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TITLE

MOLECULAR MIMICRY IN PRIMARY BILIARY CIRRHOSIS AND OTHER HEPATIC AND EXTRA-HEPATIC DISEASES

by

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Abstract

This Thesis investigates the concept that molecular mimicry may be instrumental to the appearance and/or maintenance of liver autoimmunity, with a main focus on primary biliary cirrhosis (PBC).

The first part of the study concentrates on the investigation of immunological cross-reactivity between pyruvate dehydrogenase complex E2 $(PDC\text{-}E2)_{212\text{-}226}$, the immunodominant mitochondrial autoepitope in PBC and microbial mimics. Several bacterial and viral sequences were identified that share extensive homology with $PDC\text{-}E2_{212\text{-}226}$ and while some from mycobacteria, *lactobacillus* and *E. coli*, acted as target of cross-reactivity, similarly good mimicking sequences from other micro-organisms were unreactive. The possible bearing on the mechanism of the disease of this selective cross-reactivity is discussed.

Next is the identification of cross-reactive immune responses between hepatitis C virus (HCV) polyprotein and cytochrome P450IID6 $(CYP2D6)_{252\text{-}271}$, the major autoepitope of anti-liver kidney microsomal type-1 antibody (LKM1), the serological hallmark of autoimmune hepatitis type-2. Viral/self cross-reactivity was documented only in those patients possessing the HLA B51 allele. The evolution of immunological cross-reactivity was investigated over a 10-year period in a girl who developed primary and secondary LKM1 response following HCV infection resulting in a florid autoimmune hepatitis 9 years later. A study in a cohort of subjects vaccinated against hepatitis B provided the opportunity to document the appearance of cross-reactivity between viral sequences and mimics on myelin antigens.

The findings of the present Thesis demonstrate that disease-specific microbial/self cross-reactive responses do occur and may be of pathogenic significance.

To my Ithaca

To my family

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List of Abbreviations

Aa	amino acid
Ab	Antibody
ADCC	Antibody-dependent cell-mediated cytotoxicity
ADV	Adenovirus
Ag	Antigen
AIH	Autoimmune hepatitis
AITD	Autoimmune thyroid disease
ALD	Alcoholic liver disease
ALT	Alanine aminotransferase
AMA	Anti-mitochondrial antibody
ANA	Anti-nuclear antibody
APC	Antigen presenting cells
ASC	Autoimmune sclerosing cholangitis
AST	Aspartate aminotransferase
BCOADC	Branched-chain oxo acid dehydrogenase complex
BEC	Biliary epithelial cells
BGAL	β galactosidase
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
CD	Chron's disease
Clp	Caseinolytic protease
CMV	cytomegalovirus
cpm	Counts per minute
CTL	Cytotoxic T lymphocyte
CYP1A2	Cytochrome P450IA2
CYP2D6	Cytochrome P450IID6
DAB	Diaminobenzidine
DCDA	Diaminopimelate decarboxylase
DMSO	Dimethyl sulphoxidase
DNA	Deoxyribonucleic acid
E1	Envelope 1
E3BP	E3 binding protein
EAE	Experimental autoimmune encephalomyelitis
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
ECOLI	*Escherichia coli*
EDTA	Ethylenediaminetetra-acetate
ELISA	Enzyme-labeled immunosorbent assay
FACS	Fluorescence activated cell sorter
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
h	Hour

HAEIN	*Haemophilus influenzae*
HBc	Hepatitis B core antigen
HBV	Hepatitis B virus
HBvacc	Hepatitis B vaccine
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HELHEP	*Helicobacter hepaticus*
HELPY	*Helicobacter pylori*
HLA	Human leucocyte antigens
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
HSK	Herpes simplex keratitis
Hsp	Heat shock protein
HSV1	Herpes simplex virus type 1
ICP4	Infected cell protein-4
Id	Identification
IFNγ	Interferon-γ
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHL	Intrahepatic lymphocytes
IIF	Indirect immunofluorescence
IL	Interleukin
IU	International unit
LACDE	*Lactobacillus delbrueckii*
LKM1	Liver kidney microsomal type-1
MALE	Maltose E
MBP	Myelin basic protein
mg	Milligram
MHC	Major histocompatibility complex
MHCIITA	MHC class II *trans*-activator
min	Minutes
ml	Millilitre
mm	millimetre
MMDB	Molecular modeling database
MND	Multiple nuclear dot
MOG	Myelin oligodendrocyte glycoprotein
MW	Molecular weight
MYCGO	*Mycobacterium gordonae*
NC	Nitrucellulose
NKT	Natural killer T-cells
NPC	Nuclear pore complex
NPCP	Nuclear pore complex protein

NS5B	Non-structural 5B
NT	Natural T-cells
NuMa	Nuclear mitotic apparatus
nv	Normal value
OADC	Oxo-acid dehydrogenase complex
OD	Optical density
OGDC	Oxoglutarate dehydrogenase complex
OLT	Orthotopic liver transplantation
OPD	*o*-Phenylenediamine dihydrochloride
PAGE	polyacrylamide gel electrophoresis
PBC	Primary biliary cirrhosis
PBMC	Peripheral bllod mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDC	Pyruvate dehydrogenase complex
PE	Phycoerythrin
PM-scl	Polyomyositis sclerosis antigen
PSEAE	*Pseudomonas aerigunosa*
PSI	Position specific iterative
rAI	Relative affinity index
RNA	Ribonucleic acid
rpm	Revolutions per minute
RU	Relative unit
rUTI	Recurrent urinary tract infection
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SHBsAg	Small Hepatitis B surface antigen
SI	Stimulation index
SMA	Smooth muscle antibody
TCR	T-cell receptor
Th	T-cell helper
TNF	Tumor necrosis factor
TRMA	tRNA-methyltransferase
UDCA	Ursodeoxycholic acid
UK	United Kingdom
UREA	Urease A
UREB	Urease B
UTI	Urinary tract infection
v	Volume
V	volt
w	Weight

PUBLICATIONS

The following papers have been published reporting studies of which this Thesis consists:

1. BAUM H, BOGDANOS DP, VERGANI D. (2001) Antibodies to Clp protease in primary biliary cirrhosis: possible role of a mimicking T-cell epitope (Letter). *J Hepatol* 34:785-7
2. WEN L, MA Y, BOGDANOS DP, WONG FS, DEMAINE A, MIELI-VERGANI G, VERGANI D. (2001) Pediatric autoimmune liver diseases: the molecular basis of humoral and cellular immunity (Review). *Curr Mol Med* 1:379-89
3. BOGDANOS DP, CHOUDHURI K, VERGANI D. (2001) Molecular mimicry and autoimmune liver disease: virtuous intentions, malign consequences (Review). *Liver* 21:225-32.
4. BOGDANOS DP, BAUM H, SHARMA UC, GRASSO A, MA Y, BURROUGHS AK, VERGANI D. (2002) Antibodies against homologous microbial caseinolytic proteases P characterise primary biliary cirrhosis (Original). *J Hepatol* 36:14-21
5. VERGANI D, CHOUDHURI K, BOGDANOS DP, MIELI-VERGANI G. (2002) Pathogenesis of autoimmune hepatitis (Review). *Clin Liver Dis* 6:439-49
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hepatitis C virus infected patients is associated with the possession of HLA B51 (Original). *Int J Immunopathol Pharmacol* 17:83-92

9. VERGANI D, BOGDANOS DP, BAUM H. (2004) Unusual suspects in primary biliary cirrhosis (Editorial). *Hepatology* 39:38-41
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The following papers have been submitted for publication reporting studies related to this Thesis:

1. BOGDANOS DP, BAUM H, GUNSAR F, LOPES R, LONGHI MS, MA Y, BURROUGHS AK, VERGANI D. A microbial mimic of the dominant T-cell autoepitope accounts for primary biliary cirrhosis-specific antibodies to a non-mimicking microbial antigen.
2. BOGDANOS DP, BAUM H, OKAMOTO M, MONTALTO P, SHARMA UC, VLACHOGIANNAKOS J, MA Y, BURROUGHS AK, VERGANI D. Immunoglobulin G3 antibodies to a lactobacillus mimic cross-reactive with the major immunodominant mitochondrial epitope are characteristic of primary biliary cirrhosis.
3. BOGDANOS DP, MA Y, HADZIC N, LONGHI MS, PORTMANN B, MIELI-VERGANI G, VERGANI D. Virus-self cross-reactivity leading to *de novo* autoimmune hepatitis nine-years after liver transplantation.

The following papers have been published reporting studies unrelated to this Thesis, but carried out during the period of the PhD of the candidate (05/2001 – 07/2004).

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CHAPTER 1

Introduction

1.1 The lymphoid liver

Classically, lymphoid organs were considered to be those tissues with exclusively immunological functions, for example, the thymus, lymph nodes and spleen (Miller and Davies, 1964). This definition has recently been expanded to include organs whose primary function is not immunological but which clearly require dedicated and often elaborate immunological mechanisms to mediate their functions. The gut and the uterus, for example, fall into this category; the liver is also regarded as a lymphoid organ with unique immunological properties (Head, 1996, Abreu-Martin and Targan, 1996, Doherty and O'Farrelly, 2000). Because of its location and function, the liver is continuously exposed to a large antigenic load that includes pathogens, toxins, tumor cells, dietary and self-antigens. The range of local immune mechanisms required to cope with this diverse immunological challenge has been the focus of ongoing studies. The liver has an 'epithelial constitution' and contains large numbers of phagocytic cells, antigen presenting cells (APC) and lymphocytes and is a site for the production of cytokines, complement components and acute phase proteins (Doherty and O'Farrelly, 2000).

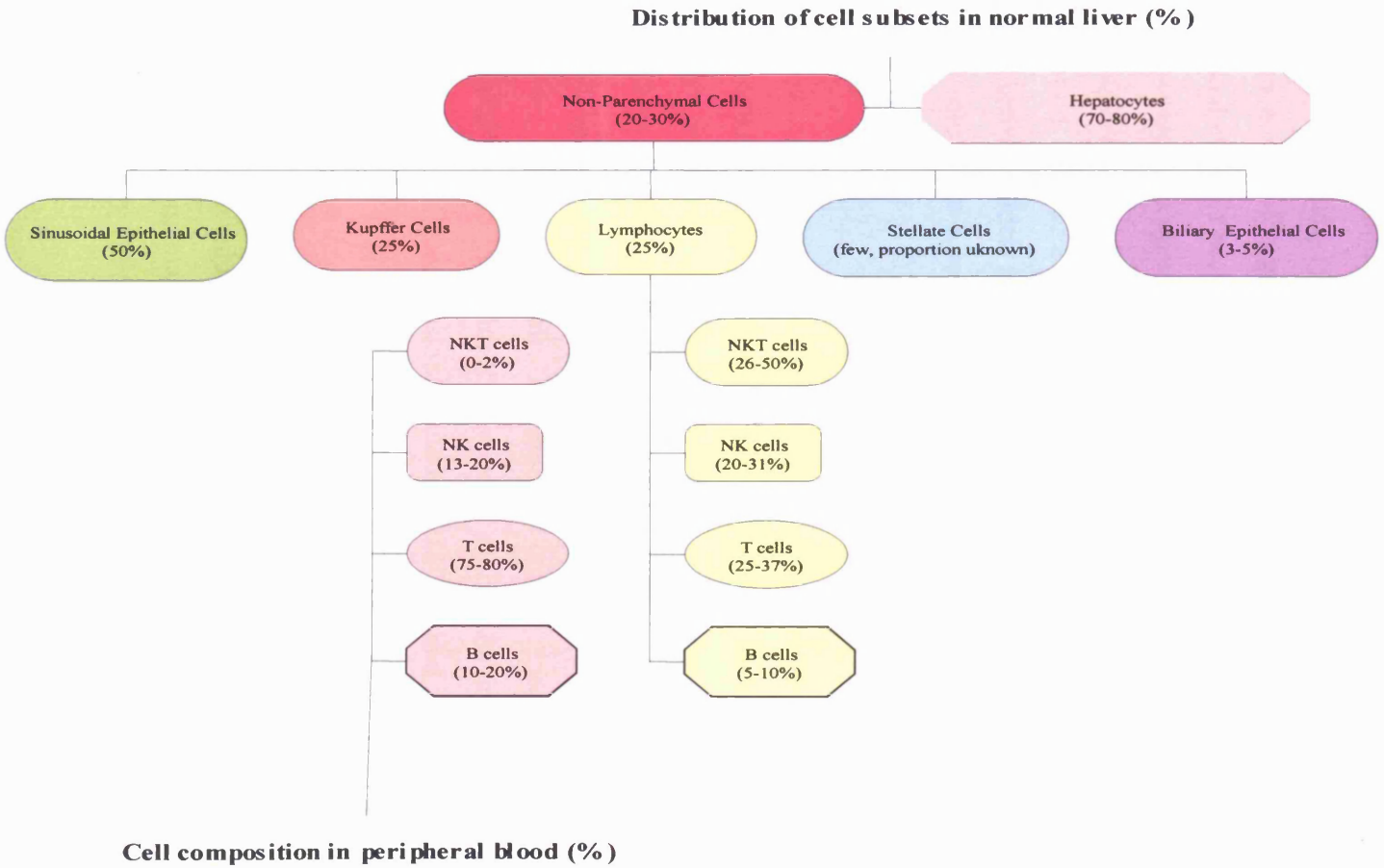
In the normal liver, the hepatocytes comprise 70% of the total number of cells. The remaining 30% cells are mostly concerned with immunological functions and include lymphocytes, sinusoidal endothelial cells, Kupffer cells, biliary epithelial cells and hepatic stellate cells (Hata *et al.*, 1990, Norris *et al.*, 1998, Norris *et al.*, 1999, Doherty *et al.*, 1999, Doherty and O'Farrelly, 2000). Cell numbers and proportions are shown in Figure 1.1.

The normal human liver has an intrinsic lymphocyte population mainly resident in the portal tract but also scattered throughout the parenchyma. One to two million of these lymphoid cells are retrievable from 200 mg of normal liver tissue, indicating that an average human liver of 1.5 kg contains approximately 1×10^{10} lymphocytes. Intrahepatic lymphocytes (IHL) consist of natural killer T (NKT) lymphocytes (50% of the IHL), T-cells (25% of IHL), natural killer (NK) cells (20%) and B lymphocytes (5%) (Figure 1.1). The relative proportions among IHL of different classes of lymphocytes differ widely from those in the peripheral blood where up to 80% are T-cells, 20% B-cells, 13% NK-cells, and 0.2-2% are NKT-cells (Hata *et al.*, 1990, Norris

et al., 1998, Norris *et al.*, 1999, Doherty *et al.*, 1999, Doherty and O'Farrelly, 2000, Mehal *et al.*, 2001).

The relative frequencies of lymphocyte subpopulations can also vary dramatically from individual to individual. This is likely to reflect differences in each individual's immunological status that would be influenced by factors such as genetic background, history of infections, and current antigenic exposure. Ongoing immune challenges to the liver would presumably affect the relative proportions of resident hepatic lymphocytes and infiltrating circulating lymphocytes that are present in the liver (Crispe, 2003, Kita *et al.*, 2001).

The liver is an organ in which blood from the intestines, rich in bacterial and dietary antigens intermingles with the circulating lymphocytes. The constitutive presence of antigenic material imposes constraints on immune responses that are generated in the liver and distinctive control mechanisms have to determine whether antigen encounter will result in immunological un-responsiveness (tolerance) (Crispe, 2003, Kita *et al.*, 2001). It is obvious that liver autoimmunity implies loss of self-tolerance, and the question arises as to how this happens. An insight to the basic characteristics of liver tolerance comes from a better understanding of aspects of immunological tolerance in general (Crispe, 2003).



Percentage proportions of different cell types in the normal liver and comparison with lymphocyte subsets in blood. NKT, natural killer T-cells; NK, natural killer (see 1.1) adopted from Mackay (2002) and modified according to Hata et al. (1990), Norris et al. (1998, 1999), Doherty and O'Farrely (2000)

1.2 Immunological tolerance and the liver

Early in the history of experimental transplantation, immunologists were surprised to discover that in many species, allogeneic liver grafts could be established and maintained with minimal immunosuppression, while skin, kidney and other allografts were rejected rapidly (Calne *et al.*, 1969, Calne, 2000). Reasons to explain why the liver is often a site of immune tolerance have not been fully clarified, but it has been suggested that it is because the liver is a site where harmless food antigens from the gut are processed and presented to the immune system (Crispe, 2003). However, the liver is also subjected to invasion by pathogens that breach the intestinal mucosa and invade the circulation (Calne, 2000). Immune tolerance towards such invaders would not be advantageous, which indicates that liver lymphocytes must be able to switch rapidly from a tolerant to a responsive state (Cantor and Dumont, 1967, Calne, 2000, Crispe, 2003).

Tolerance is the controlled inability to respond to antigens to which an individual has the potential for response (Kamradt and Mitchison, 2001). Self-tolerance refers to lack of responsiveness to an individual's antigens, and obviously it underlies our ability to live in harmony with our own cells and tissues (Miller and Basten, 1996, Kamradt and Mitchison, 2001). Several mechanisms, albeit not well understood, have been postulated to explain the tolerant state. Tolerance is antigen specific and is achieved through deletion of lymphocytes (clonal deletion) or their inactivation (clonal anergy) or through a mechanism of suppression/regulation (Miller and Basten, 1996, Kamradt and Mitchison, 2001). Clonal deletion is prominent in T lymphocyte tolerance (Akkaraju *et al.*, 1997, Ferber *et al.*, 1994, Critchfield *et al.*, 1994) whilst anergy is the main mechanism of B lymphocyte tolerance (Goodnow *et al.*, 1988).
Tolerance may be induced centrally, in the primary lymphoid organs (thymus for the T-cell tolerance, bone marrow for B-cells) or in the periphery (Miller and Morahan, 1992, Miller and Basten, 1996, Kamradt and Mitchison, 2001, Mathis and Benoist, 2004).

1.2.1 Central tolerance

In order to establish a complete immune repertoire, thymus and bone marrow generate potentially reactive T and B lymphocytes by a process referred as positive selection (Miller and Basten, 1996, Kamradt and Mitchison, 2001). The next step involves clonal

deletion of self-reactive T and B lymphocytes during their maturation in the central lymphoid organs. Clonal deletion of developing intrathymic T-cells has been extensively investigated. It is proposed that many autologous protein antigens are processed and presented by thymic antigen-presenting cells in association with self Major Histocompatibility Complex (MHC) molecules (Miller and Basten, 1996, Kamradt and Mitchison, 2001). The developing T-cells that express high-affinity receptors for such self-antigens are negatively selected, or deleted, and therefore the peripheral T-cell pool is lacking or deficient in self-reactive cells. As with T-cells, clonal deletion is also operative for B-cells (Kamradt and Mitchison, 2001). When developing B-cells encounter a membrane-bound antigen within the bone marrow, they undergo apoptosis. Clonal deletion of self-reactive lymphocytes, however, is far from perfect. Although this process eliminates a significant fraction of autoreactive lymhocytes, other potentially self reactive, naïve cells (not subject to the thymic or bone marrow negative selection process) escape and are found in the periphery. These lymphocytes 'evade' negative selection, either because their affinity to the 'self' determinants in the thymus is too low or their specific antigens are not expressed in the thymus (Miller and Basten, 1996, Kamradt and Mitchison, 2001). There is similar 'slippage' in the B-cell system as well. T and B-cells that bear receptors for a variety of self-antigens, can be found in the peripheral blood of healthy individuals (Kitze *et al.*, 1988, Naquet *et al.*, 1988, Kamradt and Mitchison, 2001).

1.2.2 Peripheral tolerance

Those self-reactive lymphocytes that escape negative selection can inflict tissue injury unless they are deleted or muzzled in the peripheral tissues (Miller and Morahan, 1992). Several 'back-up' mechanisms that silence such potentially autoreactive lymphocytes are known to exist, the major being ignorance, anergy and suppression/regulation (Miller and Morahan, 1992).

Ignorance relates to prevention of contact between autoreactive lymphocytes and self-antigens (Barker and Billingham, 1977, Butcher and Picker, 1996, Kamradt and Mitchison, 2001).

Anergy refers to prolonged or irreversible functional inactivation of lymphocytes, induced by encounter with antigens under certain conditions (Quill, 1996, Delves and Roitt, 2000a). Activation of antigen-specific T-cells requires two signals: recognition of peptide antigen in association with self MHC molecules on the surface of APC and a set

of second costimulatory signals provided by APC (Quill, 1996, Delves and Roitt, 2000b). To initiate second signals, certain T-cell-associated molecules must bind to their ligands on the APC (Lenschow *et al.*, 1996, Grewal and Flavell, 1998, Holter *et al.*, 1996). If the antigen is presented by cells that do not bear these ligands, a negative signal is delivered, and the cell becomes anergic (Lenschow *et al.*, 1996, Grewal and Flavell, 1998, Holter *et al.*, 1996, Kamradt and Mitchison, 2001). Such a cell then fails to be activated even if the relevant antigen is presented by competent APC (e.g., macrophages, dendritic cells) that can deliver costimulation (Kamradt and Mitchison, 2001). Because costimulatory molecules are not expressed or are weakly expressed on most tissues, the encounter between autoreactive lymphocytes and their specific self-antigens leads to clonal anergy. Clonal anergy therefore affects autoreactive lymphocytes not only in the thymus but also in the tissues (Miller and Morahan, 1992). It is believed that if B-cells encounter an antigen in the absence of specific helper T-cells, the antigen-receptor complex is down-regulated, and such cells never re-express their immunoglobulin receptors (Kamradt and Mitchison, 2001). Understandably, such cells are unable to respond to subsequent antigenic stimulation. In addition to antigen-induced loss of surface immunoglobulin receptors, other mechanisms of B-cell anergy are also postulated to exist (Rathmell *et al.*, 1996, Nemazee, 2000).

Although clonal deletion and anergy are the primary mechanisms of self-tolerance, it is believed that additional 'fail-safe' mechanisms must also exist. Much renewed interest is focused on regulatory T-cells with the ability to down-regulate the function of other autoreactive T-cells (Shevach, 2000). The molecular mechanisms by which suppressor T-cells recognize antigens and exert their suppressive effects have remained elusive (Shevach, 2000). There is some evidence that peripheral suppression of autoreactivity may be mediated, in part, by the regulated secretion of cytokines such as interleukin (IL)-10, transforming growth factor-β and IL-4 (Powrie *et al.*, 1996, Seddon and Mason, 1999). The behaviour and possible participation of regulatory T-cells in human autoimmune liver disease is the focus of ongoing studies (Longhi *et al.*, 2004).

