation and IFN-γ production as measured by ELISPOT assay were essentially undetectable, whereas $CD4^+$ T cells from patients during the acute or recovery phase of HCV infection exhibited strong proliferation and IFN-γ secretion. This dysfunction was restricted to HCV-specific $CD4^+$ T cells, as demonstrated by normal proliferative responses to tetanus toxoid. Different patterns in the loss of proliferative and IFN-γ secretory responses suggested that anergy arose in some patients, while exhaustion of virus-specific T cells occurred in others.

Importantly, in a recent study with HCV-infected chimpanzees, the only animal model for HCV, depletion of $CD4^+$ T cells before reinfection of immune chimpanzees resulted in persistent, low-level viremia despite functional intrahepatic memory $CD8^+$ T cell responses. Incomplete control of HCV replication by memory $CD8^+$ T cells in the absence of adequate $CD4^+$ T cell help was associated with emergence of viral escape mutations in class I MHC-restricted epitopes and failure to resolve HCV infection [18]. This result directly demonstrated the role of $CD4^+$ T cell response in the protective T cell immunity against HCV. In the chimpanzee model, it was the hepatic, not the peripheral, $CD4^+$ T cell response that was significantly correlated with either sustained or transient viral clearance [19]. Unfortunately, little information is available on intrahepatic T cell responses during the acute phase of human HCV infection since liver biopsies are unethical at that stage. Of note, HCV titers were found to rise very early and rapidly during the incubation period and to outpace the T cell response [7], an observation that was also reported in HCV-infected chimpanzees [19]. Viral replication is considerably slower in HBV infection [20,21]. Hence, the kinetics of viral replication may contribute to the much higher rate of chronicity observed in HCV infection compared to HBV infection.

2.2. HBV

As in HCV infection, MHC class II genotype has been reported to influence the outcome of HBV infection, the most consistent protective effect being associated with HLA-DR13 (HLA DRB1*1301-01 and -02) [22–24]. As for the DQB1*0301 or DRB1*1101 allele in HCV, carriage of HLA-DR13 was associated with a stronger response to a greater number of peptides than seen in patients without HLA-DR13 [24]. These findings imply an enhanced ability of these HLA molecules to present HBV-derived antigens. Interestingly, however, patients with acute and chronic disease recognized the same immunodominant epitopes, and the only peptide preferentially recognized by $HLA\text{-}DR13^+$ individuals was not HLA-DR13-restricted in any of the clones examined [24]. This suggests that a polymorphism in linkage disequilibrium with the HLA-DR13 allele may be involved in the enhanced ability of HLA-DR13 carriers to clear HBV.

Further indications for the important role of $CD4^+$ T cells in HBV infection comes from the finding that transfer of HBV envelope-specific Th1 cells into HBV transgenic mice could induce liver damage and clear the virus [25]. In addition to a direct effector functions, antigen-specific $CD4^+$ T cells appear to be essential for the activation of virus-specific $CD8^+$ T cells, as suggested by the abrogation of certain virus-specific $CD8^+$ T cell functions after depletion of $CD4^+$ T cells from the incubation [26]. This is similar to the helper function of $CD4^+$ T cells in T cell immunity to HCV [18]. Of note, intracellular IFN-γ staining reveals the presence of HBV core-specific $CD4^+$ T cells in only one of 4 patients analyzed during the incubation and clinically acute phases of HBV infection, while envelope-specific $CD4^+$ T cells could not be detected in any of the patients, even though all of them were eventually able to eliminate the virus [27]. This appears to suggest that $CD4^+$ T cells play a less significant role in HBV infection than in HCV infection.

Nonetheless, as in HCV infection, there is considerable evidence that viral clearance in HBV infection is associated with a strong and multispecific $CD4^+$ T cell response, as assessed via proliferation and/or intracellular cytokine expression, to stimulation with viral antigens [26,28–33]. In contrast, $CD4^+$ T cell proliferation and cytokine expression in response to viral antigens are frequently undetectable and, if present, weaker and more narrowly focused in patients with chronic HBV infection [26,28,29,32,33]. Similarly, PBMC (but not $CD4^+$ T cell-depleted PBMC) and liver-infiltrating T cells from sustained responders to combined ribavarin and IFN-γ therapy for chronic hepatitis C exhibited more frequent and vigorous proliferative responses to core and envelope antigens than nonresponders [34].

