Review Article

Mitochondrial antigens and antibodies in primary biliary cirrhosis

Patrice Butler, Francesca Valle and Andrew K. Burroughs

Hepato-biliary and Liver Transplantation Unit, University Department of Medicine, Royal Free Hospital and School of Medicine, Pond Street, Hampstead, London NW3 2QG, UK

Introduction

Primary biliary cirrhosis (PBC) is considered to be an autoimmune chronic liver disease. It is characterized by progressive inflammatory destruction of intrahepatic bile ducts which results in cholestasis and which may progress to cirrhosis and liver failure. It is a disease which occurs in females in 90% of cases. The diagnosis is most commonly made in middle aged women who present with itching and usually with jaundice. However, less severe cases are being diagnosed because of cholestatic liver function tests or the presence of anti-mitochondrial antibodies (AMAs) in the serum. The latter are the most characteristic feature of PBC.¹ These AMAs are non-organ- and non-species-specific and are of both the IgG and IgM classes. They were first detected in PBC patients in 1965 by Walker *et al.*² by indirect immunofluorescence tests on cryostat sections. To date, 9 different AMA types (anti-M1 to anti-M9) have been defined, using immunofluorescence and complement fixation tests (CFTs), enzyme linked immunosorbent assay (ELISA) and Western blotting.^{3–5} Four of these AMAs, anti-M2, -M4, -M8 and -M9 are associated with PBC. The others anti-M1, -M3, -M5, -M6 and -M7 are mainly associated with non-hepatic disorders.⁶ The past 4 years have seen a great expansion in publications related to the immunology of PBC due to the identification of the major M2 antigens and the use of molecular biology techniques. These give good prospects for a better understanding of the pathogenesis of the disease.

M2 – a family of autoantigens

Approximately 95% of PBC sera react with a trypsin-sensitive inner mitochondrial membrane

antigen designated M2.⁷ Early experiments using chloroform extraction showed that the M2 antigen co-purified with the F1 part of the mitochondrial H⁺-ATPase,^{8,9} but further studies using immunoblotting techniques failed to demonstrate reactivity of anti-M2 antibodies with any of the major subunits of the F1-ATPase.¹⁰ M2 analysis by Western blotting has demonstrated at least 5 antigenic bands (M2a to M2e) with approximate molecular weights 80–68 kD (M2a), 64–60 kD (M2b), 56–50 kD (M2c), 48–43 kD (M2d) and 36 kD (M2e).^{11,12} There is some confusion as to the exact molecular weights of eight of the M2 bands due to differences between laboratories with regard to species of mitochondria used, antigen preparation and techniques of detection. Recent identification of the antigens should allow pure preparations to be used in immunoblotting experiments. This would eliminate the apparent discrepancies.

In 1987 Gershwin *et al.* identified a cDNA clone isolated from a rat liver cDNA library which expressed the 70 kD M2 autoantigen.¹³ This led to the identification of the 3 major M2 antigens as the E2 components (dihydrolipoamide acetyltransferases) of the three mitochondrial 2-oxo-acid dehydrogenase multienzyme complexes: pyruvate dehydrogenase complex (PDC), 2-oxoglutarate dehydrogenase complex (OGDC) and the branched chain 2-oxo-acid dehydrogenase complex (BCOADC).^{14,15} A fourth antigen was then identified which is designated protein X.¹⁶ This was found to possess cross-reactive AMA epitopes with the E2 of PDC.^{16,17}

Each multienzyme complex consists of multiple copies of three enzymes E1, E2 and E3. The E2 component forms a central symmetrical core around which are arranged multiple copies of E1 and E3. The major immunodominant region on each of these enzymes and protein X is the lipoic acid binding site which possesses a covalently attached lysine residue.^{18,19} Up to three lipoyl moieties can be present as a part of the functional

site of the enzyme. Human PDC-E2 contains 3 autoreactive determinants, 2 cross-reactive lipoyl domains and an area surrounding the E1/E3 binding region, the dominant epitope being present on the inner lipoyl domain.²⁰ The presence of a lipoyl residue is vital for antibody recognition. Fussey *et al.* have demonstrated that unlipoylated E2, where lysine was replaced by a glutamine residue was no longer recognized by PBC antibodies. However, if octanoic acid was added to the critical lysine residue, antibody binding properties were restored because octanoic acid can mimic the unique peptide cofactor conformation.²¹