1.3 Breakdown of tolerance: mechanisms of autoimmunity

Although it would be attractive to explain all autoimmune diseases by a single mechanism, it is now clear that several different pathological processes could break tolerance, thus terminating a previously unresponsive state to autoantigens (Ohashi and DeFranco, 2002). More than one defect may be present in each disease, and the defects vary from one disorder to the other. It has been argued that the multilayered nature of self-tolerance is a 'fail-safe' mechanism: all or several control mechanisms must be infringed before disease develops (Ohashi and DeFranco, 2002). This approach may well explain several important principles regarding autoimmune disease: (1) it is usually multifactorial involving immunologic, genetic, and environmental factors interacting through complicated mechanisms that are incompletely understood (Ohashi and DeFranco, 2002); (2) it often has a slower progress than immune reactions to pathogens, suggesting that the control mechanism may continue to work up to a point; (3) it has a tendency to remit and relapse, indicating that control mechanisms may recover and at least temporarily restore tolerance (Kamradt and Mitchison, 2001, Ohashi and DeFranco, 2002).

The initiating mechanisms in autoimmunity can best be discussed in terms of those discussed for tolerance (Kamradt and Mitchison, 2001). While there is no convincing evidence of breakdown of central tolerance as a cause of autoimmunity, several mechanisms exist whereby failure of peripheral tolerance may contribute to the pathogenesis of autoimmune diseases (Kamradt and Mitchison, 2001, Ohashi and DeFranco, 2002). Most of these mechanisms have been deduced from *in vitro* work or animal model studies, since the evolution of human autoimmune disease can rarely be investigated from the onset (Kamradt and Mitchison, 2001, Ohashi and DeFranco, 2002). It appears that tolerance to self may be disrupted by several mechanisms such as: failure of T-cell anergy, activation-induced cell death and/or T-cell mediated suppression; polyclonal lymphocyte activation; release of sequestered antigens; exposure of 'hidden' or 'cryptic' self antigens; 'epitope spreading'; 'aberrant' expression of class II MHC molecules; cytokine influence; and finally microbial/self molecular mimicry.

1.3.1 Failure of T-cell anergy

Potentially autoreactive T-cells that escape clonal deletion are usually rendered anergic when they encounter self-antigens expressed on costimulator-deficient APC in the tissues (Kamradt and Mitchison, 2001, Ohashi and DeFranco, 2002). It follows that such T-cell anergy may be broken if these APC can be induced to express costimulatory molecules and to secrete cytokines that stimulate the generation of T helper cells type 1 (Th1) (Mueller *et al.*, 1989, Quill, 1996, Kamradt and Mitchison, 2001, Ohashi and DeFranco, 2002). Such induction may occur after infections with resultant tissue necrosis and local inflammation (Tough *et al.*, 1997, Kamradt and Mitchison, 2001, Ohashi and DeFranco, 2002). Breakdown of B-cell anergy is also possible (Goodnow *et al.*, 1991).

1.3.2 Failure of activation-induced cell death

Persistent activation of potentially autoreactive lymphocytes may lead to their apoptosis (Refaeli *et al.*, 1998, Rathmell and Thompson, 2002). It follows therefore that defects in apoptotic pathways may allow persistence and proliferation of autoreactive lymphocytes in the peripheral tissues (Strasser *et al.*, 1991, Martinon and Tschopp, 2004, Opferman and Korsmeyer, 2003).

1.3.3 Failure of T-cell mediated suppression

The hypothesis that loss of regulatory or suppressor T-cells that can limit the function of autoreactive cells can lead to autoimmunity is quite attractive (Strasser *et al.*, 1991). This notion has been difficult to prove, however, in large part because of the difficulty in the isolation and identification of antigen-specific regulatory T-cells.

1.3.4 Polyclonal lymphocyte activation

Autoimmunity may occur if self-reactive but anergic clones are stimulated by antigen-independent mechanisms. Several microorganisms and their products are capable of causing polyclonal (i.e., antigen nonspecific) activation of B-cells (Primi *et al.*, 1977, Hang *et al.*, 1985). Bacterial lipopolysaccharide (endotoxin), for example, can stimulate the production of anti-DNA, anti-thymocyte and anti-red cell antibodies *in vitro* (Murakami *et al.*, 1997). Other bacterial products can also bind to and activate a large pool of CD4 T-cells in an antigen-independent manner. They do so by binding to class II MHC molecules on antigen-presenting cells and the beta chains of the T-cell receptor

(TCR), outside the antigen-binding groove (Dellabona *et al.*, 1990). Because they stimulate all T-cells that express a certain set or family of the Vbeta TCR, they are called 'superantigens' (Herman *et al.*, 1991). It has been proposed that among the T-cells activated by superantigens, some may be reactive to self-antigens, leading to the induction of autoimmunity and indeed autoimmune disease, as it is for example the case for multiple sclerosis (Brocke *et al.*, 1993).

1.3.5 Release of sequestered antigens

Any self-antigen that is completely sequestered during development is likely to be viewed as foreign if introduced into the circulation (Kamradt and Mitchison, 2001). The mere release of these antigens is not sufficient to cause autoimmunity; the inflammation associated with the tissue injury is essential for up-regulation of costimulatory pathways that are critical for the induction of an immune response as it is the case of post-traumatic uveitis where an immune response is directed to sequestered ocular antigens (Caspi, 1999). Infections have been proposed to account for the release of sequestered autoantigens through tissue damage and the induction of inflammatory cytokines and costimulatory molecules (Horwitz *et al.*, 1998, Kamradt and Mitchison, 2001)

1.3.6 Exposure of 'hidden' or 'cryptic' self antigens and 'epitope spreading'

It is believed that a large number of intracellular self-determinants are not readily recognized by the immune system, and hence T-cells specific for such 'cryptic' self-epitopes are not deleted (Lipham *et al.*, 1991, Sercarz *et al.*, 1993, Markovic-Plese *et al.*, 1995). It follows that such T-cells could cause autoimmune diseases if the cryptic epitopes are somehow presented to them in an immunogenic form (Sercarz *et al.*, 1993). Several factors could be responsible for rendering an epitope cryptic, including the available amount of the determinant itself, its ability to be processed by APC, and the availability of costimulatory factors (Sercarz *et al.*, 1993). It has long being proposed that regardless of the initial trigger of an autoimmune response, the progression of the autoimmune response is maintained by recruitment of autoreactive T-cells that recognize normally cryptic self-determinants (Sercarz *et al.*, 1993). The induction of such autoreactive T-cells is sometimes referred to as epitope spreading because the immune response 'spreads' to include determinants within the same molecule (intra-molecular) or other molecules (inter-molecular) that were initially not recognized (Lehmann *et al.*, 1992). There is compelling evidence for the role of epitope spreading

in the pathogenesis of a murine model of multiple sclerosis, type 1 diabetes, and systemic lupus erythematosus (SLE) (Lehmann *et al.*, 1998, McCluskey *et al.*, 1998, Zechel *et al.*, 1998, Miller *et al.*, 1997).

1.3.7 'Aberrant' expression of class II MHC molecules

Class II expression is the medium for communication between CD4 helper T lymphocytes and APC (Delves and Roitt, 2000a). Since this step is critical in initiating an immune response, class II expression is restricted to immune-cells such as professional APC and B lymphocytes (Delves and Roitt, 2000a). 'Aberrant' or 'inappropriate' appearance of class II MHC molecules on cells that do not normally express them may be sufficient to incite an autoimmune response against that particular cell by CD4 T-cell lymphocytes as it has been shown, for example, on hepatocytes in autoimmune hepatitis and biliary epithelial cells in primary biliary cirrhosis (Ballardini *et al.*, 1984, Lobo-Yeo *et al.*, 1990).

1.3.8 Cytokine influence

Cytokines are soluble factors with pleiotropic effects that act both locally and systemically on cells of the immune system (Delves and Roitt, 2000b). Their role in the immune system is the recruitment of immunocompetent cells and modulation of their functions. In respect to their role in the induction of autoimmunity, cytokines can induce class II and enhance class I MHC expression on target cells that could eventually lead indirectly to the induction of autoimmune processes (bystander activation) (Cope, 1998, Hill and Sarvetnick, 2002, Tough *et al.*, 1996). Other possible mechanisms include cytokine-mediated breakdown of immunological tolerance and a direct cytotoxic effect on target cells (Hill and Sarvetnick, 2002).

1.3.9 Molecular mimicry between microbial and self proteins

The concept of molecular mimicry, as it will be discussed in detail, is based on the observation that some infectious agents share antigenic determinants (epitopes) with self-antigens (Oldstone, 1987, Oldstone, 1998). An immune response against such microbes may then turn against the cross-reacting self-antigen initiating an autoimmune cascade that leads to host cell destruction and eventually to autoimmune disease (Oldstone, 1987, Oldstone, 1998).

1.4 Breakdown of liver tolerance

Various mechanisms have been proposed to account for the initiation of an autoimmune liver response with no single initiating event being able to account for all instances of autoimmunity (Kita *et al.*, 2001). Two general conditions, however, must prevail: self reactive B and T lymphocytes must exist in the immunological repertoire and autoantigens must be presented in conjunction with MHC class II molecules by APC (Kita *et al.*, 2001, Mackay, 2002). The presence of lymphocytes in the liver is commonly considered to be associated with disease pathogenesis (Hata *et al.*, 1990, Doherty and O'Farrelly, 2000, Mackay, 2002). Lymphocyte infiltration is well documented in inflammatory conditions such as autoimmune and viral liver diseases (Knolle and Gerken, 2000). The liver harbours lymphoid cells that subserve innate and adaptive immune responses and, like peripheral lymphoid tissues, is a site where naive T-cells can be activated by local antigen (Hata *et al.*, 1990, Norris *et al.*, 1998, Norris *et al.*, 1999, Doherty *et al.*, 1999, Doherty and O'Farrelly, 2000, Mehal *et al.*, 2001). Hepatocytes or cholangiocytes can carry antigens perceived as foreign, and for which neither of the appropriate options, elimination or complete tolerance, has been achieved (Reynoso-Paz *et al.*, 1999, Kita *et al.*, 2001). Such antigens may be derivatives of hepatotropic viruses, drugs or xenobiotics, or the host itself, as it is the case in autoimmune liver diseases (Reynoso-Paz *et al.*, 1999, Kita *et al.*, 2001).

1.4.1 Infections and liver tolerance

Viral or other microbial infection of the liver induces an immune response to clear the pathogen, and the competence of the immune response determines whether chronic disease will develop or, as usually happens, clearance will occur (Knolle and Gerken, 2000). Hepatotropic viruses are generally non-cytopathic, and the host immune response is responsible for both viral clearance and the associated inflammatory cell injury (Chisari and Ferrari, 1995, Cerny and Chisari, 1999, Bertoletti and Ferrari, 2003). A misdirected immune response to hepatocellular or biliary ductular constituents released in the course of such injury is a postulated pathway for liver autoimmunity (Reynoso-Paz *et al.*, 1999, Kita *et al.*, 2001).

1.4.2 Drugs and liver tolerance

The liver is the major site of drug metabolism. Drugs and their metabolites can be toxic if their degradation pathways are defective (Bissell *et al.*, 2001, Liu and Kaplowitz, 2002). Thus, intracellular antigens may be released from the damaged cells (Robin *et al.*, 1997). Presentation of protein adducts may eventually lead to an immune response, including the production of autoantibodies, inflammation and hepatocyte necrosis (Robin *et al.*, 1997, Liu and Kaplowitz, 2002). In addition, the covalent binding of a drug metabolite to its enzyme may results in the modification of this enzyme and in the appearance of antibodies that recognise the alkylated form as a neo-antigen (Robin *et al.*, 1997, Liu and Kaplowitz, 2002).

1.4.3 Apoptosis and liver tolerance

Apoptosis, as the basis for programmed cell death, is responsible for the normal regular turnover of hepatocytes and the elimination of liver cells in inflammatory pathologies (Patel, 2000, Kita *et al.*, 2001, Crispe, 2003). Although less obvious in biliary epithelium, apoptosis is also present in autoimmune cholangiopathies (Reynoso-Paz *et al.*, 1999). Aside from hepatocytes and their disposal, apoptosis is relevant to the breakdown and/or maintanance of liver tolerance (Patel, 2000, Kita *et al.*, 2001, Crispe, 2003). First, death by apoptosis allows for non-inflammatory elimination of cell components in contrast to necrosis which is pro-inflammatory and potentially autoantigenic (Mackay, 2002). However, if apoptosis is overwhelming, apoptotic fragments can induce an autoimmune response and indeed autoimmune disease as it has been seen in the case of SLE (Cocca *et al.*, 2002). Second, apoptosis is a mechanism used for the removal of autoreactive T and B lymphocytes, as it is illustrated by lymphoproliferative diseases due to deficiency of apoptotic genes (Vaishnaw *et al.*, 1999).

1.4.4 Autoimmune liver diseases

Autoimmune liver diseases represent a broad spectrum of disorders that can affect one or the other of the two anatomical components, in particular the hepatocytes in autoimmune hepatitis (AIH) types 1 and 2 and *de novo* AIH following transplantation and the cholangiocytes and bile ducts in primary biliary cirrhosis (PBC), and sclerosing cholangitis (SC) (Mackay, 2002). AIH-1 is characterised by seropositivity for antibodies to nuclear (ANA) and/or smooth muscle antibodies (SMA) and AIH type 2

for anti-liver kidney microsomal type 1 antibodies (LKM1) (Alvarez *et al.*, 1999). The serology of SC is similar to that of AIH-1 (Gregorio *et al.*, 1997, Gregorio *et al.*, 2001). The serological hallmark of PBC is the presence of high-titre anti-mitochondrial antibodies (AMA) (Neuberger, 1997). AIH and PBC represent the major autoimmune liver diseases; their main clinical, serological, and immunological characteristics as well as the current knowledge of their pathogenesis will be described later in detail.

1.5 Virus, liver and autoimmunity

Several viruses home to the liver (hepatotropic viruses) and cause hepatitis (Mackay, 2002). The liver can also be affected by viruses that primarily target other tissues such as herpes simplex virus (HSV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), and measles virus (Mackay, 2002). Among hepatotropic viruses, hepatitis B and C viruses (HBV, HCV) can take up permanent residence and cause serious chronic disease (Chisari and Ferrari, 1995, Cerny and Chisari, 1999). Hepatitis viruses have been found to induce aberrant autoantibodies which in the majority are non-organ specific and usually similar to those observed in AIH (Bogdanos *et al.*, 2000a, Obermayer-Straub and Manns, 2001). Autoimmunity is therefore a common feature of HBV and HCV infection with over 50% of the infected patients being positive for autoantibodies against nuclear antigens (ANA) and smooth muscle antigens (SMA) (Gregorio *et al.*, 1996, Gregorio *et al.*, 1998, Lenzi *et al.*, 1999, Abuaf *et al.*, 1993, Cassani *et al.*, 1997, Clifford *et al.*, 1995, Bortolotti *et al.*, 1996, Muratori *et al.*, 2003b). As discussed later, LKM1 antibody is confined to patients with chronic HCV infection being found in up to 10% of them while it is absent in chronic HBV infection (Lunel *et al.*, 1992, Bortolotti *et al.*, 1996, Gregorio *et al.*, 1998, Cassani *et al.*, 1997, Nishioka *et al.*, 1997, Lenzi *et al.*, 1999). Immunopathological manifestations in HBV and especially in HCV infection are not limited to autoantibody seropositivity but embrace the full spectrum of textbook autoimmune disorders, ranging from non-organ specific cryoglobulinaemia to the exquisitely organ specific autoimmune thyroiditis and include such conditions as rheumatoid arthritis, Sjögren's syndrome, glomerulonephritis, lichen planus, polyarteritis nodosa, and type 1 diabetes (Czaja, 1997, Mehta *et al.*, 2001). The mechanisms leading to immunopathological manifestations in viral hepatitis differ. Thus in cryoglobulinaemia from HCV and glomerulonephritis from HBV formation of immune complexes is involved in the pathological damage. Hepatitis C and B viral

constituents are integral components of the immune complexes and interferon-(IFN)-α induced control of the viral infection is associated with an improvement in cryoglobulinaemia and glomerulonephritis (Czaja, 1997, Mehta *et al.*, 2001, Bogdanos *et al.*, 2000a, Obermayer-Straub and Manns, 2001). Interferon is also involved at the other end of the spectrum, but this time in unmasking or inducing *de novo* autoimmune thyroiditis (Dusheiko, 1997, Marcellin *et al.*, 1995). IFN-α may favour the development of Th1 immune reaction and thereby contribute to the development of autoimmune disease (Foster, 1997). In addition, IFN-α inhibits the apoptosis of autoreactive B-cells leading to the overproduction of autoantibodies (Su and David, 1999).

That viruses and other microbial agents have the potential to trigger autoimmune responses ultimately resulting in autoimmune disease is an accepted notion indirectly supported by epidemiological studies (Kurtzke, 1993, Gamble, 1980), geographic distribution (Ebers and Sadovnick, 1993), migration surveys (Anand *et al.*, 1996) and concordance studies between monozygotic and dizygotic twins (Ebers *et al.*, 1986, Eastmond and Woodrow, 1977, James, 1996). Attempts to establish a direct epidemiological association between viral infections and autoimmune disorders have been hampered by the fact that, frequently, patients have experienced multiple viral infections by the time their disease is diagnosed (von Herrath, 2000). Moreover, immunologically mediated damage may occur years after the causative viral infection has been cleared by the host immune response, a 'hit and run' event, so that no viral footprints are detectable in the affected organ, thus removing our ability to identify the link (Oldstone, 1987, von Herrath, 2000). Type 1 diabetes, for example, is 100 times more frequent in individuals who had congenital rubella virus infection, compared to non-infected children (Menser *et al.*, 1978). However, pancreatic β-cell destruction may become evident several years -up to the forth decade of life- after the exposure to the virus (Menser *et al.*, 1978, von Herrath, 2000).

The mechanism by which viruses could induce autoimmunity is under investigation (von Herrath *et al.*, 2003, Wucherpfennig, 2001). First, viruses can selectively infect specialised target cells, such as the pancreatic β-cells or the hepatocytes, leading directly to their destruction (von Herrath, 2000, von Herrath *et al.*, 2003, Wucherpfennig, 2001). In the case that all the cells are destroyed by a viral infection, the disease that occurs cannot be considered autoimmune in nature. This may happen in certain cases of hyperacute type 1 diabetes or in the case of fulminant hepatic failure related to viral hepatitis (von Herrath, 2000, von Herrath *et al.*, 2003, Wucherpfennig, 2001). On the

other hand, if the viral infection destroys some but not all the target cells, disease may not result until years later (von Herrath, 2000, von Herrath *et al.*, 2003, Wucherpfennig, 2001).

Another scenario implicates activation of the target cells that leads to the secretion of immune mediators, among them cytokines such as IFN-γ, tumor necrosis factor-α (TNF-α) or production of mediators such as nitric oxide that harm the surrounding cells (Wucherpfennig, 2001). This 'bystander' destruction of the cells could accompany inflammatory reactions in the liver (Knolle and Gerken, 2000, Diehl, 2000). The third possibility by which a virus can induce autoimmunity is molecular mimicry (Oldstone, 1987, Oldstone, 1998, Wucherpfennig, 2001, von Herrath *et al.*, 2003).

1.6 Viruses, molecular mimicry and autoimmunity

The hypothesis of molecular mimicry is based on the concept that an immunogenic determinant of an exogenous agent, such as a virus, incites formation of antibodies or effector T-cells, which in turn, react with homologous epitopes on a host protein (Oldstone, 1987, Oldstone, 1998). The sharing of a linear amino acid sequence or a conformation fit between a virus and a host 'self' determinant is the initial step of this process (Oldstone, 1987, Oldstone, 1998). Autoimmunity provoked by molecular mimicry should occur only when the viral and host determinants are similar enough to cross-react, yet different enough to break immunological tolerance (Oldstone, 1987, Oldstone, 1998). Antibodies or cytotoxic lymphocytes may then cross-react with a self-protein, thereby causing cellular injury leading to cell destruction. Once the infectious agent initiates this process, it needs not to be present during the autoimmune destruction that follows. In this situation, the microbial agent may have been cleared, but elements of the immune response mounted against it would lead to tissue injury that, in turn, would release more self antigens, thereby inducing more antibodies, and so on (Oldstone, 1987, Oldstone, 1998). Therefore, although viruses may initiate disease, the likehood of their recovery from tissue sites is small (Oldstone, 1987, Oldstone, 1998, von Herrath, 2000).

There are numerous examples of antigenic determinants shared between viruses, bacteria or parasites, and host cell proteins (Oldstone, 1987, Oldstone, 1998, von Herrath, 2000). In an early study, more than 3% of over 600 monoclonal antibodies raised against different viral proteins, cross-reacted with normal tissue antigens

(Srinivasappa *et al.*, 1986). Conversely, antibodies to self proteins such as hormones, cells of the nervous system or lymphocyte subsets may cross-react with viral polypeptides (Oldstone, 1987, Oldstone, 1998, von Herrath, 2000). The ability to find cross-reactive T-cells or antibodies which can recognise self-epitopes does not necessarily mean an autoimmune disease will develop (Oldstone, 1987, Oldstone, 1998, von Herrath, 2000). As it has been mentioned (*see* 1.2), regulation or suppression of self-reactive immune responses occurs when most of us encounter foreign antigenic mimics. This would explain why, although we are all commonly exposed to microbial mimics throughout our lives, autoimmunity is a relatively rare event. The transition from cross-reactive immune responses to autoimmune disease depends on numerous intrinsic and extrinsic factors such as the ability of host MHC molecules to bind and present antigens, the likelihood of a peptide being processed for binding to the MHC, the presence in the periphery of B or T-cells specific for self epitopes having escaped deletion, and the level of cytokines induced after an immune recognition event.

A long-standing paradigm in support of the molecular mimicry hypothesis is that proposed in the case of acute rheumatic fever (Cunningham *et al.*, 1989, Cunningham, 2000, Galvin *et al.*, 2002). Rheumatic fever involves autoimmune-mediated myocardial pathology that can arise following infection with group A *streptococcus* (Cunningham, 2000). At the molecular level, streptococcal surface M proteins share an amino acid sequence with cardiac myosin (Cunningham *et al.*, 1989, Cunningham, 2000, Galvin *et al.*, 2002). Serum antibodies from patients with acute rheumatic fever as well as monoclonal antibodies directed against a peptide of the streptococcal M protein cross-react with high-molecular weight cardiac and skeletal myosin (Cunningham *et al.*, 1989, Cunningham, 2000, Galvin *et al.*, 2002). Conversely, a monoclonal antibody raised to ventricular myosin binds the streptococcal M protein (Cunningham *et al.*, 1989, Cunningham, 2000, Galvin *et al.*, 2002). Help for pathogenic autoantibody development is provided by T-cell determinants that are stimulated by both streptococcal M peptides and mimics of cardiac myosin (Cunningham *et al.*, 1989, Cunningham, 2000, Galvin *et al.*, 2002). Multiple exposures to this pathogen in the susceptible host leads to progressive valvular damage mediated by cross-reactive antibodies (Cunningham *et al.*, 1989, Cunningham, 2000, Galvin *et al.*, 2002).