$CD4^+$ T cell proliferation and cytokine production in response to HBcAg/HBeAg antigens was reported to be most intense during seroconversion to anti-HBe, an event that frequently correlates with the elimination of the virus and remission of liver disease, and to be lost after viral clearance [29]. Waning of the T cell response during or shortly after the recovery phase or after recovery from acute exacerbations has also been reported by other investigators [28,35]. Yet, others found strong proliferative responses to nucleocapsid antigens, but rarely to envelope antigens, in a majority (82%) of subjects who had had acute self-limited HBV infection up to 13 years earlier [31]. Similar persistence has been described for the HBV-specific $CD8^+$ T cell response [36,37]. Both groups of investigators showed that minute amounts of viral DNA remained detectable in serum and/or PBMC [31,36]. This suggests that, as in HCV infection, the HBV-specific memory T cells are maintained by the residue viral antigen expression and may be important for long-term control of the HBV infection.

3. $CD8^+$ T cells

The studies on the $CD8^+$ cytotoxic T cell response in T cell immunity against HBV and HCV have benefited greatly from the development of direct assays for epitope-specific $CD8^+$ T cells, including peptide-MHC tetramer assay, intracellular cytokine staining and ELISPOT (Table 1). Studies employing these single cell-based assays as well as the more conventional cytotoxicity-based assays in patients and chimpanzees have revealed a central role of virus-specific $CD8^+$ T cells in the control of HCV and HBV.

Table 1
Assays for virus-specific $CD8^+$ T cells

Assay	Quantitative	T cell function-based	T cell expansion-based
Tetramer	yes	no	no
Cytokine flow cytometry	yes	yes	no
ELISPOT	yes	yes	no
CTL assay	no	yes	yes

3.1. HCV

Of the limited number of subjects studied during the early phase of HCV infection, patients who cleared virus exhibited not only a vigorous $CD4^+$ T cell response, but also an early, pronounced and sustained increase in the frequency of virus-specific $CD8^+$ T cells, as assessed through the use of HLA-A2 tetrameric complexes [7,8]. This increase occurred at the same time as the onset of hepatitis, suggesting that these cells played a major role in the destruction of hepatocytes by immune-mediated mechanisms. Yet, there was only a moderate reduction in viral titers during acute hepatitis and the $tetramer^+$ T cells initially failed to produce IFN-γ. Viral elimination was not observed until these $CD8^+$ T cells started to secrete IFN-γ, apparently after receiving the appropriate signals from $CD4^+$ T cells. In another HLA-A2 positive subject who failed to clear the virus, only a minor and transient increase in virus-specific $CD8^+$ T cell responses could be detected via tetramer binding or intracellular IFN-γ staining. This essentially confirms the results of a study using an IFN-γ ELISPOT assay for the detection of HCV-specific $CD8^+$ T cells during acute HCV infection showing that patients with self-limiting disease had a significantly higher frequency of such cells than did patients in whom the infection became chronic [38]. Similarly, another tetramer-based analysis indicated that the percentage of virus-specific $CD8^+$ T cells was markedly increased in patients during the acute phase of HCV infection compared to chronically infected patients, but decreased to similar levels during recovery [39]. Of note, HCV-specific $CD8^+$ T cells were clearly

detectable at frequencies ranging between 0.01% and 1% of total $CD8^+$ T cells in the majority of chronically infected patients examined [40], which is in agreement with reports of vigorous and multispecific CTL responses in some patients with chronic HCV infection not only in PBMC, but also in liver-infiltrating lymphocytes [41–43]. However, these responses appear to be too weak to eliminate the virus but may be sufficient to cause CTL-mediated liver cell injury. Nonetheless, the detection of more frequent and vigorous CTL responses in patients with low titers of serum HCV RNA compared to those with high titers [8,44] suggests that these $CD8^+$ T cell responses play a role in limiting viremia even in chronic HCV infection.