Recently, Fussey *et al.*²² have shown that 41% of PBC sera react with the non-lipoic acid-containing E1 alpha component (41 kD) of mammalian PDC, and 7% react with the E1 beta component (36 kD). Fregeau *et al.*²³ have shown that 66% of PBC beta react with the E1 alpha component and 2% react with the E1 beta component. Interestingly, the autoantibody specific for E1 alpha (a non-lipoic acid-containing enzyme) was able to inhibit PDC complex activity. The other AMAs have also been shown to inhibit their respective enzyme complexes – this is not too surprising as the immunoepitopes are important functional sites.^{24,25}

The specificity of antimitochondrial antibodies in 211 PBC patients has been examined: 209 (99%) were found to have antibodies against one or more autoantigens, the majority 203 (96%) having antibodies to PDC and protein X.¹⁶ Fregeau *et al.*²⁶ have shown that 10% (19/188) of PBC sera reacted to OGDC-E2 when probed against bovine heart mitochondria. However, when these sera were probed against purified OGDC-E2 an additional 54 sera (29%) exhibited weak reactivity. This implies that an increased sensitivity is obtained when pure enzyme preparations are used. Overall, this group demonstrated that 39% of PBC sera reacted with OGDC-E2. This is lower than the 72% of PBC sera that reacted with OGDC-E2 as described by Fussey *et al.*,¹⁵ and is another source of heterogeneity of results in this field. Are the different prevalences of antibodies due to sampling of different populations or due to different techniques of identification?

Van de Water *et al.*²⁷ have developed an enzyme linked immunosorbent assay (ELISA) using recombinant cloned antigens to quantify specific antimitochondrial antibodies in patients with PBC. This method has also been successfully used by Heseltine *et al.*²⁸ However, in contrast to the cloned antigens used by Gershwin *et al.* they used the purified PDH complex obtained through successive gel filtration steps. ELISA is a highly sensitive and quantitative method for detection of AMA. It should eventually substitute immunofluorescence as the standard method for detection of mitochondrial autoantibodies in clinical practice.

Anti-M4, -M8 and -M9 autoantibodies

The finding of anti-M4 antibody in PBC was originally thought to indicate an overlap disease between PBC and chronic active hepatitis as this 'mixed form' condition was usually associated with the presence of anti-M2 and anti-M4 AMA on immunofluorescence.²⁹ Whilst some PBC patients exhibit both M2 and M4 reactivity on immunofluorescence, some sera may show only the M4 pattern. A study carried out using sera positive for AMA on immunofluorescence but negative when reacted with M2 rich preparations on immunoblots showed that these sera reacted with a single antigen (MW 50–52 kD) – M4.³⁰ In this study, reactivity with the 52 kD antigen alone was constant, irrespective of disease stage, whereas the occurrence of anti-M2 and anti-M4 in sera increased with disease progression. The M4 antigen is trypsin insensitive and probably located on the outer mitochondrial membrane.

Anti-M8 antibody is only found in association with anti-M2. However, not all anti-M2 patients have anti-M8. A serial study has shown that the presence of anti-M8 when detected with anti-M2 and anti-M4 may characterize a subgroup of patients with progressive disease activity.³¹

Anti-M9 antibody is predominantly present in patients with asymptomatic and early PBC. It can occur in patients who have been clinically and histologically defined as having PBC but who are M2-negative. Interestingly, it can also occur in healthy controls exposed to PBC, i.e. relatives and laboratory workers. When purified M9 antigen (isolated from rat liver mitochondria) is subjected to electrophoresis and Western blotting, anti-M9 antibodies detect 2 antigenic determinants at molecular weights of 98 and 59 kD.³²