The initial pathogenic insult does not need to be closely temporally related to the manifestation of autoimmune disease. This is well illustrated in Chagas' disease caused

by the *Trypanosoma cruzi* (Kierszenbaum, 1999). Acute infection with *T. cruzi* causes a transient myocarditis which resolves completely. Decades later, an autoimmune myocarditis, histologically characterised by mononuclear cell infiltration, in the absence of detectable parasites, develops in the susceptible host, leading eventually to dilated cardiomyopathy (Kierszenbaum, 1999). Molecular mimicry between the B13 antigen of *T. cruzi* and cardiac myocytes is thought to be responsible for the development of the autoimmune response (Cunha-Neto *et al.*, 1996). This system, illustrates the 'hit and run' model for the development of autoimmunity following infection (Oldstone, 1987, Oldstone, 1998, von Herrath, 2000).

Another striking example of molecular mimicry is a murine model of herpes simplex keratitis (HSK), a T-cell–mediated inflammatory disease of the cornea that is induced by local application of HSV (Zhao *et al.*, 1998). In humans, HSV1-induced destruction of corneal tissue represents a leading cause of blindness (Streilein *et al.*, 1997). In the mouse model, keratogenic T-cell clones produced following corneal application of the virus cross-react with a peptide from the HSV1 UL6 protein (Zhao *et al.*, 1998) . Viruses with a mutated UL6 gene or with a single amino acid substitution at the UL6 T-cell epitope are greatly impaired in their ability to induce HSK indicating that a viral infection can trigger T-cell-mediated autoimmunity by molecular mimicry (Zhao *et al.*, 1998, Panoutsakopoulou *et al.*, 2001).

EBV infection has been implicated in the induction of anti-nuclear autoimmunity in SLE (James *et al.*, 2001b, James *et al.*, 2001a). A recurring proline-rich sequence -PPPGMRPP- is among the earliest antigenic sites to provoke a humoral autoimmune response against the B protein of the nuclear ribonucleoprotein (snRNP) autoantigenic complex (James and Harley, 1998). Immunisation of animal models with this peptide induces lupus-like autoimmunity, as does immunisation with the closely related sequence – PPPGRRP - found in the EBV nuclear antigen-1 (EBNA-1) (James and Harley, 1998). Over time epitope spreading occurs from the single octapeptide, to other snRNP proteins as has been shown by the presence of autoantibodies that recognise various snRNP proteins including A, C, D and 70kDa antigens (James and Harley, 1998). Epidemiological support to this model comes from a study demonstrating that 99% of young SLE patients have seroconverted against EBV compared to 70% of no-SLE normal controls (James *et al.*, 1997).

The majority of animal studies that have examined the issue of immunological cross-reactivity have focused on B- and CD4 T-cells. More recently, the role of CD8 T-cells

has also been investigated in a mouse model of inflammatory bowel disease using CD8 T-cell clones that recognise both mycobacterial and murine heat shock protein 60 (hsp60) (Steinhoff *et al.*, 1999). Adoptive transfer of hsp60-specific T-cells into TCRβ null mice leads to massive infiltration of these T-cells into the small intestine and the liver (Steinhoff *et al.*, 1999). Transfer of hsp60-specific T-cells into wild-type mice does not cause such pathology, possibly because in vivo expansion of these T-cells is limited in hosts that are not immunodeficient. Disease in this system is mediated by TCR recognition of the hsp60 self-antigen; a non-cross-reactive T-cell clone that only reacts with mycobacterial hsp60 does not cause disease (Steinhoff *et al.*, 1999). These results establish TCR cross-reactivity between murine and bacterial hsp60 and indicate that molecular mimicry may also be relevant for CD8 T-cell populations (Steinhoff *et al.*, 1999).

1.7 Molecular mimicry in viral hepatitis

1.7.1 Hepatitis B virus and myelin basic protein

Interestingly, the concept of molecular mimicry was for the first time tested in a rabbit animal model using a hepatitis B virus DNA polymerase peptide with six amino acids identical to the encephalitogenic region of rabbit myelin basic protein (MBP), the major autoantigen of multiple sclerosis (MS) (Fujinami and Oldstone, 1985). T-cell reactivity to MBP was observed following immunization of rabbits with this peptide, and four of eleven animals showed histological signs of experimental autoimmune encephalomyelitis (EAE), the animal model of MS (Fujinami and Oldstone, 1985). This finding raised the important question of whether infection with viral or bacterial pathogens, rather than immunization with synthetic peptides, can also induce autoimmunity (Oldstone, 1998). As it will be discussed later (*see* Chapter 10), isolated cases of demyelinating disorders such as MS have been reported, although rarely, as a result of autoimmune adverse reactions following exposure to hepatitis B surface antigen through prophylactic HBV vaccination (Cohen and Shoenfeld, 1996).

1.7.2 Hepatitis B and C viruses and non-organ specific autoantibodies

SMA and ANA are frequently found, and appear *de novo*, in the course of chronic HBV although their origin remains unclear (Gregorio *et al.*, 1996). In an attempt to explain their production Gregorio *et al.* (1999) searched for putative cross-reactive epitopes in HBV and human smooth muscle and nuclear proteins. Six human proteins were identified with a high local sequence similarity with the HBV DNA polymerase: four nuclear proteins involved in structural and regulatory functions, namely MHC class II *trans*-activator (MHCIITA), nuclear pore complex protein (NPCP), nuclear mitotic apparatus (NuMA), and polyomyositis sclerosis antigen (PM-scl) and two smooth muscle proteins involved in muscle contraction, myosin and caldesmon (Figure 1.3A) (Gregorio *et al.*, 1999). When serum samples from HBV infected patients, pathological and healthy controls were tested for reactivity to synthetic peptides spanning the homologous sequences, viral/self mimicry was observed almost exclusively in HBV infected subjects (Gregorio *et al.*, 1999). Antibody recognition of HBV-DNA polymerase/self peptide pairs was cross-reactive as shown by anti-peptide antibody competition experiments (Gregorio *et al.*, 1999). Gregorio *et al.* (1999) further demonstrated cross-reactivity between homologous peptides and whole antigen with respect to myosin and caldesmon, and identified these proteins as autoantigens in chronic HBV infection by immunoblot (Gregorio *et al.*, 1999).

More recently, the same group of investigators went on to study the role of molecular mimicry between HCV, nuclear and smooth muscle antigens (Gregorio *et al.*, 2003). Using similar methodology, they found cross-reactive antibodies between a number of HCV antigens and human nuclear and smooth muscle mimics (Figure 1.3B). Myosin also appeared to be an important molecular target in HCV infection, although a different epitope to that found in HBV infection was involved (Gregorio *et al.*, 2003).

These studies document the usefulness of the application of molecular mimicry as a tool for the identification of unknown autoantigens cross-reactively recognised by viral agents, have provided an insight into how molecular mimicry and immunological cross-reactivity may be involved in the induction of autoimmunity related to viral hepatitis, and have been the foundation on which this thesis is based (Gregorio *et al.*, 1999, Gregorio *et al.*, 2003).

		Protein	Sequence
A	i)	HBV DNA polymerase	V G L L G F A A P F T Q C G
			: : : : : : : . . :
		MHCIITA	V G L L G R A A L D S P P G
		HBV DNA polymerase	S S T V P V F N P
			: : : : : : :
		NPCP	S S T V P V S L P
		HBV DNA polymerase	G P L E E E L P R L A D
			: . : : : : : : : :
		NuMA	G S L E E E K R R A A D
		HBV DNA polymerase	N E K R R L K L
			. : : . : : : .
		Pm-scl	Q E K K R L K I
	ii)	Myosin	D E K R R L E
			. : : : : : .
		HBV DNA polymerase	N E K R R L K
			. : : : : : :
		Caldesmon	E E K R R L K
B	i)	HCV polymerase	L I S Q A E A A L E N L V
			: . : : : : : : .
		Matrin 3	L D Q E N E A A L E N G I
		HCV polymerase	A V S R T Q R R G
			: : : : . : : :
		Histone H2A	A V S R S Q R A G
		HCV polymerase	A L V A F K I M S G E
			. : : : : : : : . :
		Replication protein A	S L V A F K I M P L E
	ii)	HCV polymerase	L A T K T F G S S G S S
			. : : : : . : : . : :
		Smoothelin	M Q T K T F S S S S S S
		HCV polymerase	E G E V Q I V S T A T Q
			: : : : : : : : :
		Myosin	E L E D T L D S T A T Q
		HCV polymerase	G V R V L E D G V N F A
			: : : . : . : . : . : .
		Vimentin	G V R L L Q D S V D F S

Figure 1.2 *Amino acid sequence homology between: (A) the hepatitis B virus DNA polymerase and i) human nuclear proteins (MHCIITA, MHC class II trans-activator; NPCP, nuclear pore complex protein; NuMA, nuclear mitotic apparatus; Pm-scl; polyomyositis sclerosis antigen) and (ii) smooth muscle proteins; (B) the hepatitis C polyprotein and (i) nuclear antigens and (ii) smooth muscle proteins. Amino acids in standard single letter; Colon (:), identity; Full stop (.), conservative substitution*

1.8 Hepatitis C and autoimmune hepatitis

AIH-2 is a severe form of autoimmune liver disease affecting mainly children that requires life-long immunosuppressive treatment (Gregorio *et al.*, 1997). Progression to cirrhosis and liver failure may occur in some patients despite therapeutic intervention and usually requires liver transplantation (Gregorio *et al.*, 1997). Histologically, AIH-2 is characterised by interface hepatitis where lymphocytes, plasma cells and macrophages from the portal tract infiltrate the adjacent parenchyma, and surround dying hepatocytes (Dienes *et al.*, 1989). Serologically, AIH-2 is characterised by elevated levels of immunoglobulin G (IgG) and high titres of LKM1 autoantibodies, the name deriving from the distinctive pattern that the antibody gives on rodent liver and kidney sections (*see* 2.2.2.1) (Alvarez *et al.*, 1999). The molecular target of LKM1 has been identified as cytochrome P450 IID6 (CYP2D6), a 50 kDa microsomal enzyme involved in the metabolism of xenobiotics (Alvarez *et al.*, 1985, Gueguen *et al.*, 1989). LKM1 is also found in up to 10% of patients with chronic HCV infection but not in other viral hepatitides (Lunel *et al.*, 1992, Bortolotti *et al.*, 1996, Gregorio *et al.*, 1998, Cassani *et al.*, 1997, Nishioka *et al.*, 1997, Lenzi *et al.*, 1999). Early studies have also reported a minority of patients with AIH-2 to be infected with HCV, suggesting a causal link between this virus and AIH-2 (Lenzi *et al.*, 1990, Vergani and Mieli-Vergani, 1993).

How cytochromes and in particular CYP2D6 become 'self targets' is not yet established (Manns and Obermayer-Straub, 1997, Bogdanos and McFarlane, 2003). It has long been thought that intracellular proteins are normally 'hidden' from immune recognition and therefore immunologically privileged (*see* 1.3.5). Antigen release following hepatocyte injury could provide the stimulus for an immune response towards 'cryptic' epitopes on CYP2D6 (Manns and Obermayer-Straub, 1997). This model of anti-CYP2D6 antibody production fails to provide a convincing explanation for the highly-specific nature of the observed anti-CYP2D6 immune response (Bogdanos and McFarlane, 2003). Firstly, not every form of liver damage is associated with an anti-CYP2D6 immune response: anti-CYP2D6 antibodies are undetectable in viral hepatitis A and B and in type 1 AIH (Bogdanos and McFarlane, 2003). Secondly, in those conditions where reactivity to cytochromes is present, distinctive profiles can be recognised. Anti-CYP2D6 antibodies are found in AIH-2 and in a proportion of HCV

infected patients. In neither condition is it possible, however, to detect antibodies to cytochrome P450 1A2 (CYP1A2), the marker of the hepatitis associated with autoimmune polyendocrine syndrome type 1 (Clemente *et al.*, 1998, Manns and Obermayer-Straub, 1997, Bogdanos and McFarlane, 2003). Moreover, despite a high level of amino acid homology between CYP2D6, CYP1A2 and CYP1A1, the latter does not act as target autoantigen (Clemente *et al.*, 1998). These patterns indicate a specific, antigen-driven mechanism in the breakdown of immunological tolerance towards individual cytochromes (Bogdanos and McFarlane, 2003).

Kerkar *et al.* (2003) have identified the sequence $CYP2D6_{193\text{-}212}$ as a major B-cell epitope recognised by sera from 93% of patients with AIH-2 and 50% of those with anti-LKM1 positive HCV infection. They also found that there is extensive cross-reactivity between immune responses directed to $CYP2D6_{193\text{-}212}$ and highly homologous regions within HCV and CMV antigens (Kerkar *et al.*, 2003). Modelling studies located the $CYP2D6_{193\text{-}212}$ peptide to the surface of CYP2D6, making it accessible to antibody recognition in the context of the physiologically folded protein (Kerkar *et al.*, 2003). Alanine substitution of the homologous HCV epitope confirmed that the homology shared by HCV, CMV and $CYP2D6_{193\text{-}212}$ was responsible for the observed cross-reactivity (Kerkar *et al.*, 2003). Furthermore, these Authors have shown that purified $CYP2D6_{193\text{-}212}$ specific autoantibodies are able to inhibit the activity of the native enzyme (Kerkar *et al.*, 2003).

These findings provide suggestive evidence for molecular mimicry to be a mechanism for induction of anti-CYP2D6 autoantibodies and give support to the multi-hit theory of autoimmunity. Thus, multiple exposures to CMV, a common viral pathogen, may establish permissive immunological conditions, by priming a cross-reactive subset of autoreactive lymphocytes, in a genetically predisposed host (Kerkar *et al.*, 2003). Chronic infection with HCV may then provide the final impetus for the generation of LKM1 autoantibodies through cross-reactive mechanisms (Kerkar *et al.*, 2003). Depending on the degree of immunological priming, the degree of genetic susceptibility (particularly at the HLA locus and coding regions for 'innate' components of immunity), and antigenic dose of the infecting pathogens, a minority of individuals may progress to autoimmune disease.

Others have also invoked cross-reactive mechanisms to explain the generation of LKM1 antibodies on the basis that two sequences from the HCV polyprotein, one each from the envelope 1 (E1) and the non structural 5B (NS5B) antigens, and one from the

immediate early 175 KDa protein of HSV-1, share extensive homology with $CYP2D6_{254-271}$, the second most frequently recognised B-cell epitope of LKM1 in AIH-2 (Manns *et al.*, 1991). Although attractive, there was little experimental evidence to support this hypothesis and reactivity to the viral mimics had not been tested (Manns *et al.*, 1991).

That reactivity to viral and self peptides, as indeed to the whole CYP2D6 protein, is of the IgG isotype thus suggesting the involvement of T-cell 'help' for B-cell immunoglobulin isotype class switching (Cebra *et al.*, 1984, Snapper and Mond, 1993). This notion is further supported by the presence of autoreactive T-cells in the blood and the liver of patients with AIH-2 and the results of immunohistochemical studies demonstrating that most infiltrating T-cells are positive for the CD4 helper/inducer phenotype, and a sizeable minority are positive for the CD8 cytotoxic/suppressor phenotype (Senaldi *et al.*, 1992, Vergani and Mieli-Vergani, 2003).

Taken together, these studies indicate that an autoimmune response to CYP2D6 is likely to follow the rules of any other autoimmune response, and involve CD4, CD8, and B-lymphocytes, one of the components possibly prevailing at different stages of the pathological progress (Vergani and Mieli-Vergani, 2003, Bogdanos and McFarlane, 2003). Interestingly Cerny and Chisari (1999) have proposed a role for CD8 molecular mimicry in the causation of post-infectious autoimmunity making reference to HCV and AIH-2. They base their contention on the work of Kammer *et al.* (1999), showing induction of cytotoxic T-cells (CTL) reactive with a decamer (amino acids 8-17) of CYP2A6 and CYP2A7 after exposure to the homologous HCV core sequence (amino acids 178-187), the relevant peptides being totally unreactive at the B-cell level (Bogdanos *et al.*, 2000b). These authors proposed that liver cell damage mediated by HCV-specific CTL leads to the release of intracellular proteins like CYP2A6 and CYP2D6, uptake of these autoantigens by professional APCs, and autoantibody formation in the presence of autoreactive CD4 and CD8 T-cells (Kammer *et al.*, 1999). After viral clearance, the autoimmune disease would be upheld due to ongoing hepatocyte lysis by cross-reactive, HCV-induced CTL maintained by autoreactive helper T-cells (Kammer *et al.*, 1999). The interaction of HCV, autoreactive T-cells, and autoreactive CD8 and CD4 T-cells would thus be required for the induction of HCV-associated autoimmunity directed to cytochromes. The need of this complex interaction would explain the relatively low frequency of anti-CYP2D6 immune responses, and indeed of AIH, among HCV patients despite the high number of individuals with cross-

reactive CTL (Bogdanos *et al.*, 2000b). Kammer *et al.* (1999) findings demonstrate the potential of HCV to induce autoreactive CD8 CTL by a molecular mimicry mechanism involving viral/self mimics and therefore show a possible mechanism by which a virus can trigger AIH.

A feature of AIH-2 is the possible coexistence of other autoimmune diseases, in particular type 1 diabetes, Addison's disease, hypoparathyroidism and autoimmune thyroiditis (Gregorio *et al.*, 1997). Choudhuri *et al.* (1998) have demonstrated cross-reactive LKM1 antibody responses between $CYP2D6_{321-351}$, the third most frequently recognised epitope of CYP2D6, and structurally similar regions of carboxypeptidase H, an autoantigen in type 1 diabetes, and 21-hydroxylase, the major autoantigen in Addison's disease. These Investigators proposed that autoimmunity once induced against one self-antigen may spread via molecular mimicry to other homologous self antigens, and, in genetically predisposed individuals, lead to overt autoimmune disease (Choudhuri *et al.*, 1998). Although intermolecular epitope spreading is a feature of other autoimmune disease such as type 1 diabetes and connective tissue disorders, the mechanistic explanation for this phenomenon has relied on molecular association or tissue colocalisation for the diversification of autoimmunity (James and Harley, 1998). The hypothesis of Choudhuri *et al.* (1998) although able in principle to explain the spread of autoimmunity to anatomically distant tissues through immunological cross-reactivity, has not yet been confirmed.

It is now becoming clear that molecular mimicry studies have been based either on epidemiological association of infectious agents with specific autoimmune manifestations or experimental evidence demonstrating cross-reactivity between microbial and self antigens (Oldstone, 1998, von Herrath, 2000, von Herrath *et al.*, 2003). In either case, the microbial trigger as well as the self target (or at least its origin) are known and an investigation is carried out to identify the microbial/self determinants that serve as targets of the cross-reactive immune responses (Oldstone, 1998). Several studies have provided knowledge of the fine specificity of immune responses towards specific antigenic regions (Oldstone, 1998, von Herrath, 2000, von Herrath *et al.*, 2003). These regions have been used through molecular mimicry studies as tools for the identification of mimicking microbial agents, which could be pathogenetically linked with the autoimmune disease (Oldstone, 1998, von Herrath, 2000, von Herrath *et al.*,

2003). Primary biliary cirrhosis is one of these autoimmune diseases whereby molecular mimicry has been investigated in detail taking into account epidemiological considerations linking specific microbial agents with the disease, evidence of microbial/self cross-reactitivity but also identification of putative microbial mimics through search of homologies with the disease's single immunodominant epitope (Van de Water *et al.*, 2001).

1.9 Primary biliary cirrhosis

Primary biliary cirrhosis is an immune-mediated chronic cholestatic liver disease characterised by inflammatory destruction of the small intrahepatic bile ducts, progressing to cirrhosis and subsequent liver failure (Kaplan, 1996, Neuberger, 1997). The disease affects mainly middle-aged women, with most cases occurring between the ages of 40 and 60 years (Sherlock and Scheuer, 1973, Mahl *et al.*, 1994). There is a wide variation in the recorded incidence between areas, ranging from 30 per 100,000 of the population in high prevalence areas like Northern England, rising to 1 in 1,500 in women over the age of 40 in the health district of Newcastle and North Tyneside, to less than 25 cases per million in Canada and Australia and almost no cases in sub-Saharan Africa or India (Witt-Sullivan *et al.*, 1990, Watson *et al.*, 1995, Kingham and Parker, 1998, James *et al.*, 1999, Metcalf and James, 1997). Two recent, large, epidemiological studies in UK and USA have indicated an upward trend of the prevalence rates of PBC, but it is not yet clear whether this increase is authentic and/or reflects greater awareness of the disease, in combination with an increased availability and better application of improved serological diagnostic tools (Kim *et al.*, 2000, Prince *et al.*, 2001).

The clinical spectrum of PBC ranges from asymptomatic anicteric cholestasis with or without extrahepatic manifestations to severe cholestasis with cirrhosis and subsequent liver failure, the disease being along with chronic hepatitis C virus infection the commonest reasons for liver transplantation in the developed World (Neuberger, 1997). While ursodeoxycholic acid (UDCA) may slow progression, there is yet no curative therapy (Neuberger, 1997). Immunosuppressive therapy is relatively ineffective (Neuberger, 1997).

1.9.1 Anti-mitochondrial antibody

The nature of PBC became clearer around the 1960s when first its apparent and then its unequivocal association with an antibody directed to mitochondrial antigens (AMA) was described (Mackay, 1958, Walker *et al.*, 1965). A milestone for the clinical hepatologist was the observation in 1965 by Walker, Doniach, Roitt and Sherlock that AMA was present in all their patients with PBC and in none of the controls which included patients with extra-hepatic bile duct obstruction, drug induced cholestasis and viral hepatitis (Doniach *et al.*, 1966). Ever since the description of this close association, AMA has acted as a powerful diagnostic tool since positivity for AMA was able to direct towards the correct diagnosis in the work up of cholestatic conditions, avoiding at times invasive procedures such as explorative laparotomy (Sherlock and Scheuer, 1973). In addition to revealing a diagnostic marker, the paper by Walker *et al.* (1965) showed what anti-mitochondrial reactivity looks like under a fluorescent microscope and provided a straightforward technique for its detection. Almost forty years on, the detection of AMA is routinely done as originally described in the laboratory of Professor Deborah Doniach.

1.9.2 M2 antigens

In 1967 Berg *et al.* demonstrated that PBC sera reacted in vitro with isolated mitochondria. PBC specific AMA were shown to react with trypsin-sensitive antigens named mitochondrial type 2 (M2) to be differentiated from M1, the target antigen of anti-cardiolipin antibodies found in a proportion of patients with syphilis (Berg *et al.*, 1982). The M2 antigen was found to be closely associated with the inner surface of the inner membrane of all mitochondria tested, from human to fungal, and anti-M2 antibodies were shown to cross-react with several bacteria, anti-mitochondrial activity, for example, being absorbed out of PBC sera by *E. coli* (Berg *et al.*, 1967, Lindenborn-Fotinos *et al.*, 1985, Teoh *et al.*, 1994).