Studies in chimpanzees also indicated that $CD8^+$ T cell response is required for protection against persistent HCV infection. Antibody-mediated depletion of $CD8^+$ T cells before reinfection of immune animal was found to result in prolonged virus replication despite the presence of memory $CD4^+$ T helper cells primed by the prior infections. The virus replication was not terminated until HCV-specific $CD8^+$ T cells recovered in the liver [45]. These results demonstrate an essential role for memory $CD8^+$ T cells in long-term protection from chronic hepatitis C.

Although there are reports of persisting $CD8^+$ T cell responses in subjects after viral elimination [2,8,9], the frequency of HCV-specific $CD8^+$ T cells has been shown to decline rapidly whether or not viral clearance occurred [46] [38]. These findings agree with the detection of a significantly lower frequency of $tetramer^+CD8^+$ T cells in the circulation of recovered, compared to chronically infected, patients or chimpanzees [9,19,40]. In another study, the percentage of HCV-specific $CD8^+$ T cells staining positive for intracellular IFN-γ was shown to be higher in chronically infected than in recovered subjects, but the difference failed to reach statistical significance [15]. In that study, peptide-stimulated $CD8^+$ T cell lines from patients with chronic HCV infection exhibited significantly stronger and more frequent cytolytic activity compared to those obtained from recovered subjects.

In contrast, virus-specific $CD8^+$ T cells were detected via IFN-γ ELISPOT at low frequencies in subjects who had had acute self-limiting HCV up to 20 years earlier, but not in subjects with chronic infection [2]. After in vitro expansion, $CD8^+$ T cells from 11 of 12 of recovered subjects, but only 1 of 6 chronically infected patients, showed evidence of a significant CTL response to stimulation with HCV peptides. This cytotoxic activity was attributable to the DR^- T cell population, indicating that no recent antigen contact had occurred. Indeed, HCV RNA could not be detected by nested PCR in 10 of the 12 recovered individuals. Similarly, the phenotype of virus-specific $CD8^+$ T cells from a patient recovered from HCV infection was found to be $CD45RO^+RA^-$ with high levels of expression of CD27 and CD28, but low levels of HLA-DR, i.e., a resting memory phenotype [9]. In contrast, $tetramer^+CD8^+$ T cells from patients with chronic hepatitis C showed more frequent expression of both CD27 and HLA-DR compared to HCV-nonspecific cells [47]. Interestingly, the specific phenotype of $tetramer^+$ T cells varied not only between patients but also between specificities within the same patient. Others also noted considerable variation in the phenotype of peripheral HCV-specific $CD8^+$ T cells, with $CD45RO^+$ $CD45RA^-CD27^+$ predominating in two patients and $CD45RO^-CD45RA^+$ with infrequent expression of CD27 in another [40]. Expression of CD69 was detected on <10% of peripheral, but on all liver-infiltrating, virus-specific CD8 T cells.

In a recent investigation of 7 patients with acute HCV infection, virus-specific CD8 T cells were found to be impaired in their capacity for expansion, CTL activity, IFN-γ production and intracellular perforin content [48]. Improvement in all CD8 functions was seen in three HCV-infected patients with self-limiting disease, whereas these functions remained impaired in four other patients with a chronic disease outcome. The same functional impairments were observed in $tetramer^+$ HCV-specific CD8 T cells from patients with chronic HCV, but not in those from recovered patients [9]. A transient failure to secrete IFN-γ that subsided as viremia declined was also reported in another patient who was eventually able to clear the virus [8]. In contrast, other investigators found the reduction in the secretion of IFN-γ and TNF-α of $tetramer^+$ T cells after stimulation with virus-specific peptides or mitogens to be sustained regardless of whether the patients ultimately cleared the virus or developed chronic hepatitis [49]. Functional impairments of HCV-specific $CD8^+$ T cells may at least