There is dispute as to whether the type and titre of mitochondrial antibodies have prognostic value in PBC.³³ However, Weber *et al.* have recently suggested that AMA profiles are predictors of prognosis. They found that the presence of anti-M8 with anti-M2 (Profile II) and anti-M4 with anti-M8 and anti-M2 (Profile III) is associated with a more advanced histological stage at the time of diagnosis and a more progressive course than in patients with anti-M2 alone (Profile I). After following these patients for up to 16 years it was found that only 24% of patients with Profile I had reached stage III or IV (fibrosis or cirrhosis of the liver) while 75% of patients with Profile II or III had a progression to stage III or IV.³¹ Klein *et al.* have shown that AMA profiles determined at early stages of PBC differentiate between a benign and a progressive course of the disease.³⁴ This finding, if confirmed, should be of great importance in the design of therapeutic trials, clinical monitoring and decision making regarding liver transplantation. At present the M4,

M8 and M9 (and M3, M5, M6 and M7) remain uncharacterized.

The role mitochondrial antibodies play, if any, in the pathogenesis of PBC is still unclear. However, Heseltine *et al.* have recently shown that the titre of immunoglobulin G autoantibodies to purified PDC-E2 and X increases significantly not only with histological progression of disease but also with established prognostic 'markers' i.e. higher serum bilirubin and lower serum albumin levels.²⁸

Peptide specific and cross-reactive M2 autoantibodies

As all of the M2 antigens identified so far except for E1 possess at least one lipoyl domain, it is possible that each of these antigens may possess cross reactive epitopes. Cross-reactivity between PDC-E2 and protein X has been confirmed by several groups working in the field. However, there have been conflicting reports on the cross-reactivity of the other M2 antigens. Surh *et al.*¹⁶ have shown that AMA cross-reactivity is limited to PDC-E2 and protein X, as reactivity with OGDC and BCOADC was not affected by absorption with recombinant PDC-E2. In addition, Fregeau *et al.*²⁶ have demonstrated that affinity purified antisera from the OGDC-E2 did not react on immunoblot with any component enzymes of PDC or BCOADC. Fussey *et al.*³⁵ have demonstrated that neither PDC-E2 nor protein X antibodies recognize OGDC-E2, BCOADC-E2 or PDC-E1 alpha/beta. However, in 12 of 12 PBC sera, antibodies eluted from either OGDC-E2 or BCOADC-E2 showed complete cross-reactivity with each other. Eluted AMA from PDC-E1 alpha/beta showed complete specificity for each polypeptide.

In contrast, our group have demonstrated unequivocal cross-reactivity between each of the major M2 antigens using affinity purified antibodies from mitochondrial proteins immobilized on nitrocellulose blots.³⁶ This has been independently confirmed by Fusconi *et al.*³⁷ using the same techniques. They have demonstrated that both cross-reactive and peptide specific antibodies are present in PBC sera. They have shown three epitopes to be present on both PDC-E2 and protein X, two epitopes on the BCOADC-E2 and three on the OGDC-E2: one of these epitopes was common to all bands.

We have suggested that there are both peptide-specific and cross-reactive AMA. Thus, different sera may have different proportions of antibodies of these two kinds. Relative affinities of AMAs for antigens may be an important factor in explaining apparent discrepancies in results. One group has shown a considerable heterogeneity of relative affinities of AMA in PBC.³⁸

The conformation of epitopes on the lipoic acid binding site may be another factor in determining their antigenicity. Our group have shown that neither lipoate nor lipoamide react with PBC sera using direct and competitive enzyme linked immunosorbent assays.³⁶ Fussey *et al.*¹⁹ have shown that preincubation of PBC sera with 1 mM-lipoate, 1 mM-lipoamide alone or in combination failed to absorb out the reactivity of PBC sera against PDC-E2 in immunoblotting experiments. Surh *et al.*³⁹ have demonstrated that a minimum of 75 amino acids is required to detect autoantibody binding on the inner lipoyl domain of PDH-E2, and that 93 amino acids are required for strong AMA activity. Because such a large region is required for reactivity, it is thought, and it is probable, that a conformational epitope is important in autoreactivity. Leung *et al.*⁴⁰ have designed mutant structures to replace the lysine residue in the lipoyl domain. They used glutamine, a negatively charged amino acid; histidine, a positively charged amino acid; and tyrosine, an aromatic amino acid. In contrast to the findings by Fussey *et al.*,²¹ they found that a variation in the charge distribution at the lipoyl domain of PDC-E2 and the removal of lipoic acid by the change of a single amino acid did not significantly alter antibody binding at the lipoyl domain, i.e. the reactivity is not influenced by the presence of lipoic acid. They suggest that PBC autoantibodies are recognizing a specific amino acid sequence and its associated secondary or even tertiary structure.