Cloning and identification of the autoantigenic targets of AMA represent a major step forward in the study of PBC. Reactivity against several mitochondrial antigens was noted in early studies, a 74 kDa protein present in mitochondrial preparations from a variety of species being the most frequently recognised antigen (Lindenborn-Fotinos *et al.*, 1985, Frazer *et al.*, 1985). The precise molecular weight was species-, but not organ-specific (Baum and Berg, 1981). Less frequent was reactivity against antigens of 56, 48, 41 and 36 kDa (Baum and Berg, 1981).

The M2 antigens have been identified as components of the 2-oxo-acid dehydrogenase multienzyme complexes (OADC): pyruvate, 2-oxoglutarate and branched-chain 2-oxo acid dehydogenase complexes (PDC, OGDC and BCOADC, respectively) (Bassendine *et al.*, 1997). Each of these three multifunctional complexes catalyses a set of linked reactions and occupies a key position in energy metabolism in a cell (Yeaman, 1989, Yeaman *et al.*, 2000). PDC links glycolysis to the Krebs cycle, OGDC is in the Krebs cycle itself and BCOADC is involved in the regulation of the oxidation of the branched-chain amino acids (Yeaman, 1989, Yeaman *et al.*, 2000). Each complex consists of multiple copies of at least three enzymes (E1, E2 and E3), which are encoded by genes in the nucleus and separately imported into mitochondria for assembly into high molecular weight multimers localised to the matrix aspect of the inner membrane (Yeaman, 1989, Yeaman *et al.*, 2000). The structural core of these complexes is provided by E2, to which multiple copies of E1 and E3 are non-covalently bound (Yeaman, 1989, Yeaman *et al.*, 2000). The E2 enzymes have a common structure, which consists of the N-terminal domain containing the lysine-bound lipoyl groups, which have a central role in the catalytic cycle; the peripheral subunit binding domain, responsible, at least in part, for binding the E1 and E3 components together; and the C-terminal inner core, which houses the active site responsible for the acyltransferase activity. E3 is common to all 3 complexes whereas E1 and E2 are unique to each complex. PDC contains a fourth polypeptide with a structural role, the E3 binding protein (E3BP), once termed protein X (De Marcucci and Lindsay, 1985).

It took a surprisingly long time after their original detection and characterisation for the molecular identities of the members of the M2 family to be established. The reason for the delay in realising that the major M2 antigen was a key mitochondrial constituent was primarily a trivial one. M2 co-purified with the inner mitochondrial membrane-albeit it was well known that it could readily be detached therefrom (Baum, 1995). In the mind-set of most investigators, PDC was a constituent of the matrix, and so its possible identity with M2 was never considered.

It was in 1987 that a cDNA for the 74 kDa mitochondrial autoantigen was cloned and sequenced leading to the identification of the E2 subunit of PDC-E2 as the major autoantigen in PBC (Gershwin *et al.*, 1987, Van de Water *et al.*, 1988b, Yeaman *et al.*, 1988). This significant development was accompanied by considerable efforts devoted to the characterization of the AMA responses by PBC sera. It was established that serum autoantibodies from more than 95% of patients with PBC react with PDC-E2 by

immunoblotting or ELISA, whereas the frequency of reactivity against E2 subunit of OGDC and BCOADC is 50-70% (Leung *et al.*, 1997). A few AMA seropositive patients react with PDC-E2 alone, while a majority also show reactivity against OGDC-E2 or BCOADC-E2 (Leung *et al.*, 1997). Reactivity against BCOADC-E2 or OGDC-E2 alone is less common. As in the case of PDC, the predominant antibody reactivity to the BCOADC and OGDC enzyme complexes is against the E2 component (Yeaman *et al.*, 2000). The E2 components of the OADC are evolutionarily highly conserved, both between species and between the different complexes (Baum, 1995, Yeaman *et al.*, 2000).

Of note, a single PBC serum can react with several mitochondrial autoantigens and each autoantigen seems to be recognised by a distinct population of autoantibodies, albeit there is some cross-reactivity between them (Baum, 1995, Leung *et al.*, 1997). Major cross-reactivity occurs between PDC-E2 and PDC-E3BP: all PBC sera reactive with PDC-E2 also react with a peptide of 50-56 kDa, shown to be identical to the E3BP protein of PDC (Surh *et al.*, 1989). That this is due to cross-reactivity, was documented by absorption studies where removal from PBC sera of reactivity to human PDC-E2 with recombinant PDC-E2 also removed reactivity against the 56 kDa peptide (Surh *et al.*, 1989). It is unclear whether the initial breakdown of self tolerance is to PDC-E2 with epitope spreading to E3BP, to E3BP with spreading to E2, to both proteins simultaneously, or to either E2 or E3BP with immunologic cross-reactivity to the other (Dubel *et al.*, 1999, Palmer *et al.*, 1999).

Multiple bands on immunoblot could be due either to antibodies recognising different epitopes, each being characteristic for a different antigen or to the presence of a single cross-reactive epitope on different molecules. Through inhibition, biochemical and epitope mapping studies, it is now clear that more than 90% of PBC patients have antibodies to PDC-E2 and PDC-E3BP, but only 50% of patients have AMA that recognize all three oxo-acid complexes suggesting that epitopes in BCOADC and ODGDC are independent of the epitopes on PDC-E2 (Yeaman *et al.*, 2000).

The use of oligopeptides and recombinant antigens has shown that the predominant epitope of PDC-E2 is located within the lipoyl domain of PDC-E2, but that at a 100-fold higher concentration, AMA can also react with the outer domain (Surh *et al.*, 1990). In view of the sequence similarity between inner and outer regions, this differential reactivity has been interpreted as the result of cross-reactivity between the

two lipoyl binding domains, the priming immunogenic region being the inner lipoyl domain (Surh *et al.*, 1990).

B-cell epitope mapping based on the use of truncated constructs of peptides has shown that AMA reactive with BCOADC-E2 and OGDC-E2 are each directed against a conformational epitope that includes the inner lipoyl domain (Leung *et al.*, 1995, Moteki *et al.*, 1996b). The highly conserved structure in the E2 subunit of 2-OADC and their lipoyl domains indicates that lipoic acid may be part of the immunodominant epitope. Studies investigating the contribution of lipoic acid to the autoantibody reactivity have, nonetheless, shown that AMA are capable of binding to both lipoylated and unlipoylated PDC-E2 (Flannery *et al.*, 1989, Fussey *et al.*, 1990, Leung *et al.*, 1990, Quinn *et al.*, 1993).

A notable characteristic of AMA in PBC sera is their capacity to block the catalytic function of the 2-OAD multienzyme complexes in vitro (Van de Water *et al.*, 1988a, Sundin, 1990). There is, however, no proof that any of these autoantibodies can interfere with PDC activity in vivo.

Autoantibodies to the 2-OADC enzymes are detected not only in serum, but also in body fluids into which serum immunoglobulins can be transferred. Interestingly, IgA AMA have been detected in the bile, saliva, and urine of patients with PBC, suggesting that AMA are secreted into the luminal fluid across bile ducts, salivary glands and uroepithelium of patients with PBC (Nishio *et al.*, 1997, Tanaka *et al.*, 2000, Reynoso-Paz *et al.*, 2000). Reactivity against a band on immunoblot characteristic for PDC-E2 has been reported in the cerebrospinal fluid of a patient with PBC and a demyelinating disorder (Mackay *et al.*, 2000).

1.9.3 AMA as predictors of disease

Seropositivity for AMA is reported to be much higher than the prevalence of PBC in the general population, suggesting that AMA may precede the clinical onset of the disease (Metcalf and James, 1997). Evidence relating to the earliest events in the natural history of PBC has been derived from studies of patients with positive AMA, who were discovered accidentally when they were tested for an 'autoantibody profile' (Mitchison *et al.*, 1986, Metcalf *et al.*, 1996, Kisand *et al.*, 2001). In 1986, Mitchison *et al.* (1986) of the Newcastle group, found liver histology diagnostic of or compatible with PBC in 24 (83%) of 29 patients 'accidentally' found to be seropositive with an AMA titre of

1/40 or greater, with normal conventional liver function tests and no symptoms of liver disease. Only two had normal liver histology (Mitchison *et al.*, 1986). When the same subjects were re-assessed some ten years later, three quarters of them had become clinically symptomatic with persistently cholestatic liver function tests (Metcalf *et al.*, 1996). The median follow up in this cohort was 17.8 years, with one subject positive for AMA 23 years before the diagnosis of PBC was made (Metcalf *et al.*, 1996). Interestingly, none of the patients in this cohort had progressed to cirrhosis (Metcalf *et al.*, 1996). These findings indicate that, first, the progression of the disease can be very slow, second, identification of patients through an autoantibody screening of asymptomatic subjects may select particularly mild cases, third, AMA is a very powerful predictor of future development of PBC. A study by Kisand *et al.* (2001) provides support to the Newcastle experience reporting development of abnormal liver function tests, during a nine-year period of observation, in three of eight asymptomatic individuals positive for antibodies to PDC by ELISA.

AMA have also been investigated for their ability to predict disease progression (Christensen *et al.*, 1980, Roll *et al.*, 1983, Heseltine *et al.*, 1990, Kisand *et al.*, 1998). Heseltine *et al.* (1990) reported that IgG AMA titres correlated with histological stage of disease and with prognostic biochemical variables, including serum bilirubin and albumin levels. This report supports the earlier observation that IgG AMA titres, as assessed by immunofluorescence, increase with disease progression (Christensen *et al.*, 1980, Roll *et al.*, 1983). An association between serum AMA titre and liver inflammatory activity has been reported in both untreated and UDCA treated PBC patients (Kisand *et al.*, 1998).

More recently, van Nostrand *et al.* (1997) have analysed the relationship between an established indicator of disease progression, such as the Mayo Risk Score, and levels of AMA measured by quantitative enzyme immunoassays, where recombinant OADC proteins served as substrate. The levels of AMA to 2-OADC proteins varied by more than 200-fold between patients but remained relatively constant over time in individual patients. Despite being positively correlated with the Mayo Risk Score, the levels of AMA to 2-OADC proteins were not useful in predicting disease progression in the individual PBC patient (Van Norstrand *et al.*, 1997). In addition, these authors went on to show that there were no significant differences in the levels of autoantibodies to 2-OADC proteins among patients at different histological stages of the disease (Van

Norstrand *et al.*, 1997). In view of the contrasting experimental evidence alluded to above, the role of AMA as predictor of disease progression remains to be clarified.

1.9.4 Detection of AMA

AMA are commonly detected by indirect immunofluorescence (IFL), immunoblotting, or ELISA and an important decision for diagnostic laboratories and clinicians is the choice of assay for the detection of AMA (Leung *et al.*, 1997). IFL using rodent tissue sections or immobilized HEp-2 cells is by far the most commonly used serological test for AMA (Leung *et al.*, 1997). This technique, especially when used on a composite substrate, i.e. a substrate composed of a combination of rodent tissues, enables the detection of a large variety of autoantibodies including SMA, ANA, and LKM1, all important in the diagnosis of autoimmune liver disease (Leung *et al.*, 1997). AMA stains the distal renal tubuli and the gastric parietal cells of the stomach, and the dual renal-gastric positivity gives a clear, unmistakable immunofluorescent picture. The identification of AMA target antigens as subunits of the 2-OADC has led to the development of immunoblotting for the detection of specific mammalian mitochondrial antigens (Leung *et al.*, 1997). Mitochondrial preparations derived from bovine or porcine heart, rat liver, human placenta, or other tissues are electrophoretically separated on SDS-polyacrylamide gel, tranferred onto nitrocellulose filters, and assayed with sera from patients with PBC and controls (Leung *et al.*, 1997). AMA reactivity against these antigens is visualised by addition of an enzyme-labelled antihuman immunoglogulin and a chromogenic enzymatic substrate (Leung *et al.*, 1997). The presence in the test serum of antibodies to any of the electrophoretically separated mitochondrial components results in bands identified by their molecular weight (Leung *et al.*, 1997).

In addition to immunoblotting, which is time consuming, labour intensive, and requiring, as immunofluorescence, a degree of subjective interpretation, other approaches such as ELISA are gaining momentum since, at least for those ELISA kits produced commercially, the need for a skilled observer in the interpretation of the results is reduced to a minimum, making them ideal for use in non specialised centres (Van de Water *et al.*, 1989, Moteki *et al.*, 1996a, Miyakawa *et al.*, 2001). Moreover, ELISA permit the evaluation of numerous sera in a single run. ELISA are widely used for the detection of antibodies to purified porcine or bovine M2 antigen or recombinant OADC proteins (Czaja and Homburger, 2001).

1.9.5 AMA negative PBC

The pathognomonic connotation of AMA in the diagnosis of PBC started being questioned when Brunner and Klinge (1987) described four female patients with 'immunocholangitis', a condition characterized by the typical biochemical and histological features of PBC, but with AMA seronegativity (Brunner and Klinge, 1987). A similar presentation characterised patients suffering from 'primary autoimmune cholangitis', 'autoimmune cholangiopathy', and 'AMA-negative PBC' (Goodman *et al.*, 1995, Taylor *et al.*, 1994, Michieletti *et al.*, 1994, Ben-Ari *et al.*, 1993, Lacerda *et al.*, 1995, Sanchez-Pobre *et al.*, 1996, Invernizzi *et al.*, 1997). Though in one study AMA negativity has been reported in one third of PBC patients, a large series from the Mayo Clinic, shows only 6% AMA negativity amongst 597 cases of PBC analysed (Goodman *et al.*, 1995, Lacerda *et al.*, 1995). A similarly low proportion was noted in an earlier study by Berg *et al* (Berg *et al.*, 1986).

The proportion of AMA negative PBC patients as ascertained by conventional immunofluorescence decreases when AMA is tested using more sensitive techniques (Nakanuma *et al.*, 1997, Kitami *et al.*, 1995, Kinoshita *et al.*, 1999, Nakajima *et al.*, 1999).

Care must, therefore, be exercised when labelling patients as AMA negative, because AMA, and in particular the PBC-specific anti-M2, not detected by immunofluorescence, may be detectable by other means, such as ELISA or immunoblot using purified bovine or porcine heart mitochondria (Nakanuma *et al.*, 1997, Kitami *et al.*, 1995, Kinoshita *et al.*, 1999, Nakajima *et al.*, 1999). There is an increasing amount of evidence for this notion. Of 20 PBC patients AMA negative by immunofluorescence, 3 were found to have M2 specific antibodies by ELISA and immunoblot (Michieletti *et al.*, 1994). Similarly, Invernizzi *et al.* (1997) reported a positive M2 specific band by immunoblotting in 6 of 25 (24%) IFL AMA-negative PBC patients. Muratori *et al.* noted that six out of 19 (32%) IFL-AMA negative patients recognised AMA-specific bands when tested by immunoblot against beef heart mithochondria. In other studies, up to 100% of patients AMA negative by immunofluorescence were found to be positive when tested by a variety of techniques including immunoblotting, ELISA using recombinant polypeptides spanning M2-related epitopic regions, and enzyme inhibition assays (Nakanuma *et al.*, 1997, Kitami *et al.*, 1995, Kinoshita *et al.*, 1999, Nakajima *et al.*, 1999).

Of interest are the studies by the group of Gershwin who make use of cloned mitochondrial antigens, at times combined in hybrid recombinant molecules, as targets of AMA reactivity in ELISA and immunoblot (Moteki *et al.*, 1996b, Miyakawa *et al.*, 2001). One of such molecules named rMIT-3 contains three key autoepitopes of M2, present on PDC-E2, BCOAD-E2, and OGDC-E2 (Miyakawa *et al.*, 2001). In 1996, using these recombinant molecules they identified the presence of AMA in 67% of patients diagnosed as AMA negative by IFL (Moteki *et al.*, 1996b). More recently, Miyakawa *et al.* (2001) from the same group, reported that, using an ELISA based on an advanced version of the recombinant mitochondrial preparation, 73% of IFL-AMA negative PBC patients were AMA-positive. When reactivity was investigated against the individual components of the trimeric recombinant molecule, it was found that antibodies to at least one of its components were present, by immunoblot, in 90% of 30 IFL AMA-negative PBC patients (Miyakawa *et al.*, 2001).

1.9.6 PBC-specific ANA

There are two main types of ANA in PBC: those that are not disease-specific, directed against targets such as centromere, Ro/SSA, La/SSB, Scl-70 and histones and those which are highly PBC-specific (Vergani and Bogdanos, 2003). The existence of ANA specific for PBC is in contrast to AIH-1, a disease also characterized by the presence of ANA (alone or in association with anti-smooth muscle antibody), but in which no specific ANA have yet been identified (Vergani and Bogdanos, 2003).

Two distinct immunofluorescence patterns characterise PBC-specific ANA, namely the perinuclear (rimlike) and the multiple nuclear dot (MND) patterns (Courvalin and Worman, 1997, Szostecki *et al.*, 1997). Their molecular targets have been identified, with the antibodies responsible for the rimlike pattern recognising constituents of the nuclear envelope including gp210, a 210 kDa transmembrane glycoprotein of the nuclear pore complex (NPC), lamin B receptor, and nucleoporin p62, while those responsible for the MND pattern recognise the sp100 and promyelocytic leukaemia proteins (Courvalin and Worman, 1997, Szostecki *et al.*, 1997). These two latter auto-antigens colocalize and the corresponding autoantibodies tend to occur together (Szostecki *et al.*, 1997).

The PBC-specific ANA are not confined to AMA negative PBC, even though they are over-represented in this form of PBC (Vergani and Bogdanos, 2003). This over-

representation is partially due to technical reasons since ANA positivity can be obscured by the confounding simultaneous presence of AMA (Vergani and Bogdanos, 2003). When analysed in immunofluorescence, ANA become more visible if AMA is absent or weak (Vergani and Bogdanos, 2003).

The potential of using observer-independent assays such as ELISA has been recently outlined (Muratori *et al.*, 2003a). Measuring simultaneously reactivity to 3 PBC-specific ANA targets, namely anti-sp100, anti-gp210 and anti-lamin B receptor in PBC by ELISA, it was found that 85% of the AMA negative patients investigated were positive for one or more of the ANA specificities, suggesting that the clinicians provided with a panel of PBC specific ANA results may have at their disposal a 'positive' tool in the diagnosis of AMA negative PBC (Muratori *et al.*, 2003a, Vergani and Bogdanos, 2003). The validity of these markers in the diagnosis of PBC in AMA negative patients needs confirmation in further studies.

Earlier immunofluoscence studies in which the majority or the totality of AMA negative PBC patients were found to be ANA positive, give support to the notion that PBC-specific ANAs may become an essential marker for AMA-negative PBC (Michieletti *et al.*, 1994, Lacerda *et al.*, 1995). Of note is the observation that patients negative for MND or rimlike reactivity by immunofluorescence may be positive when tested by ELISA for reactivity to sp100 and gp210 - their molecular equivalents (Muratori *et al.*, 2003a).

1.9.7 Pathogenicity of AMA

Anti-M2 AMA are so disease-specific that it may be assumed that they are relevant to disease pathogenesis (Neuberger and Thomson, 1999). Most workers, however, have concluded that T-cells rather than the antibodies themselves are the direct cause of tissue damage (Gershwin and Mackay, 1991, Jones *et al.*, 1995, Gershwin *et al.*, 2000). For example, high titres of AMA can be raised in experimental animals, e.g. by immunization with recombinant human PDC-E2, with no evidence of liver damage even after a prolonged period, and there is no universal recurrence of PBC following liver transplantation, despite the persistence of AMA (Krams *et al.*, 1989, Neuberger and Thomson, 1999). Anti-PDC-E2 AMA are able to inhibit *in vitro* the enzymatic activity of PDC-E2. However, there is no evidence that AMA interfere with PDC activity *in vivo* (Van de Water *et al.*, 1988a, Sundin, 1990. The immunodominant antigenic regions recognised by T lymphocytes on PDC-E2 have been defined

[Shimoda, 1995 #240, Shimoda *et al.*, 1998, Kita *et al.*, 2002, Shimoda *et al.*, 1995). These comprise a region within the inner lipoyl-binding domain of the subunit, overlapping amino acid 212-226 (PDC-$E2_{212-226}$) (Shimoda *et al.*, 1995, Shimoda *et al.*, 1998, Kita *et al.*, 2002) and targeted by both CD4 and CD8 T-cells. The same region is also the core target of the receptors of B-lymphocytes, which in their soluble form are antibodies (Van de Water *et al.*, 1988b, Tuaillon *et al.*, 1992, Matsui *et al.*, 1993). It is conceivable that helper T-cells, cytotoxic T-cells and B-cells act in concert in inflicting damage to the liver ductules in the pathogenic process of PBC (Gershwin *et al.*, 2000). In support of this view is the recent finding that soluble PDC-E2 complexed with a PDC-E2-specific human monoclonal antibody favours generation of PDC-E2-specific cytotoxic cells at a 100-fold lower concentration than otherwise required in the presence of the soluble antigen alone (Kita *et al.*, 2002). Thus, AMA may play a pathogenic role in facilitating the development of a PDC-E2 specific CD8 T-cell immune response (Kita *et al.*, 2002). That AMA may have pathogenic properties has been indirectly suggested by a recent clinical report where two infants, who received AMA transplacentally from their mothers, developed liver pathology that lasted as long as the autoantibody persisted in their serum (Hannam *et al.*, 2002).

1.9.8 PDC-$E2_{212-226}$ is physically exposed on the molecule's surface

Determination of the three-dimensional structure of the inner lipoyl domain of human PDC-E2 has revealed that PDC-$E2_{212-226}$ is physically exposed on the molecule's surface which may partially explain its particular antigenicity (Howard *et al.*, 1998). However, the mechanism by which this short sequence becomes the focus of PBC-specific anti-mitochondrial immune responses remains obscure.

Assuming that a concerted autoimmune attack is the ultimate mechanism of damage in PBC, the question as to what triggers this autoimmune attack remains without an answer (Baum, 1995). Many of the epidemiological, clinical and serological features of PBC are compatible with an environmental trigger (Jones, 2003).

1.9.9 Geographical clustering and PBC

An early study by Triger (1980) demonstrated that within the city of Sheffield in Northern England, 88% of the PBC patients received their water from the Revelin reservoir which accounts for 40% of the total water supply for the local population

(Jones, 2003). Geographical clustering within a UK health administration of Northern-Eastern England has recently been demonstrated (Prince *et al.*, 2001). Prince *et al* (2001) found that the risk for PBC was higher in the urban area of Tyneside than in the surrounding rural area. Within the rural area, there was significant clustering of cases. PBC occurred to a density of 10.7 cases/km^2 in the highest risk areas (Prince *et al.*, 2001).

1.9.10 Familial clustering and PBC

Several studies have suggested that PBC cases tend to cluster within families, the prevalence of PBC being higher among family members of individuals with the disease (Bach and Schaffner, 1994, Brind *et al.*, 1995, Tsuji *et al.*, 1999, Agarwal *et al.*, 2002, Abu-Mouch *et al.*, 2003). In a study from the North-East of England the sibling relative risk for PBC was estimated at 10.5, suggesting a familial risk similar to that seen for other autoimmune diseases such as rheumatoid arthritis (Jones *et al.*, 1999). Interestingly, when two members of the same family are affected, the disease tends to occur at the same time suggesting the existence of an external trigger (Neuberger and Thomson, 1999).

1.9.11 Migration studies and PBC

People migrating between different geographical regions seem to acquire the prevalence of the host population, thus while in the UK, the prevalence is about 150–240 per million, the prevalence among British immigrants to Australia is 47 per million and for the Australian community only 19 per million. Similarly, while PBC is almost absent in India, the prevalence of end stage PBC among first generation migrants to the UK from the Indian subcontinent is 14 per million (Watson *et al.*, 1995, Anand *et al.*, 1996)

One interpretation of these observations would be that the affected individuals within the same family or the same region are exposed to common environmental triggers (Jones, 2003). Environmental risk for a disease may be attributable either to infectious agents, for example viruses or bacteria, or non infectious agents such as chemicals and drugs. A number of putative infective organisms have been implicated in the pathogenesis of PBC, including *Escherichia coli*, mycobacteria and retroviral agent (Burroughs *et al.*, 1984, Burroughs *et al.*, 1992, Vilagut *et al.*, 1994, Xu *et al.*, 2003).

1.10 PBC, bacterial infection and molecular mimicry

Hopf *et al.* (1989) reported an association between PBC and the presence of rough form mutants of *E. coli* in the patients' faecal samples, whilst Butler *et al.* (1995) reported reactivity to PDC-E2 in 52% of sera from patients with chronic urinary tract infections (UTI). Conversely, patients with PBC have a higher incidence of recurrent UTI (Butler *et al.*, 1995b). Recently, Mayo *et al.* (2000) reported that antibodies directed to an 18aa (177-194) sequence within the proteolytic subunit of the ATP-dependent Clp protease of *E. coli* are present in a third of sera from patients with PBC but in less than 1% of sera from patients with other autoimmune diseases. Further support to these reports was recently provided by Parikh-Patel *et al.* (2001) in the first controlled epidemiological analysis to show a positive association of PBC with UTI. These findings suggest an association between *E. coli* or other bacteria causing UTI and AMA responses (Hopf *et al.*, 1989, Burroughs *et al.*, 1992, Butler *et al.*, 1995a, Butler *et al.*, 1995b, Parikh-Patel *et al.*, 2001).

Granulomatous lesions identifiable either as aggregates of histiocytes or as non-caseating lesions adjacent to damaged bile ducts are frequently observed in the liver of patients with PBC, and the hypothesis that mycobacterial infection (able to induce granulomas) may be involved in the pathogenesis of PBC has been forwarded (Kaplan, 1996). Early studies have reported AMA detection by ELISA and immunoblotting in up to 43% of patients with active pulmonary tuberculosis and in patients with leprosy (Klein *et al.*, 1993, Gilburd *et al.*, 1994).

A study by Vilagut *et al.* (1994) showed that all of 19 sera from Spanish patients with PBC reacted specifically with an extract of *Mycobacterium gordonae* (MYCGO), consequently identified as the 65-kDa heat shock protein (hsp65) of MYCGO (Vilagut *et al.*, 1997). There were no other reactions with nine other atypical mycobacteria (Vilagut *et al.*, 1994). Furthermore, they demonstrated that anti-hsp65 MYCGO antibodies, cross-reacted with PDC-E2 (Vilagut *et al.*, 1994). In fact, cross-reactivity of AMA against prokaryotic antigens has been reported for a number of microbes, including *E. coli*, *Klebsiella pneumoniae, Proteus mirabilis, Staphylococcus aureus* and *Salmonella minnesota*, although the target components of AMA in these bacteria have not been fully determined (Sayers and Baum, 1976, Fussey *et al.*, 1991, Baum, 1995). Several arguments have been proposed in support for a role of molecular mimicry in PBC (Van de Water *et al.*, 2001). Many suggestions are based on the observation that

pyruvate dehydrogenase-E2 (PDC-E2), the major mitochondrial autoantigen recognized by both B- and T-cells from patients with PBC, is a well-conserved molecule among various species, including bacteria (Van de Water *et al.*, 2001). Sera and T-cells from patients with PBC react with both human and *Escherichia coli* PDC-E2, and the epitope of *E. coli* PDC-E2 also maps to similar lipoyl domains (Fussey *et al.*, 1991, Fussey *et al.*, 1990, Shimoda *et al.*, 1998). Further evidence of cross-reactivity is that antibodies raised in rabbits against rough mutants of bacteria (which have fragile cell walls) were reported to react with a typical M2 pattern on immunoblotting against mitochondria (Stemerowicz *et al.*, 1988).

In support of the role of molecular mimicry and immunological cross-reactivity in PBC is the finding by Shimoda *et al.* (1997) that human PDC-E2 and *E. coli* PDC-E2 cross-react at the T-cell level. One T-cell clone, specific to the lipoyl domain of human PDC-E2 (GDLLAEIETDKATI) was shown to cross-react with the exogenous peptide derived from the lipoyl domain of *E. coli* PDC-E2 (EQSLITVEGDKASM) and the ExDK motif was found to be essential for the T-cell epitope recognition (Shimoda *et al.*, 1998). The sequence ExDK is also found in *E. coli* PDC-E2 (Shimoda *et al.*, 1998). In a recent study, the same group of investigators was able to cross-reactively activate PDC-E2 specific clones with 7 additional microbial mimics, all but one originated from OADC, such as *Neurospora crassa* PDC-E2, *E. coli* OGDC-E2, *Neisseria meningitis* outer membrane protein P64K, *Acholeplasma laidlawii* PDC-E2, *Azotobacter vinelandii* PDC-E2 and *Pseudomonas putida* PDC-E2 (Shimoda *et al.*, 2000).

Taken together, these data suggest that the initial stimulus for an immune reaction directed specifically at the E2's of the OADC, (and E3BP of PDC), may be a chronic exposure, in the context of costimulatory signals, and perhaps a particular genetic background, to microbial peptides that share strong sequence similarities with the lipoyl-binding peptides at the core of the dominant epitopes (Burroughs *et al.*, 1992, Van de Water *et al.*, 2001). Expansion of reactivity and specificity to incorporate the entire domain, and then also to recognize E1 sub-units, would be by epitope-spreading (*see* 1.3.6). Because of the highly conserved nature of PDC-E2 among species, microbial mimics not of an OADC origin would be more convincing as playing a role in the pathogenesis of PBC. Such mimics had not been reported at the time the work described in this Thesis started.

1.11 Hypothesis

The hypothesis of the present thesis is that molecular mimicry and immunological cross-reactivity play a key role in the development or maintenance of liver autoimmunity with a special focus on PBC.

1.12 Aims

1) Humoral and cellular micro-organism/self crossreactive responses will be studied in primary biliary cirrhosis, a liver disease considered to have an autoimmune pathogenesis and epidemiologically linked to micro-organism exposure.
2) The appearance and evolution of cross-reactive immune responses will be investigated in liver kidney microsomal-1 (LKM1) antibody positive hepatitis C virus (HCV) infected patients.
3) Molecular mimicry before and after exposure to a specific viral antigen (hepatitis B surface antigen-HBsAg) will be investigated in vaccinees before and after immunisation against HBsAg.

CHAPTER 2

Material and Methods

2.1 MATERIALS

2.1.1 Commercially available chemicals, reagents, buffers and media

^{3}H-thymidine	Amersham Pharmacia Biotech Limited, Little Chalfont, Buckinghamshire, UK
β-mercaptoethanol (C_2H_6OS)	Bio-Rad Laboratories, Hertfordshire, UK
Acetic acid	BDH, VWR International Ltd, Poole, Dorset, UK
Acetonitrile	BDH
Bovine Serum Albumin (BSA) (Fraction V)	Sigma Aldrich, Gillingham, Dorset, UK
Brefeldin A	Becton Dickinson, Franklin Lakes, New Jersey, USA
Bromophenol Blue	Sigma Aldrich
Cytofix/Cytoperm	Becton Dickinson
Diaminobenzidine (DAB) ($C_{12}H_{14}N_4$ .4HCl)	Sigma Aldrich
Dimethyl sulfoxide (DMSO) (C_2H_6SO)	Sigma Aldrich
Fungizone	Invitrogen Life Technologies, Paisley, UK
Glycerol ($C_3H_8O_3$)	BDH
Heat inactivated foetal calf serum	Sigma Aldrich
Heparin (preservative free)	Leo Laboratories Ltd., Princes Risborough, Leicestershire, UK
Hydrogen peroxide (H_2O_2)	BDH
Lymphoprep (Ficoll)	Axis-Shield, Oslo, Norway
Methanol (CH_3OH)	BDH
Milk powder (fat free)	Nestlé, Vevey, Switzerland

Molecular weight rainbow coloured protein markers	Amersham Pharmacia Biotech Limited
o-Phenylenediamine dihydrochloride (OPD)	Sigma Aldrich
Penicillin/streptomycin	Life Technologies
Phosphate Buffered Saline (PBS) tablets	Sigma Aldrich
Polyoxyethylene-sorbitan monolaurate (Tween20)	Sigma Aldrich
Recombinant human interleukin-2	EuroCetus, Amsterdam, Netherlands
Ready mini-gels (15% Tris-HCl) for immunoblotting	Bio-Rad Laboratories
RPMI 1640 medium with glutamine	Life Technologies
Sodium azide (NaN_3)	BDH
Sodium dodecyl sulphate (SDS) ($C_{12}H_{25}O_4SNa$)	Bio-Rad Laboratories
Sodium perborate (0.03%) in phosphate citrate buffer (0.05 M, pH 5.0)	Sigma Aldrich
Streptavidin coated 96-well microplates	Mimotopes, Clayton, Victoria, Australia
Sulfuric acid (H_2SO_4)	BDH
Tris (0.25M)/Glycine (1.92M)/SDS (1%)	National Diagnostics, Hessle Hulk, UK
Trypan-blue (0.4%)	Sigma Aldrich
Urea (CH_4NO_2)	Bio-Rad Laboratories

2.1.2 Antigens, peptides

Biotinylated peptides	Mimotopes
Cytochrome P450IID6 (CYP2D6) recombinant (baculovirus)	Pharmacia
Herpes Simplex Virus-1 (HSV1) extract	Virusys, North Berwick, Maine, USA

Pyruvate dehydrogenase complex (PDC) purified from porcine heart	Sigma Aldrich
Small hepatitis B surface antigen (SHBsAg) recombinant (yeast)	Research Diagnostics, Flanders NJ, USA

2.1.3 Antibodies

FITC conjugated rabbit anti-human total IgG	Dako, Ely, UK
QRed conjugated anti-CD4	Sigma Aldrich
PE conjugated anti-IFNγ	Dako
Peroxidase conjugated goat anti-human IgA	Southern Biotechnology Associates Inc., Birmingham, Alabama, USA
Peroxidase conjugated goat anti-human IgG	*ibid*
Peroxidase conjugated goat anti-human IgM	*ibid*
Peroxidase conjugated mouse anti-human IgG1, IgG2, IgG3, IgG4	*ibid*
Peroxidase conjugated rabbit anti-human IgA, IgG, IgM, κ, λ	Dako

2.1.4 Non-commercially available antigens

Periplasmic maltose *E. coli*	Professor Winfried Boos, Konstanz, Germany

2.1.5 Plastics, consumables

Centrifuge tubes (50 ml; 15 ml)	Falcon, Becton Dickinson
Cryovials	Nunc, Roskilde, Denmark
Cryo 1°C Freezing Container	Nalgene, Nalge Europe Ltd., Hereford, UK
Disposable plastic pipettes	Bibby Sterilin Limited., Store Staffs, UK

FACScan tubes	Becton Dickinson
Filter and pipette tips	Starlab, Milton Keynes, UK
Laboratory film 'parafilm'	American National Can, Chicago, Illinois, USA
Microcentrifuge tubes	Thermo Life Sciences, Basingstoke, Hampshire, UK
Pasteur pipettes	Greiner Labortechnik Ltd., Darsley, Gloustershire, UK
Precision weighing boats	Thermo Life Sciences
Syringes	Becton Dickinson
Test tube racks	Thermo Life Sciences
Tissue culture plates; 96-, 48-, 24-well	Nunc
Trays	Euroimmun
Universal containers 20 ml	Bibby Sterilin Limited
Whatman paper	Biorad Laboratories

2.1.6 Commercial assays

2.1.6.1 ELISA

Anti-cytomegalovirus (CMV)	Euroimmun, Montypool, UK
Anti-HSV1	*ibid*
Anti-*helicobacter pylori* (HELPY)	*ibid*
Anti-liver kidney microsomal-1 (LKM1) (CYP2D6)	Pharmacia
Anti-mitochondrial-2 (M2)	Pharmacia

2.1.6.2 Immunoblot

Epstein-Barr virus (EBV)	Euroimmun
HSV1	*ibid*
HELPY	*ibid*

Liver (human) mitochondrial, microsomal and cytosolic antigen	*ibid*

2.1.7 Instrumentation

Alarm timer	Sharp Electronics, Manchester, UK
Autoclave (series 290)	Harvard LTE, Greenfield, Oldham, UK
Beta counter (model 1500)	Canberra Packard Ltd., Pangbourne, Berks, UK
Benchtop centrifuge (model Labofuge 400R)	Heraeus Instruments, Osterode, Germany
Cell harvester (model 200A/290)	Cambridge Technology Inc., Massachusetts, USA
Electronic calculator	Sharp Electronics
ELISA microplate reader (model MRX)	Dynex Technologies, West Sussex, UK
ELISA washer (MRW model)	Thermo Life Sciences
Epifluorescence microscope (Aristoplan)	Leica, Germany
FACScan flow cytometer	Becton Dickinson Immunochemistry Systems San José, California, USA
Finnpipettes	Thermo Life Sceinces
Gamma irradiator (model Gammacell-1000)	AECL Industrial, Atomic Energy of Canada Ltd., Ontario, Canada
Incubator (37 °C)	Jouan, Tring, Hertfordshire, UK
Incubator (CO_2) (model MCO-17AIC)	Sanyo, Jencons Scientific Ltd, Bedfordshire, UK
Laminar flow hood	Howorth

	Farnworth, Bolton, UK
Microcentrifuge (model IEC Centra-M)	Heraeus Instruments
Electrophoresis system (model MINI-PROTEAN II)	Bio-Rad Laboratories
Neubauer counting chamber	Weber Scientific International Ltd., London, UK
Pipette controller	Starlab
Semi-dry electrophoretic transfer cell (model Trans-Blot SD)	Bio-Rad Laboratories
Shaker	Dynex Technologies,
Spectrophotometer (model DU-62)	Beckman Instruments Ltd., High Wycombe, Buckinghamshire, UK
Thermometer	Themo Life Sciences
Vortex (model Genie 2)	Scientific industries (Si), New York, USA
Water-bath	Grant Instruments Limited, Nerts, UK

2.2 METHODS

2.2.1 Bioinformatics tools

2.2.1.1 PROTEININFO 'advanced sequence search'

ProteinInfo (http://129.85.19.192/prowl/proteininfo.html) is a search engine from Proteometrics (www.proteometrics.com) that enables detection of sequences that match a particular sequence motif (Altschul, 1991). Fragments of sequence motifs can be entered and all of the sequences matching that pattern in a particular database are retrieved. Proteinfo provides the ability to choose a search among three databases, namely SWISS-PROT, NCBInr and dbEST. An entry in a database can be found by selecting keywords or sequence motifs after selecting a species or taxa (Altschul, 1991). Proteinfo's 'analysis amino acid sequence' is a tool that gives the ability to perform a variety of analyses on an amino acid sequence or an entry in the database, including amino acid composition, hydrophobicity, disulfide maps and patterns of enzymatic digestion and mass spectrometric fragmentation (Altschul, 1991).

The PROTEININFO 'advanced sequence search' tool allows identification of sequence motifs with pre-selected amino acid substitutions in desirable positions of the sequence (Altschul, 1991). Instead of looking for entries in the database that contain the EITDKA sequence of human PDC-$E2_{222-227}$, for example, it is possible to search for proteins that possess the ExTDK[STAG] motif where x, corresponds to any of the 20 amino acids; the [STAG] preference searches for entries with S or T or A or G amino acid at this position i.e. for motifs such as ExTDKS or ExTDKT or ExTDKA or ExTDKG. The PROTEININFO 'advanced sequence search' has also the ability to perform searches investigating entries with a partial identity (for example 70% or 82%) with the sequence under investigation. For example, it is possible to search for *E. coli* proteins with more than 85% identity with human PDC-E2 -EITDKA- i.e. *E. coli* sequences that have an identity of at least 5 amino acids of any possible 5 amino acid combination with EITDKA.

2.2.1.2 BLAST

2.2.1.2.1 PSI-BLASTp

The Basic Local Alignment Search Tool (BLAST) provides a method for rapid searching of nucleotide (BLASTn) and protein (BLASTp) databases. Position Specific Iterative (PSI) BLAST refers to a feature of BLAST in which a profile table that lists the frequencies of each amino acid in each position of protein sequence is automatically constructed from a multiple alignment of the highest scoring hits in an initial BLAST search (Schaffer *et al.*, 1999, Schaffer *et al.*, 2001). PSI-BLAST calculates the number of different alignments that are expected to occur in a database search by chance. The lower the expected (E) value the more significant the score (Schaffer *et al.*, 1999, Schaffer *et al.*, 2001). PSI-BLASTp filters out those cases where the pattern occurrence is probably random and not indicative of homology and permits calculated expected (E)-values to take into account the amino acid composition of the individual database sequences involved, achieved with a scaling statistical procedure which provides a scoring system for each alignment (Schaffer *et al.*, 1999, Schaffer *et al.*, 2001). By applying this programme, alignments from different proteins with equal number of identities can receive different scores, based upon their amino acid composition (Schaffer *et al.*, 1999, Schaffer *et al.*, 2001).

2.2.1.2.2 BLASTp 2 sequences

'BLASTp 2 sequences' is an interactive tool that utilizes the BLAST engine for pairwise protein-protein sequence comparison based on the same algorithm and statistics of local alignments that have been described for PSI-BLAST (Tatusova and Madden, 1999).

Different scoring matrices are provided for protein-protein comparisons; each matrix is most sensitive at finding similarities at a specific evolutionary distance (Tatusova and Madden, 1999). The default matrix, BLOSUM62 is generally considered to be the best for a wide variety of distances; PAM70 is suitable for comparison of short sequences (Tatusova and Madden, 1999).

2.1.2.3 Cn3D visualisation tool

Cn3D is a three-dimensional visualization tool for biomolecular structures, sequences, and sequence alignments (http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml). Cn3D displays structure-structure alignments along with their structure-based sequence alignments, to emphasize what regions of a group of related proteins which are most conserved in structure and sequence. Cn3D, version 4 is a complete multiple alignment editor giving the ability to create and even annotate multiple alignments. With the help of Cn3D, the three dimensional structure of short sequences, motifs or domains of a protein can be further analysed based on the structure of the whole protein deposited in the database (Figure 2.1).

Cn3D View menu contains items that control how the structure as a whole is displayed. The Show/Hide menu contains operations that allow certain substructures, chains, and domains to be shown or hidden. The choices in the Style menu affect the shape and colour of the different parts of the structure, for example, green for helices, orange for strands, and blue for coil.

Cn3D intentionally keeps colour separate from drawing style. The Style: Colouring/Shortcuts menu choices determine what properties are used to map different parts of the structure to different colours. Using the choices of the menu, it is for example easy to visualize a structure's NCBI-determined domain composition (Domain), or crystallographic temperatures (Temperature). Rendering Shortcuts choices determine the shape of the various parts of the structures, like Worms or Ball and Stick. When a single structure is loaded into Cn3D, the sequence viewer shows the sequences of all protein and nucleic acid chains in the structure. The colour of each residue is coordinated between the structure and sequence windows: each letter of the sequence represents a residue in the structure, and always adopts the colour of the backbone's alpha carbon, even if side chains are coloured differently from backbone in the structure window.

The most powerful application of the sequence window in Cn3D is to allow easy correlation of residues in the sequence with atoms in the structure. Thus, the user can quickly locate and highlight selected parts of the protein from the sequence window.

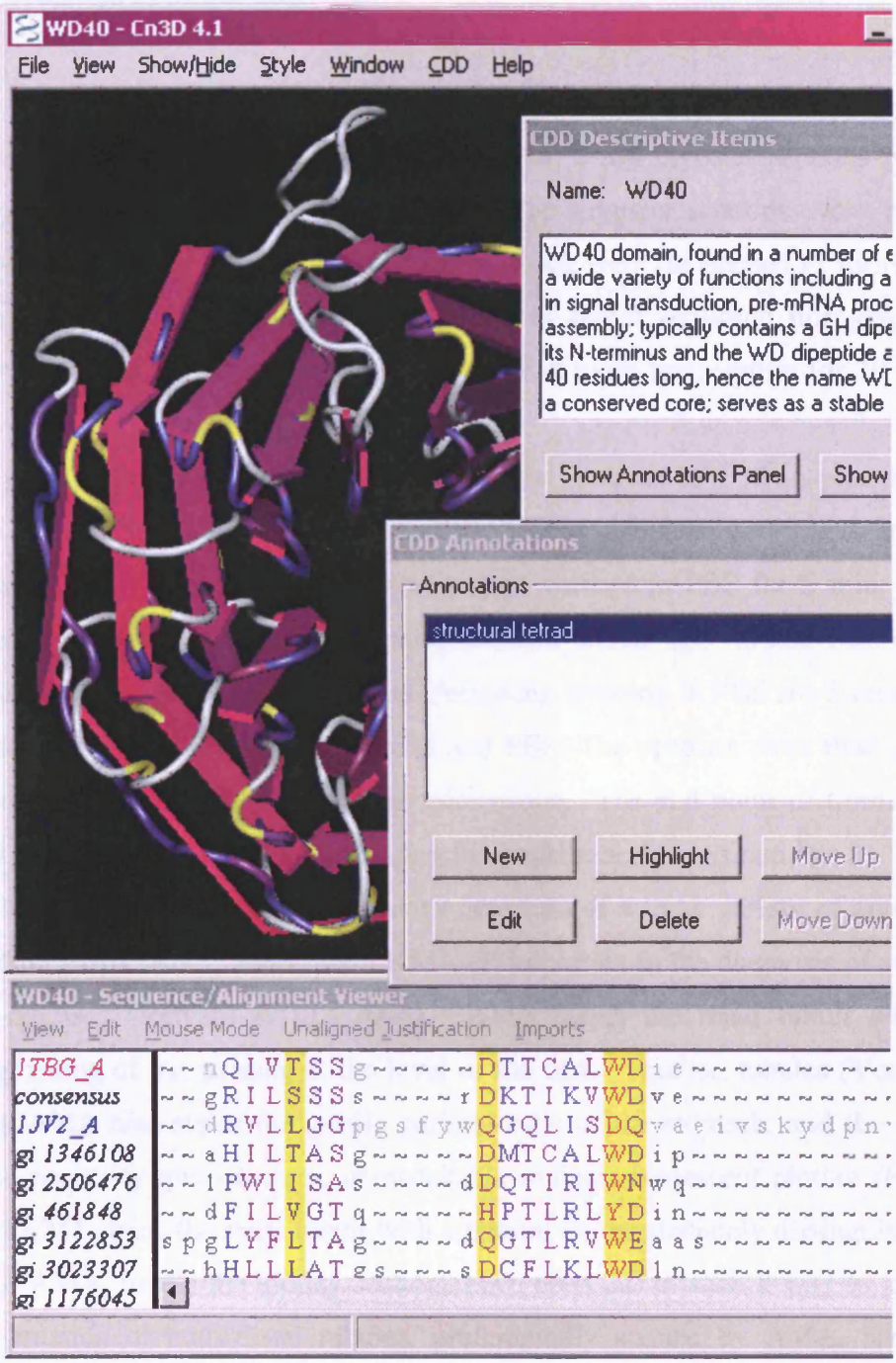


Figure 2.1 *A representative protein structure shown by Cn3D. This example is the WD40 domain, which is a multi-functional 7-fold beta propeller. Cn3D is showing a representative protein structure, the family alignment, and annotation panels with information about annotated features of this protein family. Highlighted in both structure and sequence windows are the conserved residues in a pattern characteristic to this domain*

2.2.2 Detection of antibodies

2.2.2.1 Indirect Immunofluorescence

Tissue autoantibodies were detected by IIFL on 5-μm cryostat sections of rat liver, kidney and stomach (Smith *et al.*, 1974). The cryostat sections were prepared as follows: segments of rat liver, kidney and stomach were juxtaposed to form a block of 0.3 cm on a cryotome chuck and immersed in fumes of liquid nitrogen. After the cryostat sections were cut, they are fixed in air at room temperature for 30 min before use.