Injection of purified recombinant dihydrolipoamide acetyltransferase into animals generates mitochondrial autoantibodies, but their specificity is not the same as in PBC patients, as there is no cross-reactivity with protein X, or the outer lipoyl domain and no enzyme inhibition *in vitro*.^{41,42}

Antimitochondrial activity with prokaryotes

It is known that the AMAs of PBC patients react with certain bacterial proteins. Cross-reactivity has been demonstrated between PBC sera and a range of microorganisms, *Paracoccus denitrificans*,⁴³ *E. coli*, *Klebsiella pneumoniae*,^{36,44} *Proteus mirabilis*, *Citrobacter freundii*, *Staphylococcus aureus*³⁶ and Enterobacteriaceae.⁴⁵ This is hardly surprising considering that bacterial PDC-E2 exhibits a high degree of homology with its mammalian counterparts, particularly at the highly conserved region around the lysyl binding site for lipoate. *E. coli* PDC-E2 contains three lipoyl domains,⁴⁶ reactivity being confined to the inner regions. However, there are differences in the organization of the trans-acetylase, with the mammalian E2 existing as a core of 60 copies in an icosahedral symmetry, and the bacterial one as 24 copies in an octahedral array.

PBC sera have been shown not to react with bacterial PDC-E1. The protein sequence analysis of human E1 shows no similarity with any sequence deduced from *E. coli* PDC-E1.²²

Reactivity of PBC serum with polypeptides from *E. coli* was initially described by Lindenborn-Fotinos *et al.*¹⁰ and confirmed by Frazer *et al.*⁴⁷ by means of immunoblot techniques. Reactivity on immunoblots is with a 70–80 kD and a 52 kD membrane fraction. It has been shown that antibodies against both determinants can be absorbed with beef heart submitochondrial particles, showing that *E. coli* shares cross-reacting determinants with mitochondria. We have shown that by eluting antibodies from these bacterial bands on immunoblots and reacting them back with mitochondria unequivocal cross-reactivity can be obtained.³⁶ Stemerowicz *et al.*⁴⁵ have demonstrated that AMA production can be induced in rabbits by intravenously injecting them with rough forms of laboratory strains of *Salmonella minnesota*. Rabbit antisera raised against *Salmonella minnesota* Rb and Rc mutants recognized the PBC specific M2 antigens 74 kD and 52 kD. However, antisera against wild-type Enterobacteriaceae did not react with M2.

Fussey *et al.*²¹ have characterized the main immunogenic region in *E. coli*. They have shown that, in addition to recognizing *E. coli* E2-PDC, PBC antibodies also recognize *E. coli* E2 OGDC, the main immunogenic region lying within the lipoyl domains. The BCOADC is not present in *E. coli*.

Bacteriuria and PBC

We have found that women with PBC are much more susceptible to recurrent significant bacteriuria than women with other types of chronic liver disease.⁴⁸ In our population of PBC patients, significant bacteriuria defined a specific sub-group of PBC patients with an increased risk of death.⁴⁹

In light of this we decided to look at a group of patients suffering from recurrent significant bacteriuria with no clinical or biochemical evidence of chronic liver disease and on long term prophylactic antibiotic treatment. We found that 69% of these patients exhibited weak AMA reactivity on immunoblots, reactivity with the 74 kD M2 fraction being the most common. When antibody to the 74 kD was affinity purified it was found to cross-react with bacterial membrane fractions.^{50,51}