Fifty μl of patient sera were tested at the initial dilution of 1/10 in phosphate buffer saline (PBS, 0.15 M, pH 7.2). Tissue sections were incubated with the diluted sera for 30 min at room temperature. Slides were then washed in PBS for 5 min. Fifty μl of fluorescein isothiocyanate (FITC)-conjugated anti-human IgG diluted 1/50 were added and the sections incubated for 30 min. Following washing in PBS for 5 min, the slides are mounted in 9:1 solution of glycerol and PBS. The sections were finally examined for flourescent staining by ultraviolet microscope. The end point of titration for sera tested was determined by double diluting the positive sera to extinction.

This technique enables the simultaneous detection of a large variety of autoantibodies including AMA, ANA, SMA, and LKM1, all important in the diagnosis of autoimmune liver diseases (Vergani *et al.*, 2004). AMA stains the renal tubuli with a clear strengthening of the staining at the level of the distal, smaller, tubules (Vergani *et al.*, 2004). AMA also stains the gastric parietal cells of the stomach, and the dual renal-gastric positivity gives a clear, unmistakable immunofluorescent picture (Figure 2.2). Also LKM1 stains the renal tubuli with a pattern not immediately distinguishable from that of AMA, unless the kidney sections have been cut in such a way to give a good representation of both distal tubules, preferentially stained by AMA, and proximal tubules, preferentially stained by LKM1 (Figure 2.3). The presence of the liver in the composite substrate helps further in the differential diagnosis, since LKM1 gives a brilliant staining of the hepatocyte cytoplasm with contrastingly black, punched out nuclei, while AMA gives a much duller, granular cytoplasmic hue (Vergani *et al.*, 2004).

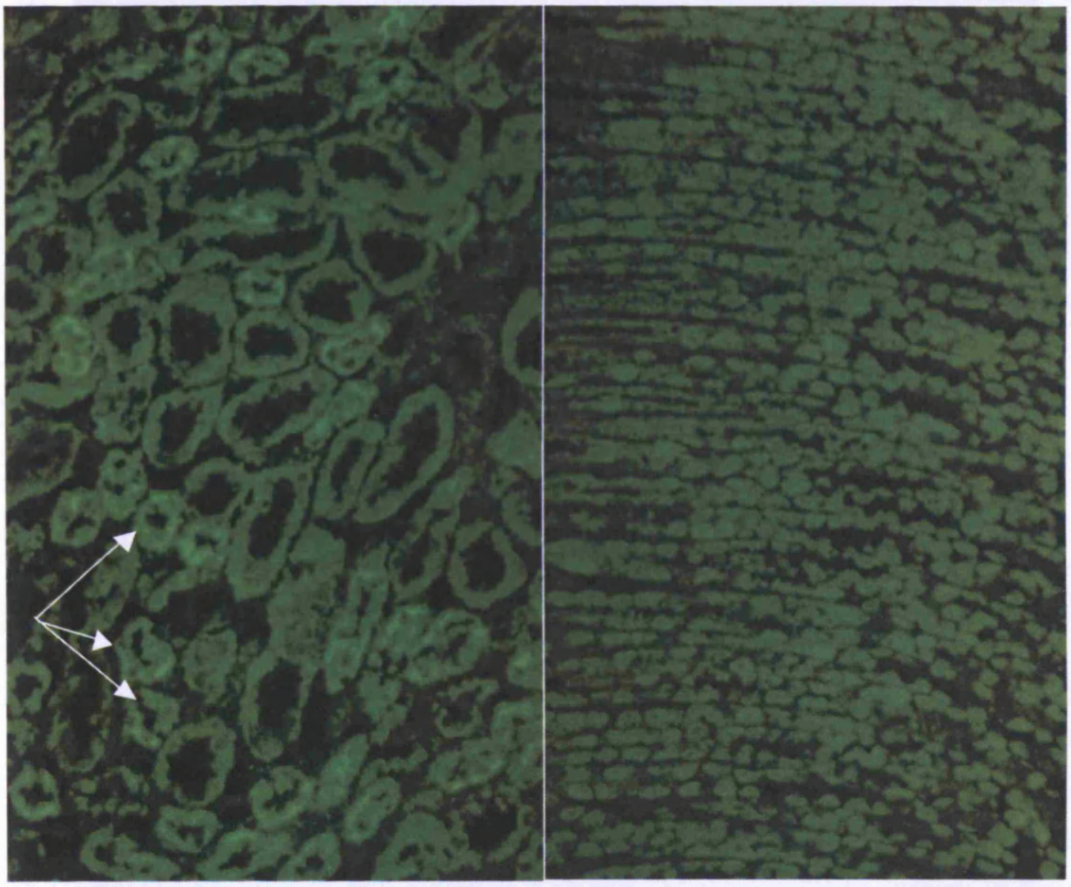


Figure 2.2 *Immunofluorescence appearance of anti-mitochondrial antibodies. Substrate: rat kidney (left), rat stomach (right). AMA stains the renal tubuli with stronger staining of the distal tubules (arrows). These are readily recognisable because smaller. The diagnosis is confirmed in the stomach, where AMA characteristically stains the gastric parietal cells*

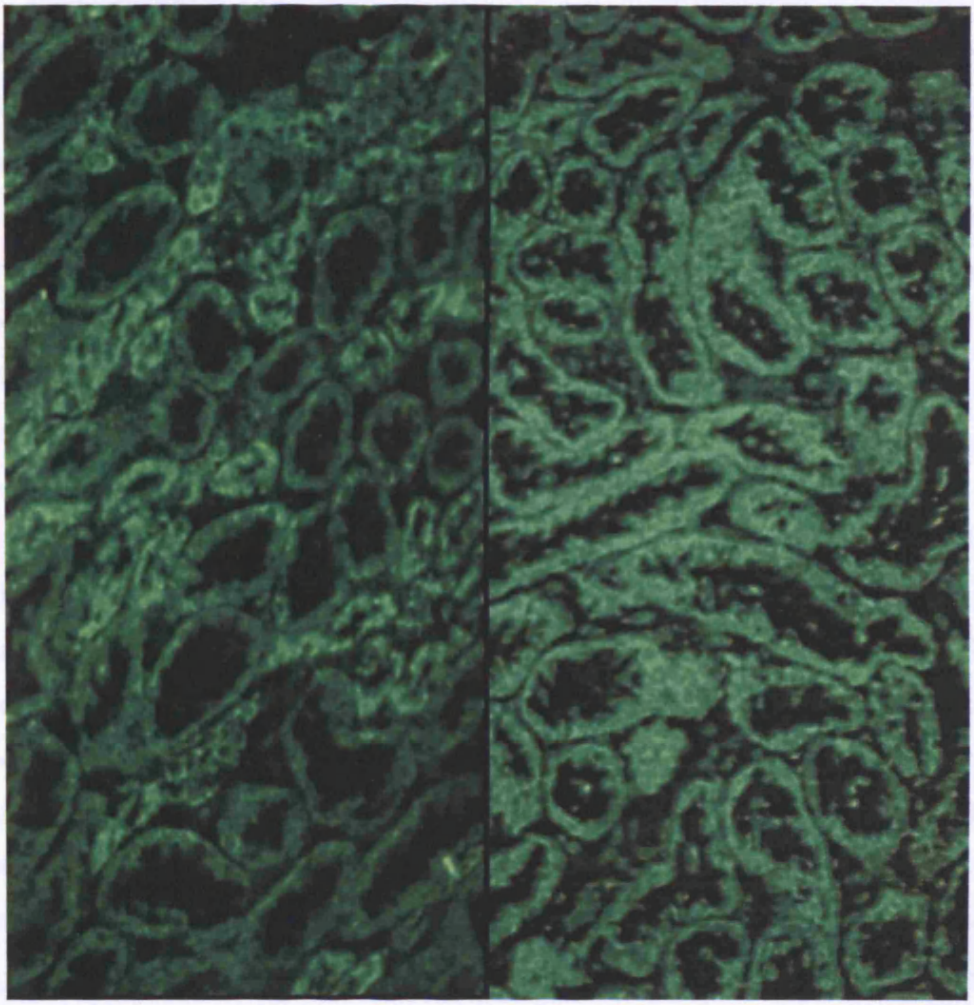


Figure 2.3 *Immunofluorescence appearance of anti-mitochondrial (AMA) (left) and liver kidney microsomal antibody (LKM1) on rat kidney. The two autoantibodies are readily distinguishable here, since AMA shows a stronger staining of the smaller, distal tubules. LKM1 and AMA are frequently misdiagnosed, especially when only the kidney substrate is used and the sections do not contain both proximal and distal tubules. The use of other tissues such as stomach and liver is very important to facilitate the diagnosis, since they give different patterns characteristic for each of the two autoantibodies (Vergani et al., 2004)*

2.2.2.2 Immunoblotting

Identification of antigen by immunoblotting requires the protein to be immobilized on a membrane, where it is identified by a specific antibody or human serum samples containing antibodies specific for this antigen. Following solubilization, the protein sample is separated by discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) system (Laemmli, 1970). This system uses one-dimension polyacrylamide mini-gel electrophoresis to separate proteins under denaturing conditions, i.e., in the presence of 0.1% SDS. Blotting is performed in a tank of buffer with the gel completely submerged between two electrode panels. In this setting, proteins are separated only according to their molecular size as they move through a polyacrylamide gel matrix toward the anode. The unique combination of speed and high resolution is the foremost advantage of mini-gel electrophoresis.

A ready-made 2-well (one large for the protein sample and one smaller for the molecular weight marker) mini (7.0 cm height x 8.3 cm width 1.0 mm thickness) 15% gel was used for the separation of proteins with a molecular size up to 100 kDa; a 7% gel was used for the resolution of proteins with a molecular size ranging from 100 kDa to 200 kDa. The protein sample to be analysed was placed in sample buffer and boiled at 100 °C for 6 min. Ready-made mini gels are loaded with 25 μg/membrane of protein. The gels were run at 200 volt (V), using constant voltage mode for 45 min. Preliminary experiments using a series of different concentrations of antigen (5 – 50 μg/membrane) have been performed in the present study to define the optimal conditions of the assay. A given amount of protein was chosen when it was immunofixed as a clear band by a positive serum, but was not detected by 7 serum samples from healthy controls and 12 pathological controls. Biotinylated molecular weight standards ranging from 14.4 to 205 kDa were used in adjacent wells to provide a reference against which to compare the proteins to be analysed. After electrophoresis, the stacking gel was removed and placed in transfer buffer. Blotting transfer buffer must allow effective elution from the gel matrix as well as effective binding of the proteins to the membrane. The commonly used buffer to transfer from SDS gels to nitrocellulose is 39 mM glycine, 48 mM Tris, pH 9.2 containing 0.0375% (w/v) SDS and 20% (v/v) methanol. This buffer maintains the negative charge of SDS-bound proteins, and the methanol increases the binding capacity of nitrocellulose. The proteins separated by SDS-PAGE were transferred on top of the nitrocellulose filter paper in a semi-dry electrophoretic transfer cell in transfer buffer at 13v constant voltage for 1 h. A transfer blotting sandwich was assembled as

shown schematically in Figure 2.4. On one half of the transfer cassette, a scouring pad soaked in buffer was placed followed by two pieces of pre-wetted Whatman 540 paper cut to the same size of the gel. A transfer membrane also cut to the same size of the gel and pre-wetted with transfer buffer, was placed on the top of the filter, the gel being placed on the top of the membrane making sure there were no bubbles between the gel and the membrane (It is important not to trap bubbles at any stage as they block protein transfer). In the case of bubbles, these were removed by rolling a pipette smoothly across the surface. The sandwich was completed with the other two layers of Whatman paper and scouring pad, respectively (Figure 2.4). After transfer, the apparatus was dismantled and the transfer membrane was removed, noting the orientation of the blot by cutting off one corner of the membrane.

The part of the membrane which contained the molecular weight standards, was cut out and reacted with peroxidase-conjugated avidin to visualize the bands. The remaining membrane was incubated in blocking buffer for 1 h to absorb non-specific binding. The membrane was washed once for 15 min, and twice for 5 min, with fresh changes of washing buffer, at room temperature, on a rocking platform. The washed membrane was cut into strips and incubated with serum samples from patients and controls at dilutions ranging between 1/200 and 1/5,000 for 2 h at room temperature. The strips were washed as previously described and incubated for 90 minutes with the labelled peroxidase-conjugated anti-human total Ig or IgG or individual IgG subclasses antisera, respectively diluted 1/1000 – 1/4000 in blocking buffer according to the manufacturer's guidelines.

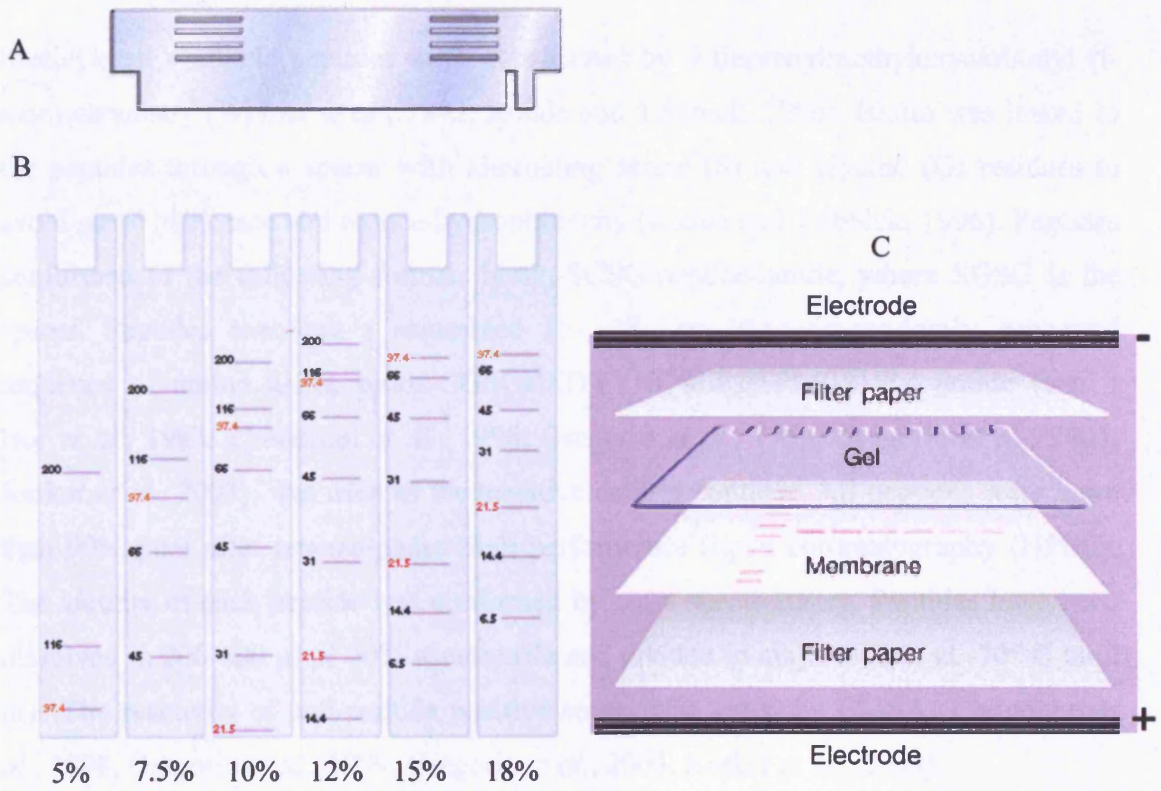


Figure 2.4 *Material specifications for ready precast gels (A-B) and schematic representation of the blotting sandwich (C). If the same protein sample is to be probed with multiple serum samples, a 2-well mini gel consisting of a large well for the protein sample and a smaller one for the molecular weight standard can be used as indicated by the type of comb of the ready precast gel (A). Migrations are based on the leading dye front being run to the bottom of a gel (B). Single-percentage gels produce the greatest resolution between any 2 bands that are close in molecular weight, whereas a linear gradient gel allows both high and low molecular weight bands to be visualized on the same gel (B). A blotting membrane (nitrocellulose) and 2 sheets of thick filter paper were preassembled into a blotting membrane/filter paper sandwich, to fit the ready Gel/Mini-PROTEAN-size gels (C)*

2.2.2.3 ELISA

Biotinylated synthetic peptides were constructed by 9-fluorenylmethyloxycarbonyl (f-moc) chemistry (Weiner *et al.*, 1992, Rodda and Tribbick, 1996). Biotin was linked to the peptides through a spacer with alternating serine (S) and glycine (G) residues to avoid steric hindrance and reduce hydrophobicity (Rodda and Tribbick, 1996). Peptides conformed to the following format: biotin-SGSG-peptide-amide, where SGSG is the spacer. Peptides encoding a scrambled 15-, 18-, or 20-meric randomly generated sequence of amino acids, biotin-SGSGHEDYVNQSLRPTPLEISVRA-amide (van 't Hof *et al.*, 1993, Choudhuri *et al.*, 1998, Gregorio *et al.*, 1999, Gregorio *et al.*, 2003, Kerkar *et al.*, 2003), was used as the negative control peptides. All peptides were more than 90% pure after reverse-phase high performance liquid chromatography (HPLC). The identity of each peptide was confirmed by mass spectrometry. Peptides have been dissolved in 200-600 µl of 40% acetonitrile and divided in aliquots kept at -70° C until use. The reactivity of anti-peptide positive serum was tested by ELISA (Choudhuri *et al.*, 1998, Gregorio *et al.*, 1999, Gregorio *et al.*, 2003, Kerkar *et al.*, 2003).

A total of 200 ml/well of 2% BSA/PBS was added for 1 h at 20°C to a 96-well polysterene plate pre-coated with 5 mg/ml streptavidin to prevent non-specific binding. The optimum concentrations of reagents at various steps of each immunoassay were determined in preliminary experiments by checkerboard titration. Two serum samples giving high anti-peptide readings were tested at dilutions of 1/100, 1/200, 1/300, 1/500, 1/1000 to define the dilution giving the lowest background noise and the optimal anti-peptide binding value using different concentrations of peptide (2, 5, 15, 30 µg/ml) (Kerkar *et al.*, 2003). After addition to each well of 100 ml of biotinylated peptide diluted to 1/1000 in PBS containing 0.1% sodium azide and 0.1% BSA, the plate was mildly agitated in a shaker for 1 h at 20°C. One hundred µl of patient serum diluted to 1/200 (except otherwise indicated) in 2% BSA/PBS containing 0.1% sodium azide were added to each well and incubated under mild agitation at 20°C for 90 minutes. After 90 minutes incubation, plates were incubated with 100 µl/well of serum diluted in 2% BSA/PBS containing 0.1% sodium azide. After each of the above steps, the plates were washed five times with PBS containing 0.1% Tween 20. After washing, 100 µl/well of horseradish peroxidase (HRP)-conjugated rabbit anti-total human Ig, or goat IgG, or mouse anti-human IgG subclasses (G1, G2, G3, G4) (Santiago *et al.*, 1997) diluted in 2% BSA/PBS were added. The reaction was detected by the addition of 100 µl/well of

freshly-prepared *o*-phenylenediamine (OPD) solution containing 0.4 mg/ml of 3% hydrogen peroxide in citrate phosphate buffer terminated with 100 µl/well of 4N sulfuric acid. Absorbance (optical density, OD) was read in a microplate reader at 490 nm. In each plate, one well was used as blank, in which serum and peptide were omitted, and two additional wells were used for a positive and a negative control. The positive control consisted of an LKM1-positive serum (titre 1/5120), which in preliminary experiments was shown to react with $CYP2D6_{253-272}$, that is a well-defined immunodominant epitope on CYP2D6 (Kerkar *et al.*, 2003). Randomly generated scrambled control peptides incubated with serum from a healthy individual were used as negative control. Each serum tested against experimental peptides was also tested against the control peptide. Reaction for a given peptide was considered positive when $OD^{test}/ OD^{control}$ peptide was ≥2, this cut off representing values higher than mean ±5 SD of anti-peptide readings of serum samples from up to 11 healthy subjects against 7 randomly selected peptides, the number of serum samples and peptides tested depending on the condition of the experiment and the total number of peptides and serum samples under investigation (Choudhuri *et al.*, 1998, Gregorio *et al.*, 1999, Gregorio *et al.*, 2003, Kerkar *et al.*, 2003).

All experiments were performed in triplicate. Repeated experiments gave variations <10%.

2.2.2.4 Inhibition studies

2.2.2.4.1 Liquid phase

To investigate whether the reactivity to microbial and homologous self peptides was due to cross-reactivity, competition ELISA were performed coating the plate with a microbial or self homologue and using the relevant microbial and self peptides and antigens, a control peptide and a control antigen as competitors in liquid phase (Choudhuri *et al.*, 1998, Gregorio *et al.*, 1999, Gregorio *et al.*, 2003, Kerkar *et al.*, 2003). A predetermined diluted serum was mixed with peptide solutions to give final peptide concentrations of 1000, 500, 250, 100, 65, 30, 15, 7.5, 3.8 µg/ml, respectively (except otherwise indicated). The solutions were incubated at room temperature for 90 minutes and then tested for residual anti-peptide antibody reactivity by ELISA.

2.2.2.4.2 Solid phase

Inhibition studies were also done using solid phase inhibitors (peptides or antigens). Briefly, 200µl of a test serum diluted in a predetermined dilution were added to the first well of an 8-well row pre-coated with either the test peptide or antigen, or control peptide or antigen. After 15 minutes incubation at 20°C on a shaker, the serum was sequentially transferred to wells 2-8, with a 15-minute incubation in each well. One hundred µl of absorbed serum were then tested for anti-peptide antigen and compared with antibody reactivity before absorption. Antibody detection was carried out under identical conditions to those described in *ELISA*.

2.2.2.5 Relative affinity of anti-peptide antibody

The relative affinity of antibody binding to microbial and self homologues was tested according to Quinn *et al* (1993) with slight modifications such as urea being used as a chaotropic agent instead of potassium thiocyanate (KSCN) (Hedman *et al.*, 1991). Microplate wells were coated with 100 µl of peptides in final concentrations as for the determination of anti-peptide antibody binding (Quinn *et al.*, 1993). After blocking for an hour and washing, wells were incubated with serum samples at a dilution of 1/1000 or 1/10000 (except otherwise indicated) for 2 hours at room temperature. Sera positive and negative for individual anti-peptide reactivities where used as controls. After washing, graded concentrations of urea (1M- 8M) were added to each well for 3 minutes to inhibit antigen-antibody interactions (Hedman *et al.*, 1991). After washing, the substrate solution was added and the reaction was stopped as previously described. Absorbance values in the absence and presence of urea were used to calculate the percentage (%) of inhibition of anti-peptide antibody binding by different concentrations of urea (Hedman *et al.*, 1991, Quinn *et al.*, 1993). Results were expressed as the mean ±SD of three experiments using individual serum samples.

2.2.3 Cellular studies

2.2.3.1 Separation of peripheral blood mononuclear cells

Ten to 40 ml of peripheral blood were obtained by venepuncture, and mixed with preservative-free heparin in a sterile syringe, to a final concentration of 10 U/ml (Plebanski, 2000). Aliquots of whole blood were layered on the top of an equal volume of Lymphoprep (Ficoll) density gradient solution of density 1.077 g/ml in a 20 or 40 ml sterile tube and centrifuged at 800*g* for 20 min at 20 °C, without brake (Plebanski, 2000). The milky-white layer of PBMC at the interface between the Ficoll (transparent) and the remaining plasma (yellow) was collected by aspirating it gently from the top with a sterile pipette to a fresh tube and washed at 300*g* for 7 min with RPMI 1640 medium without glutamine (Plebanski, 2000). In the presence of red cells, the flicked pellet of cell preparation was mixed gently with 10 ml of red blood cell lysis solution (ACK buffer containing 0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2 EDTA; pH 7.2 and left at room temperature). After 5 minutes, the solution was diluted with 25 ml of RPMI and 2 ml of foetal calf serum (FCS) to underlay the cells in the red blood cell lysis solution and centrifuged at 600*g* for 7 minutes at room temperature (Plebanski, 2000). After discarding the supernatant (including the FCS), the pellet was flicked and resuspended in 5 ml RPMI and washed by centrifuging at 600*g* for 7 minutes at room temperature. After discarding the supernatant, the flicked cells, clean of red cells (white pellet of cells), were washed again in RPMI and resuspended in the appropriate medium at the required cell concentration for further cell culture studies or diluted in frozen buffer (90% heat inactivated FCS and 10% DMSO) and stored in cryovials for 48 h at -70 °C and then transferred to a liquid nitrogen tank (Plebanski, 2000).

2.2.3.2 Thawing cryopreserved cells

Cryopreserved cells were thawed from the nitrogen tank by first transferring the vials to -70 °C for 48 h (Plebanski, 2000). Cells were then quickly thawed by immersion and gentle shaking of the vials in a 37 °C water bath (Plebanski, 2000). Cells were transferred from the vial to a 15 ml sterile Universal tube; initially slowly with gentle shaking (0.5 ml/min for the first 2 min), followed by 2 ml/min for the next 2 min and then topped up with 7 ml RPMI with glutamine (Plebanski, 2000). After centrifugation at 600*g* for 7 min at room temperature with brake, the supernatant was discarded and 12 ml of RPMI with glutamine were added for a final spin, as before (Plebanski, 2000). A

cell count was performed using a haemocytometer and lymphocyte viability was determined at the same time by Trypan-blue exclusion (Plebanski, 2000). Equal volumes of Trypan-blue solution and lymphocyte suspension were mixed, then put into a haemocytometer chamber and examined under a light microscope. The cell viability was consistently over 90% (Plebanski, 2000).

2.2.3.3 T-cell proliferation assay

The T-cell proliferation assay is based on the principle that engagement of the TCR by the HLA-antigen complex on the surface of autologous APC, together with the second signal results in T-cell activation, cytokine secretion, and cell division (Lenschow *et al.*, 1996). The level of antigen-induced T-cell proliferation, which is quantified by the incorporation or radiolabelled thymidine, gives a measure of the *in vivo* priming of T-cells specific for that antigen (Vyakarnam *et al.*, 2000). Since PBMC contain professional APC as well as T-cells, these cells can be used in proliferation assays without separation or further addition (Vyakarnam *et al.*, 2000). 1 x 10^6/ml cryopreserved or fresh PBMC were diluted in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 25 mM HEPES, 100 U/ml benzylpenicillin and 0.1 mg/ml streptomycin, 2.5 µg/ml amphotericin B (Fungizone) (complete culture medium), without IL-2 and 100 µl/well were added in a 96-well round bottom plate in the presence of peptide, full-length antigen or non-antigen specific stimuli (Vyakarnam *et al.*, 2000, Plebanski, 2000). After a 7-day culture at 37 °C in a humidified 5% CO_2 atmosphere, the microcultures were pulsed with 0.5 µCi/well of ^{3}H-thymidine for 18 h and harvested onto glass-fibre filter papers using a multichannel harvester (Vyakarnam *et al.*, 2000). Filters were discharged automatically into scintillation vials, allowed to air-dry, 1 ml scintillant added and the amount of incorporated ^{3}H-thymidine was determined by liquid scintillation spectroscopy (Vyakarnam *et al.*, 2000). In each experiment, 5-8 wells containing PBMC, but no antigen served as negative controls (Vyakarnam *et al.*, 2000). Each peptide or full-length antigen was tested in triplicate (Vyakarnam *et al.*, 2000). Results were expressed as the stimulation index (SI) which is the ratio of the mean counts per min (cpm) from triplicate determinations in the presence of antigen to the mean cpm obtained in the absence of antigen. The wells exhibiting an SI >3 were considered positive in T-cell

proliferation experiments (Shimoda *et al.*, 1995, Shimoda *et al.*, 1998, Shimoda *et al.*, 2000, Vyakarnam *et al.*, 2000).

2.2.3.4 Intracellular cytokine staining

Flow cytometry is a powerful analytical technique in which individual cells can be simultaneously analysed for several parameters, including size and granularity, as well as the expression of surface and intracellular markers defined by fluorochrome labelled antibodies (*see* 2.1.3) (Prussin and Metcalfe, 1995, Openshaw *et al.*, 1995, Carter and Swain, 1997). Fluorescent anti-cytokine monoclonal antibodies have become very useful for the intracellular staining and multiparameter flow cytometric analysis of individual cytokine-producing cells within mixed cell populations (Jung *et al.*, 1993, Vikingsson *et al.*, 1994, Prussin and Metcalfe, 1995). Multicolour immunofluorescent staining with antibodies against intracellular cytokines and cell surface markers provides a high resolution method to identify the nature and frequency of cells which express a particular cytokine(s) (Jung *et al.*, 1993, Vikingsson *et al.*, 1994, Prussin and Metcalfe, 1995, Openshaw *et al.*, 1995, Nicholson *et al.*, 1996, Picker *et al.*, 1995). For example, multicolour immunofluorescent staining of an individual cell surface antigen and two cytoplasmic cytokines has been used to identify and enumerate cell types which express cytokines in either a restricted (e.g., Th1- versus Th2-like cells) or unrestricted (e.g., Th0-like cells) pattern (Prussin and Metcalfe, 1995, Openshaw *et al.*, 1995, Nicholson *et al.*, 1996, Austrup *et al.*, 1997). In addition to enabling highly specific and sensitive measurements of several parameters for individual cells simultaneously, this method has the capacity for rapid analysis of large numbers of cells which are required for making statistically significant measurements (Roederer *et al.*, 1997, Herzenberg *et al.*, 2002).

Staining of intracellular cytokines depends on the identification of cytokine specific monoclonal antibodies which are compatible with a fixation-permeabilization procedure (Sander *et al.*, 1991). Optimal intracellular cytokine staining has been reported using a combination of fixation with paraformaldehyde and subsequent permeabilization of cell membranes with the detergent saponin. Paraformaldehyde fixation allows preservation of cell morphology and intracellular antigenicity, while also enabling the cells to withstand permeabilization by detergent (Sander *et al.*, 1991). Membrane permeabilization by saponin allows the cytokine-specific monoclonal antibody to

penetrate the cell membrane, cytosol, and membranes of the endoplasmic reticulum and Golgi apparatus (Sander *et al.*, 1991).

A direct two-colour immunofluorescent staining method was used in the present study to characterise T lymphocytes. Piars of different fluorochrome-conjugated antibodies able to bind to distinct cell surface determinants were used. Two lymphocyte markers could therefore be determined at one time. 0.5 x 10^6 cells/ml were incubated for 6 hours at 37°C in 10% FCS in RPMI, with the relevant peptides, in the presence of 20 μg/mL Brefeldin A, after the first 1 hour (Maini *et al.*, 1999, Maini *et al.*, 2000, Lopes *et al.*, 2003). Brefeldin A is used to block cytokine secretion and to induce intracellular accumulation (Yewdell and Bennink, 1989). Incubation without antigen was used as negative control. After incubation, cells were transferred to fluorescence activated cell sorter (FACS) tubes in the presence of 400 μl of PBS 1% FCS. After a centrifugation at 600g for 5 minutes at room temperature, the supernatant was removed and cells were gently vortexed. Cells were then stained with QRed-conjugated anti-CD4 antibody (1/100) and incubated for 20 minutes in 4 °C (Maini *et al.*, 1999, Maini *et al.*, 2000, Lopes *et al.*, 2003). After centrifugation, removal of the medium and vortexing as previously described, 100 μl of Cytofix/Cytoperm were added to permeabilize and fix the cell membranes, for 20 minutes at 4 °C. After centrifugation and removal of the medium, cells were stained with PE-conjugated anti-IFNγ in 100 μl of 0.1 saponin in PBS and incubated for 30 minutes in 4 °C. Following two 5 min washes with pre-cooled 0.1% FCS phosphate-buffered saline at pH 7.2, the cell pellet was resuspended in 400 μl of fixation buffer and analyzed by flow cytometry. Lymphocytes were identified by their characteristic forward and 90° light scatter properties and a gate is set to exclude debris. Fluorescence signal overlap was subtracted by electronic compensation using lymphocytes stained with a single fluorochrome. A minimum of 5,000 events was acquired for each sample and analyzed using the *FACScan Research* software (Becton Dickinson). Quadrant markers were set using preparations of lymphocytes stained with mAbs conjugated with either QRed or PE alone or in combination, such that cells in quadrant 1 stain with PE-conjugated antibody alone, in quadrant 2 with both fluorochromes, in quadrant 3 with neither and in quadrant 4 with QRed-conjugated antibody alone. After washing, cells were analyzed by flow cytometry.

2.2.4 T-cell 'help' for B-cell antibody production

For the study of ClpX specific T-cell 'help' in the production of anti-ClpP antibodies in patients with PBC, 1 x 10^6/ml cryopreserved or fresh PBMC were stimulated with PDC antigen (15μg/ml) in complete medium with IL-2 (10 U/ml) in the presence of 0.5-1 x 10^6 irradiated (3,000 rad) autologous PBMC (feeder cells) as APC (Stevens *et al.*, 1988, Mosmann and Coffman, 1989, Mahon *et al.*, 1995). Ionising radiation was used to abolish selectively the functions of radiosensitive subpopulations of immunocompetent cells. Irradiation prevents replication of feeder cells and antigen presenting cells. Ionising radiations are quantitated in Gray (Gy) or rads. One Gy is equal to 100 rads. A Gammacell-1,000 irradiator was used in this study. The gamma irradiation source used was caesium 137, which has a half-life of 30.2 years.

Every 3 days, 0.5 ml/ well of the cell-free supernatants were aspirated and the cultures replenished with fresh complete medium for 2 weeks in the presence of IL-2. At day 14, cells were washed and 1 x 10^6/ml PBMC were cultured for 72 hours in 24-well plates with individual human PDC-$E2_{212-226}$, ClpX ECOLI, ClpP ECOLI, ClpP human peptides (15μg/ml) in complete medium without IL-2 and in the presence of 0.5 x 10^6/ml irradiated autologous PBMC. At the end of the culture, the cell-free supernatant was collected and tested by ELISA (*see* 2.2.2.3) for anti-peptide antibody reactivity while the cells were washed and subjected to intracellular cytokine staining.

2.2.5 Patients

Details of patients, pathological and normal controls studied in the present Thesis are given in individual Chapters. All subjects consented to participate in the study. The Local Ethical Committees approved the study protocol.

2.2.6 Statistical analysis

Occasionally results are expressed as individual numerical values and are given in a tabulated form. More commonly and when data conform to a normal distribution, results are expressed as means and their variability around the mean is illustrated by the standard deviation (SD) of the mean. Standard error of the mean is given instead when this notation is necessary for further statistical computation eg Student's t test. The unpaired Student's t test is employed to assess differences between means of the two

samples while the paired t test is used to ascertain differences between the means of paired observations. The correlation between two variables is analysed by the use of the Pearson coefficient of correlation (r), while the distribution of discrete variables in two sets of data are compared with the Chi square (χ^2) with the Yates's correction. When dealing with small numbers, the Fisher's exact test was used instead.

Data were also analysed using non-parametric statistical computations equivalent to the parametric ones. The non-parametric analysis applies equally well to date non-normally distributed as to those following a Gaussian distribution. In normally distributed sets of data, however, parametric tests tend to be more powerful discriminations than their non-parametric counterparts. For this reason, results of non-parametric tests (such as the Wilcoxon's rank sum test and the (R) Spearman's coefficient of correlation) are given only in those cases in which data cannot be reconciled with a normal distribution. In such cases the average value is given as the median, while the extent of variation in data distribution is indicated by the range. *P* values less than 0.05 are considered significant. Statistical analyses were performed using the SPSS (SPSS inc., Chicago, Illinois, USA) statistical package.

The candidate has performed all the experiments described in this Thesis, the only exception being the IIFL for autoantibody detection performed by Dr Edward T Davies, Immunology Department, Rayne Institute, King's College.

CHAPTER 3

Mycobacteria and PBC

3.1 BACKGROUND

PBC-specific AMA have been shown to cross-react with PDC-E2 of a variety of micro-organisms (Sayers and Baum, 1976, Fussey *et al.*, 1991, Baum, 1995). A study by Vilagut *et al.* (1994) showed that sera from Spanish patients with PBC reacted specifically with an extract of *Mycobacterium gordonae* (MYCGO). This reactivity was restricted to PBC, being absent in pathological controls including patients with HBV, HCV, AIH and normal controls (Vilagut *et al.*, 1994). Furthermore, these investigators demonstrated that this reactivity reflected antibodies directed to the 65-kDa heat shock protein (hsp65) of MYCGO, that cross-reacts with PDC-E2 (Vilagut *et al.*, 1997). Vilagut *et al.* (1997) went on to suggest the existence of a shared epitope between MYCGO hsp65 and human mitochondrial antigens as the reason for the observed cross-reaction. Following a protein database search showing that there is an excellent and almost unique match between part of the dominant B-and T-cell epitope of PDC-E2 and a peptide sequence in MYCGO and other mycobacterial hsp65s, the present study investigated whether the peptides showing such molecular mimicry are, firstly, targets of a PBC-specific humoral immune response and, secondly whether they are also targets of a cross-reactive immune response. A third aim was to compare such immune responses in Spanish and in British PBC populations in view of the different PBC-specificity of reactivity against MYCGO reported in a previous study (O'Donohue *et al.*, 1994).

3.2 METHODS

3.2.1 Subjects

Serum samples were obtained from 90 patients with PBC: 40 followed up at the Liver Unit, Hospital Clinic, Barcelona, Spain (38 female, mean age 55.3 years±12SD), all but one AMA positive by IIFL (median titer: 1/400, range 1/50 – 1/800), mean duration of disease 83 months (range 5 –221) and 50 attending the out-patient clinic of the Liver Transplantation and Hepatobiliary Medicine Unit, Royal Free Hospital, London, United Kingdom (44 female, mean age 54.9 years±11SD), all but two AMA positive by IIFL (median titre: 1/640, range 1/40 – 1/10240), mean duration of disease 49 months (range 1-180). Demographic, biochemical and clinical characteristics of the patients are given in Table 3.1. Ten of 40 sera from Spain were aliquots stored at –70°C from the original study by Vilagut *et al.* (1994). All sera were tested under code.

Eighty-four chronic HCV infected subjects, all HCV RNA positive, 44 from Spain (16 female, mean age 39±13.2SD) and 40 from UK (28 female, mean age 47±11SD), all AMA negative, were tested as pathological controls.

3.2.2 Detection of AMA

For the present study, AMA was retested by conventional IIFL (*see* 2.2.2.1) and was further evaluated by immunoblotting (*see* 2.2.2.2) and by ELISA (*see* 2.2.2.3) using as antigen purified PDC from porcine heart mitochondria (*see* 2.1.2).

3.2.3 Protein database search and analysis

Identification of MYCGO hsp65 sequences homologous to human PDC-E2 was carried out through two complementary approaches. First, the complete 120 aa MYCGO hsp65 protein fragment was serially divided into 6 aa segments, each overlapping the preceding segment by 1 aa. The resulting set of 115 hexamers was used to scan for sequence-sharing motifs between MYCGO hsp65 and the 614 aa human PDC-E2 protein using the '*ProteinInfo*' computer-assisted programme (*see* 2.2.1.1). A similar investigation was carried out by dissecting the MYCGO hsp65 protein fragment into 5 and 4 aa motifs that were used as sequence probes for comparison to human PDC-E2. Second, the resulting homologies were further evaluated by comparing the MYCGO

hsp65 and human PDC-E2 using the computer-assisted protein-protein sequence comparison programme '*BLASTp 2 sequences*' (*see* 2.2.1.2.2).

3.2.4 Peptide synthesis

Six biotinylated (*see* 2.2.2.3) 15-mer regions containing the relevant sequences: MYCGO $hsp65_{90\text{-}104}$ (-DQSIGDLIAEAMDKV-), human $PDC\text{-}E2_{208\text{-}222}$ (-KVGEKLSEGDLLAEI -), $PDC\text{-}E2_{212\text{-}226}$ (-KLSEGDLLAEIETDK-), $PDC\text{-}E2_{216\text{-}230}$ (-GDLLAEIETDKATIG-), and $PDC\text{-}E2_{220\text{-}234}$ (-AEIETDKATIGFEVQ-), human $hsp60_{179\text{-}193}$ (–DKEIGNIISDAMKKV-) and an irrelevant 15-mer control peptide were synthesized (*see* 2.2.2.3).

3.2.5 ELISA

Antibody binding to the peptides was determined by ELISA (*see* 2.2.2.3). The final peptide concentration was 15μg/ml and the dilution of serum samples was 1/200 (*see* 2.2.2.3).

3.2.6 Inhibition studies

To investigate whether the simultaneous reactivity to MYCGO $hsp65_{90\text{-}104}$ and $PDC\text{-}E2_{212\text{-}226}$ was due to cross-reactivity, competition ELISA were performed (*see* 2.2.2.4.1) measuring residual anti-MYCGO $hsp65_{90\text{-}104}$ antibody reactivity after incubation with MYCGO $hsp65_{90\text{-}104}$, and $PDC\text{-}E2_{212\text{-}226}$, PDC antigen, control peptide and control antigen (cytochrome P4502D6) as liquid phase competitors (final concentrations: 1000, 500, 250, 100, 65, 30, 15, 7.5, 3.75 μg/ml).

3.2.7 Anti-peptide antibody affinity

The relative affinity of antibody binding to MYCGO $hsp65_{90\text{-}104}$ and $PDC\text{-}E2_{212\text{-}226}$ was also investigated (*see* 2.2.2.5).

3.2.8 IgG subclass of anti-PDC-E2 antibodies

IgG subclasses of PDC-E2 specific autoantibodies were determined by immunoblotting (*see* 2.2.2.2) in serum samples from 30 PBC patients including 10 MYCGO $hsp65_{90\text{-}104}$ reactive Spanish cases and 20 MYCGO $hsp65_{90\text{-}104}$ unreactive cases (10 Spanish and 10 British), all reactive with PDC-E2 antigen and peptide.

3.3 RESULTS

3.3.1 Protein database search and analysis

Amino acid comparison revealed a 7/8 (88%) aa similarity (6 identities, 1 conservative substitution) between MYCGO hsp65$_{92-99}$ (**SIGDL*I*AE**) and human PDC-E2$_{214-221}$ (**SEGDL*L*AE**) (identities in **bold**, conservative substitutions in *italic*). No other penta- or tetrameric sequences are shared in common between MYCGO hsp65 and human PDC-E2. Database searches confirmed that, in bacteria, the motif SxGDL[IL]AE is virtually unique to mycobacterial hsps (SIGDLIAE), the only exception being SEGDLVAE in β galactosidase of *Lactobacillus delbrueckii* (*see* 4..3.1). The only human sequence containing that motif is the inner lipoyl domain of PDC-E2.

In summary, there is an excellent and almost unique match between sequences of the dominant epitope of PDC-E2 and of mycobacterial hsp65.

3.3.2 ELISA

To delineate the most antigenically relevant sequence within PDC-E2$_{208-234,}$ - the core sequence of the inner lipoyl domain surrounding the key lysine residue (K_{226}), site of attachment of lipoic acid (Yeaman *et al.*, 2000) -, 14 randomly selected PBC sera were tested against four 15-mer peptides overlapping by 11 aa spanning the full PDC-E2$_{208-234}$. Reactivity to PDC-E2$_{208-222}$, PDC-E2$_{212-226}$, PDC-E2$_{216-230}$, and PDC-E2$_{220-234}$ was present in 3, 14, 6 and 7 of the 14 PBC patients, respectively, absorbance values being highest against PDC-E2$_{212-226}$ in 11 of the 14 cases. Thereafter, studies focused on the PDC-E2$_{212-226}$ sequence.

3.3.2.1 Single anti-peptide antibody reactivity

Table 3.1 summarises demographic, clinical, immunological and peptide antibody binding details of the 40 Spanish and 50 British patients with PBC.

Reactivity to MYCGO hsp65$_{90-104}$ was found in 20 (50%) of the 40 Spanish PBC patients and in 2 (4%) of the British PBC patients (p=0.000002). None of the HCV infected subjects reacted with MYCGO hsp65$_{90-104}$.