We have been studying the relationships between significant bacteriuria, R mutants and M2 antibodies in PBC. We have found a high percentage (40%) of rough forms of bacteria in infected urine obtained from PBC and recurrent urinary tract infection patients with no evidence of liver disease⁵²

but not in women with non-PBC chronic liver disease. Rough forms of bacteria have defective polysaccharide synthesis leading to an abnormal outer coat. Hopf *et al.*⁵³ have shown that *E. coli* R forms constituted an average of 5% (range 1–50%) of the total amount of *E. coli* present in the stool samples from 21/21 PBC patients.⁵³ This is low compared to the percentage we found in infected urine of PBC patients considering that in the majority of cases organisms causing urinary infections originate in the gut. This implies that there might be 'selection' of the rough mutant bacteria causing urinary infections. Deposits of lipid A which is a component of the bacterial cell wall were found by Hopf *et al.*⁵³ in the cytoplasm of hepatocytes in 11/21 of the PBC patients mentioned above but not in the liver sections of patients with chronic viral hepatitis.⁵³ These lipid A containing hepatocytes were located around the central veins and not the portal tracts. This would imply that bacteria did not arrive via the portal system from the gut but via the arterial blood supply. When one takes into account that many urinary tract infections in PBC are associated with the upper urinary tract,⁵⁴ haematogenous spread to the liver is a possibility.

Thus we hypothesize that M2 autoantibodies may be an immune response to rough forms of urinary bacteria, as microbial peptides may become more easily exposed due to their defective outer coat and act as antigens. Perhaps the paracrystalline arrangement of the E2 core may render it especially antigenic as it resembles a viral structure. This hypothetical sequence of events may be specific to urinary infection, as *E. coli* antibodies are found very frequently in chronic liver disease,⁵⁵ but AMA are specific to PBC.

PBC – the autoimmune disease

The chronic liver damage in PBC is thought to be mediated by T lymphocytes infiltrating the biliary epithelium either by cytotoxic activity or by releasing lymphokines that attract bystander T cells. Major histocompatibility complex (MHC) class I antigens HLA A, B and C are cell surface glycoproteins normally found on all nucleated cells. MHC class II antigens HLA DP, DQ and DR are usually found on a limited number of cells including macrophages, B lymphocytes, dendritic cells and vascular endothelial cells: they are important in antigen presentation. MHC class II expression is a prerequisite of antigen recognition by class II restricted CD4 positive T lymphocytes. Normal bile duct epithelium possesses MHC class I but not class II antigens. However, several groups have now demonstrated MHC class II expression on bile duct epithelium in PBC. In 1984 Ballardini *et al.*⁵⁶

showed MHC class II expression in 8/10 PBC liver biopsies. In 1986 Pisi *et al.*⁵⁷ demonstrated HLA-DR antigen expression in 50% of bile ducts in early PBC and in 100% in late stage PBC. Gores *et al.*⁵⁸ carried out a study of 114 PBC patients and found that those possessing the HLA-DRw8 antigen were at an increased risk of death compared to those possessing the HLA-DR5 antigen.

However, almost all cells can be induced to express class II antigens by cytokines, in particular interferon gamma,⁵⁹ which is also a trigger for intercellular adhesion molecule-1 (ICAM-1) which is essential for many immunological functions.⁶⁰ MHC class II expression has also been shown in rejection following liver transplantation. In hepatic graft-versus-host disease the intrahepatic bile ducts express HLA-DR antigens after bone marrow transplantation.^{61,62} Focal HLA-DR expression on bile duct epithelium has also been reported in large bile duct obstruction and in liver tissue adjacent to metastatic carcinoma as well as in cases of intrahepatic cholestasis, granulomatous hepatitis, autoimmune hepatitis, cryptogenic cirrhosis and alcoholic hepatitis.^{63–65} Thus HLA II expression is cytokine mediated and this in turn can be due to a variety of causes.

The CD3 +, CD4 +, CD8 – T lymphocyte is the predominant cell type surrounding the portal tracts in late stage PBC.^{66,67} Other studies show that CD8 + T lymphocyte is predominant^{68–70} but one of the studies showed an increased tendency toward CD4 + in late stage disease.⁷⁰ Even though T cells are the predominant cell type infiltrating the portal tracts in PBC it is still not clear whether these cells are actually causing the damage.

In autoimmune diseases like PBC it is unknown how intracellular antigens (i.e. mitochondrial PDC-E2) present themselves to the immune system. Studies on surface expression of antigenicity have demonstrated that PBC sera can react at the surface of teased out hepatocytes and intact hepatoma cells.⁷² However, these studies have been criticized as it was possible that the cells studied did not retain their integrity, thus exposing intracellular components.⁷³ Another possibility is that intracellular antigens may be released during natural cell death and turnover, enabling them to bind to MHC molecules and be presented on the surface of the cell (i.e. biliary epithelial cell or hepatocyte). CD4 + T lymphocytes can then recognize this MHC-antigen complex and can thus provide help for autoimmune B cells with a corresponding antigen activity. There is also evidence that MHC molecules may normally present self-peptides in their binding site;⁷⁴ these peptides are probably intracellular proteins. It is not known if PDC-E2 or other mitochondrial antigens are exposed in this fashion. Other studies have shown that cytochrome P-450 isoenzymes present in the

cytoplasm are recognized by specific antibodies (anti-LKM 1 and 2) as some epitopes are localized on the outer surface,⁷⁵ these antibodies characterize specific subtypes of autoimmune chronic active hepatitis.

A further hypothesis is that T lymphocytes are stimulated by E3 components on bacterial cell membranes. These T lymphocytes recognize cross-reacting determinants on biliary epithelial cell (BEC) plasma membranes as these are thought to possess E3. Bile duct destruction ensues, E2 becomes exposed and in association with MHC II can then be recognized by CD4 + T lymphocytes.¹⁶

Molecular mimicry and PBC

We think antigenic mimicry between foreign and self antigen may be an important factor in the induction of M2 autoantibodies in PBC. Molecular mimicry has been indicated as a possible mechanism for autoimmunity in some diseases. In ankylosing spondylitis (AS) approximately 90% of patients are known to possess HLA B27. This HLA subtype cross-reacts with antigens found in *Klebsiella* which can be isolated in faecal cultures obtained from AS patients during active phases of disease.⁷⁶ In reactive arthritis, *Salmonella* antigens (O-polysaccharide of bacterial lipopolysaccharide) have been found in joint material from patients with *Salmonella*-triggered reactive arthritis. In an animal model, the lipid A part of LPS is responsible for inducing arthritis. Processed LPS is very effective at stimulating lymphocytes which in turn leads to the production of cytokines and hence inflammation.⁷⁷ We hypothesize that cytokine induction and MHC II expression in PBC may be a response to infections with R forms of bacteria. In *Yersinia*-triggered reactive arthritis, non-viable structures of *Yersinia enterocolitica* O:3 have been shown at the site of inflammation within mononuclear cells in the synovial membrane of 80% of patients.⁷⁸ Specific anti-*Proteus* antibodies have been shown to be present in active rheumatoid arthritis. Anti-HLA-DR4 tissue typing sera have been shown to bind to *Proteus* microorganisms.⁷⁹

In myasthenia gravis the circulating antibodies directed against the nicotinic acetylcholine receptor are pathogenetic. Monoclonal antibodies raised against this receptor were found to bind to outer membrane proteins from *E. coli*, *Klebsiella pneumoniae* and *Proteus vulgaris*.⁸⁰ Recently it has been shown that sera from PBC patients possess antibodies against the alpha and probably the beta and gamma subunits of nicotinic acetylcholine receptor^{81,82} yet no patient had any clinical or electromyographic evidence of myasthenia gravis. This would indicate that 'host response' is important in the generation of autoimmune disease. In PBC,

mitochondrial antibodies could be pathogenetic if the host immune environment facilitates this. The recent great strides in our knowledge of the mitochondrial antigens outlined in this review should facilitate study of the interactions between antigens and lymphocytes, and hopefully map out the pathogenesis and progression of PBC, deter-

mining whether anti-mitochondrial antibodies cause disease in PBC.

Note added in proof: Klein, R. and Berg, P.A. have recently identified the epitope reacting with anti-M9 as to be part of glycogen phosphorylase (*Clin Exp Immunol* 1990, **81**: 65–71) and anti-M4 reacting with sulphite oxidase (*Clin Exp Immunol* 1991, **84**: 445–448).

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