Reactivity to the human PDC-E2$_{212-226}$ was found in 38 (95%) of 40 Spanish PBC patients and in 48 (96%) of 50 British PBC patients. None of the Spanish or British HCV infected subjects reacted with PDC-E2$_{212-226}$.

3.3.2.2 Double anti-peptide antibody reactivity

Double reactivity to the MYCGO hsp65$_{90\text{-}104}$/human PDC-E2$_{212\text{-}226}$ pair was present in 19 (47.5%) Spanish PBC patients and in 2 (4%) of the 50 British PBC patients ($p=0.000004$). No sera were found to react with human hsp60$_{179\text{-}193}$.

Among the 10 serum samples from the original study of Vilagut *et al.* with an evident anti-MYCGO hsp65 antibody reactivity, 9 (90%) were MYCGO hsp65$_{90\text{-}104}$/human PDC-E2$_{212\text{-}226}$ double reactive.

There were no differences in anti-PDC antibody titres or other clinical, biochemical or histological parameters between the Spanish PBC patients with or without MYCGO hsp65$_{90\text{-}104}$/human PDC-E2$_{212\text{-}226}$ double reactivity, as well as between the Spanish (double reactive or unreactive) and British PBC patients

Table 3.1 Demographic, immunological features and anti-peptide reactivity details of the 40 Spanish and the 50 British PBC patients

	Spanish PBC (n=40)	British PBC (n=50)	*p*
Female/Male	38/2	44/6	ns
Age (years)	55.3±1.27	54.9±1.6	ns
Duration of disease (months)	83.3±7.5	79.3±5.8	ns
AMA IIFL (pos/neg)	39/1	48/2	ns
AMA IIFL titres, median (range)	1/400 (1/50-1/800)	1/640 (1/40-1/5120)	ns
AMA ELISA titres (U/ml)	84.4± 6.1	78.3±3.14	ns
Anti-$MYCGO_{90-104}$ (Mean $*OD^{test/control}$ ±SEM)	20/40 (50%) 3.4±0.37	2/50 (4%)	0.000002
Human $PDC\text{-}E2_{212-226}$ (Mean $OD^{test/control}$ ±SEM)	38/40 (95%) 3.34±0.18	48/50 (96%) 3.44±0.19	ns ns
$MYCGO_{90-104}$/$PDC\text{-}E2_{212-226}$	19/40 (47.5%)	2/50 (4%)	0.000004

*PBC, primary biliary cirrhosis; AMA, anti-mitochondrial antibody; IIFL, indirect immunofluorescence; MYCGO, Mycobacterium gordonae; PDC-E2, pyruvate dehydrogenase complex-E2 subunit; *, absorbance values are expressed as optical density* $(OD)^{test/control\ peptide}$ *and considered positive ≥2 (see 2.2.2.3); SEM, standard error of the mean; n, number; ns, not significant*

3.3.3 Inhibition studies

3.3.3.1 Inhibition of anti-MYCGO antibody reactivity

Inhibition of anti-MYCGO hsp65$_{90\text{-}104}$ antibody binding by pre-incubation with relevant and control peptides/antigens are given in Table 3.2 and Figure 2.1.

Antibody binding to MYCGO hsp65$_{90\text{-}104}$ was inhibited by pre-incubation with the MYCGO hsp65$_{90\text{-}104}$ by 67.8-85.2% in all 10 MYCGO hsp65$_{90\text{-}104}$/PDC-E2$_{212\text{-}226}$ double-reactive cases where competition experiments were performed (Table 3.2). Among these, in cases 9, 10, 16, 17 (Figure 3.1A), and 35 of Spanish and case 11 of British serum samples (Figure 3.1B) antibody binding to MYCGO hsp65$_{90\text{-}104}$ was inhibited 68-77% by pre-incubation with human PDC-E2$_{212\text{-}226}$ and 65-78.1% by pre-incubation with PDC antigen (Table 3.2).

In cases 21, 34 and 36 of Spanish and 23 of British serum samples anti-MYCGO hsp65$_{90\text{-}104}$ antibody binding was not inhibited much beyond what was observed with control antigen or peptide, by pre-incubation with human PDC-E2$_{212\text{-}226}$ (12-23.6% inhibition) or PDC antigen (13.4-19.4% inhibition) (Table 3.2).

The level of inhibition in case 23 of the British serum samples reached 60.7% when 62.5 μg/ml of PDC antigen was used as liquid phase competitor; the inhibition decreasing thereafter when up to 1000 μg/ml PDC antigen was used for competition (Table 3.2).

3.3.3.2 Inhibition of anti-PDC-E2 antibody reactivity

The inhibition experiments were also performed in reverse. Anti-PDC-E2$_{212\text{-}226}$ antibody reactivity was tested after pre-incubation of individual serum samples from the 6 microbial/self cross-reactive cases with relevant and control peptides/antigens. In all cases (9, 10, 16, 17, 35 of Spanish and 11 of British serum samples) (Table 3.2B and Figure 3.1C-D), antibody binding to human PDC-E2$_{212\text{-}226}$ was inhibited up to 78.1%, 77% and 77% by pre-incubation with PDC-E2$_{212\text{-}226}$, MYCGO hsp65$_{90\text{-}104}$, and the PDC antigen respectively.

Table 3.2 Inhibition of anti-MYCGO $hsp65_{90-104}$ (A) and anti-PDC-$E2_{212-226}$ (B) antibody reactivity by pre-incubation with relevant and control peptides and antigens of MYCGO $hsp65_{90-104}$/PDC-$E2_{212-226}$ double reactive PBC cases

Case #	Origin	Maximum Inhibition (%)				
A.		MYCGO $hsp65_{90-104}$	Human PDC-$E2_{212-226}$	PDC antigen	Control antigen	Control peptide
9	Spanish	76.2	77	72.5	11.2	6.7
10	Spanish	72.3	69.2	72	7.8	11.2
16	Spanish	79.1	72.1	78.1	13	8.2
17*	Spanish	82.8	71.2	77.2	14.2	9.2
21	Spanish	69	17	14.2	15.1	4.5
35	Spanish	79.9	69.2	65	13.2	11.2
34	Spanish	84.9	12	13.4	11.2	9.2
36	Spanish	75.1	21.2	19.4	11.9	5.9
11**	British	85.2	68	67	13.8	9.2
23	British	67.8	23.6	60.7***	7.2	4.5
B.		Human PDC-$E2_{212-226}$	MYCGO $hsp65_{90-104}$	PDC antigen	Control antigen	Control peptide
9	Spanish	76	75.2	77	4.2	3.1
10	Spanish	73.9	73	71.3	8.2	7.1
16	Spanish	78.1	69.8	72	8	3
17*	Spanish	72	77	77	6	4
35	Spanish	67.7	72.8	69.1	10.1	2.8
11**	British	70	72	71	11	2

* *See Figure 3.1A-B;* ** *See Figure 3.1C-D;* *** *Inhibition observed with 62.5μg/ml of PDC antigen as liquid phase competitor. This declined to 23% when 1000 μg/ml was used; MYCGO, Mycobacterium gordonae; hsp, heat shock protein PDC, pyruvate dehydrogenase complex; PBC, primary biliary cirrhosis*

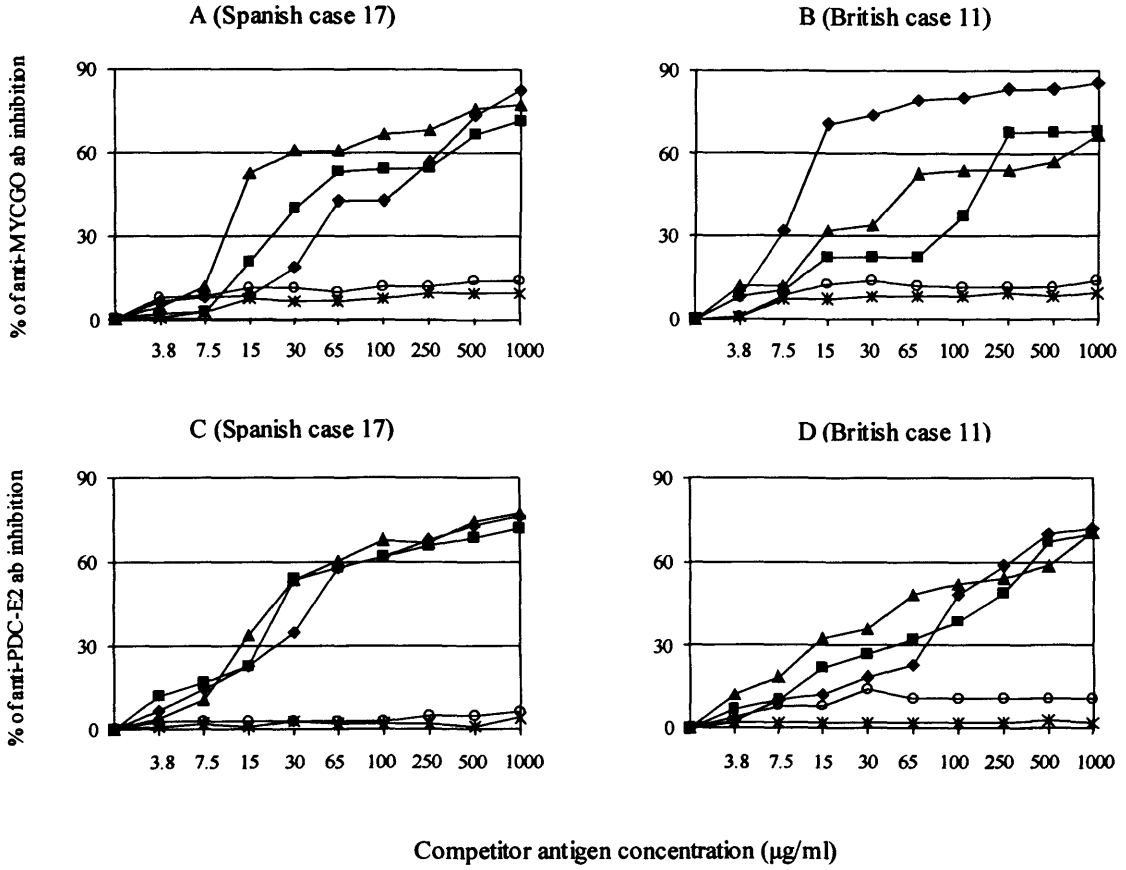


Figure 3.1 *A-D: Inhibition of antibody binding against MYCGO hsp65$_{90\text{-}104}$ (A-B) and PDC-E2$_{212\text{-}226}$ (C-D) by pre-incubation with MYCGO hsp65$_{90\text{-}104}$ (◆), human PDC-E2$_{212\text{-}226}$ (■), porcine PDC antigen (▲), control antigen (○) and control peptide (*) of Spanish case 17 (A,C), and British case 11 (B,D). Antibody binding is represented as a percentage of inhibition of binding to the peptide MYCGO hsp65$_{90\text{-}104}$ in the presence of competitor peptide at different concentrations; MYCGO, Mycobacterium gordonae; hsp, heat shock protein; PDC, pyruvate dehydrogenase complex; ab, antibody*

3.3.4 Affinity of anti-peptide antibody

The relative affinity of antibody binding to MYCGO hsp65$_{90\text{-}104}$ and PDC-E2$_{212\text{-}226}$ for Spanish case 9 and British case 11 is illustrated in Figure 3.2 demonstrating in each case higher relative antibody affinity to MYCGO hsp65$_{90\text{-}104}$ compared to PDC-E2$_{212\text{-}226}$, the avidity indices being 0.66 *vs* 0.47 and 1.1 *vs* 0.53 mol/L, respectively (Figure 3.2A-B).

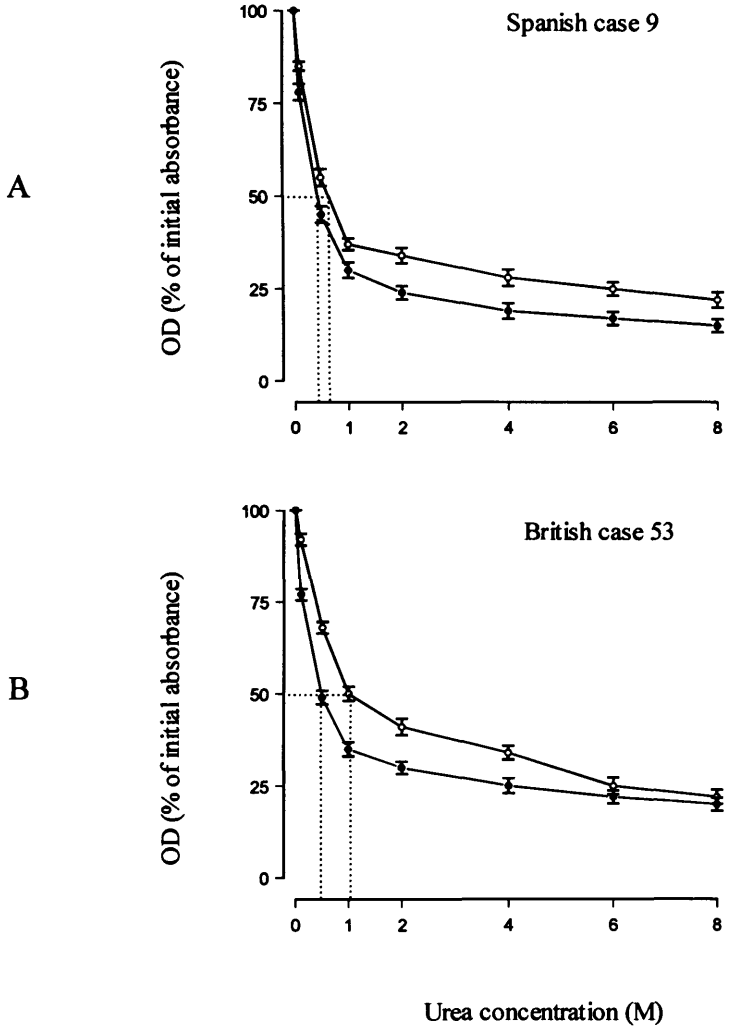


***Figure 3.2** A-B. Affinity profile of anti-peptide antibody. ELISA microplate wells were coated with Mycobacterium gordonae heat shock protein (hsp)65$_{90\text{-}104}$ and human pyruvate dehydrogenase complex (PDC)-E2$_{212\text{-}226}$. After incubation with individual serum samples of Spanish case 9 (A) and British case 53 (B) at a dilution of 1/10,000, urea was added at various concentrations and allowed to inhibit antibody-antigen interaction for 15 minutes. Absorbance values were plotted as the percentage of initial absorbance against urea concentration (mean ± SD). Each point is the average of 3 observations. From this the relative affinity index, defined as the concentration of urea that elutes 50% of the antibody reactivity, for both MYCGO hsp65$_{90\text{-}10}$ (○) and human PDC-E2$_{212\text{-}226}$ (●) can be extrapolated (-----). OD, optical density*

3.3.5 IgG subclass of anti-PDC-E2 autoantibodies

Anti-PDC-E2 reactivity was predominantly of the IgG3 subclass alone or in combination with either IgG1 or to a lesser extent IgG2 subclass, in each of the 3 groups (Table 3.3). No significant differences were found between the 10 MYCGO $hsp65_{90-104}$ reactive Spanish PBC cases and the 10 MYCGO $hsp65_{90-104}$ unreactive Spanish or the 10 MYCGO $hsp65_{90-104}$ unreactive British cases. No reactivity to PDC-E2 of the IgG4 subclass was present.

Table 3.3 Patterns of anti-PDC-E2 antibody IgG subclass specific reactivity in: (A) MYCGO $hsp65_{90-104}$ reactive Spanish PBC cases; (B) MYCGO $hsp65_{90-104}$ unreactive Spanish PBC cases; (C) MYCGO $hsp65_{90-104}$ unreactive British PBC cases. All serum samples were reactive for PDC-$E2_{212-226}$

	Pattern	IgG3	IgG1/IgG3	IgG2/IgG3
A	10 Spanish MYCGO $hsp65_{90-104}$ reactivive PBC cases	7/10	2/10	1/10
B	10 Spanish MYCGO $hsp65_{90-104}$ unreactivive PBC cases	6/10	3/10	1/10
C	10 British MYCGO $hsp65_{90-104}$ reactivive PBC cases	7/10	2/10	1/10

PDC-E2, pyruvate dehydrogenase complex-E2 subunit; MYCGO, Mycobacterium gordonae; hsp65, heat shock protein 65 kDa; PBC, primary biliary cirrhosis

3.4 DISCUSSION

In this section of the thesis, an attempt was made to see whether there is molecular mimicry at the level of mimicking peptidyl epitopes, for the previously described cross-reactivity between MYCGO hsp65 and human PDC-E2, the major target antigen of antimitochondrial immune response in patients with PBC (Vilagut *et al.*, 1994, Vilagut *et al.*, 1997, Yeaman *et al.*, 2000). The Spanish investigators found a species-specific reaction of sera from patients with PBC with membranes from MYCGO. In a subsequent study the same group was able to recognize MYCGO hsp65 as a target of PBC-specific cross-reactive antibodies (Vilagut *et al.*, 1994, Vilagut *et al.*, 1997). The authors suggested a shared epitope between MYCGO hsp65 and human mitochondrial antigens as the reason for the observed cross-reaction (Vilagut *et al.*, 1997). Starting from this point, the present study has identified a striking aa similarity between MYCGO $hsp65_{90\text{-}104}$ and human PDC-$E2_{212\text{-}226}$, the unique peptide sequence shared by the respective dominant epitopes of AMA and PDC-E2 specific CD4 and CD8 cells (Van de Water *et al.*, 1988, Tuaillon *et al.*, 1992, Matsui *et al.*, 1993, Shimoda *et al.*, 1995, Shimoda *et al.*, 1998, Kita *et al.*, 2002).

Anti-PDC-E2 antibody response is polyclonal and includes a wide range of specificities, targeting, to a greater or lesser extent, the sequence 212-226 on PDC-E2 and various flanking sequences extending out to include almost the entire inner-lipoyl domain of PDC-E2 (Van de Water *et al.*, 1988, Tuaillon *et al.*, 1992, Matsui *et al.*, 1993). The focus of autoantibody reactivity can be a linear sequence, the critical residues being contiguous, located on a short segment of the antigenic protein (Geysen *et al.*, 1985, Dyrberg *et al.*, 1990, Novotny, 1991, Wucherpfennig *et al.*, 1997, Craig *et al.*, 1998). Three-dimensional studies show PDC-$E2_{212\text{-}226}$ to be physically exposed on the molecule's surface making it a potential target of microbial/self cross-reactive humoral responses (Howard *et al.*, 1998).

The PDC-$E2_{212\text{-}226}$ mimicking motif SxGDL(IL)AE is virtually unique to mycobacterial hsps, being expressed in practically all mycobacterial hsp65s, including those of *M. tuberculosis* and *M. leprae*. In man the only full match of SxGDLIAE is the inner lipoyl domain of PDC-E2, while human hsp60, the evolutionary equivalent of hsp65, has only 2 identities at the corresponding sequence (ExGNIISD). Hence, an excellent and almost

unique match between this part of the E2 epitope and mycobacterial hsp has been identified.

The present work, involving the construction of the corresponding peptides, and testing their reactivity on relevant sera by ELISA, has led to three major findings. Firstly, 50% of the Spanish PBC patients reacted with MYCGO hsp65$_{90\text{-}104}$, this recognition being virtually absent among British PBC patients, even though the groups were equally reactive against PDC-E2$_{212\text{-}226}$. Second, within the Spanish serum samples, anti-MYCGO hsp65$_{90\text{-}104}$ reactivity was restricted to PBC, not being found in any of the 44 Spanish HCV infected controls. Thirdly, double-reactivity against MYCGO hsp65$_{90\text{-}104}$ and PDC-E2$_{212\text{-}226}$ usually reflected cross-reactivity, as demonstrated by inhibition studies.

These results agree with those of O'Donohue *et al.* (1994) in finding no difference in anti-hsp65 mycobacterial reactivity in UK between PBC patients and controls. The question then arises as to why differential reactivity to hsp65$_{90\text{-}104}$ is limited to Spanish patients with PBC. Is this because exposure to mycobacteria in general, or to a specific, atypical mycobacterium is particularly elevated in Spain? Distribution of atypical mycobacteria varies between different geographic regions; interestingly, Catalonia, the region from where the Spanish cohort originates, has one of the highest colonisation rates in Spain for atypical mycobacteria (Falkinham, 1996, Le Dantec *et al.*, 2002, de March Ayuela, 2000, Martin Casabona and Rossello Urgell, 2000). In support to an epidemiological link is the observation that reactivity to hsp65$_{90\text{-}104}$ is present in 9 of the 10 serum samples conserved from the 1993-1994 Vilagut study, a percentage close to the 100% originally reported for reactivity to full-length hsp65, while this is only 37% for the more recent sera suggesting a shift in the epidemiology over time. Thus, the differential responses to hsp65$_{90\text{-}104}$ in the two European countries may be due to different epidemiological patterns (Falkinham, 1996, Le Dantec *et al.*, 2002, de March Ayuela, 2000, Martin Casabona and Rossello Urgell, 2000). The assumption, however, that anti-hsp65$_{90\text{-}104}$ reactivity in Spanish PBC patients simply reflects a widespread exposure to mycobacteria in the Iberian country is questioned by the finding that none of 44 HCV infected patients, originating from the same region, have antibodies to hsp65$_{90\text{-}104}$.

In analysing the response to microbial and self sequences, it is evident that reactivity to PDC-$E2_{212-226}$, present in practically all the PBC patients studied, does not equate with reactivity to the highly homologous $hsp65_{90-104}$, this being confined to a set of patients within a geographically-defined subgroup. This indicates that molecular mimicry does not automatically coincide with antigenic mimicry (Oldstone, 1987, Quaratino *et al.*, 1995, Bogdanos *et al.*, 2000). In those patients who were simultaneously reactive to PDC-$E2_{212-226}$ and $hsp65_{90-104}$, double reactivity was shown to be in most cases cross-reactive, by inhibition studies.

Moreover, the antibody affinity to $hsp65_{90-104}$ was higher than that to PDC-$E2_{212-226}$, suggesting that the response to the mycobacterial sequence may precede that to PDC-E2 (Figure 3.2) (Quinn *et al.*, 1993).

A pathogenic scenario may then be proposed where exposure to a PDC-$E2_{212-226}$ mimicking microbial sequence, such as that contained in various mycobacterial hsp65, primes a potentially cross-reactive lymphocyte population (Oldstone, 1998, Liang and Mamula, 2000, von Herrath *et al.*, 2003). This view is consistent with the findings that sera from patients with infectious diseases such as tuberculosis and leprosy, in which immune responses to mycobacterial stress proteins have been demonstrated, present antibodies that recognise the main mitochondrial autoantigen of PBC (Klein *et al.*, 1993, Gilburd *et al.*, 1994). Exposure to identical or very similar sequences present in other microbes could further expand this cross-reactive population, thus resulting in emergence of autoimmunity in genetically predisposed individuals (Kerkar *et al.*, 2003). If this is the case, insignificant differences in prevalence of PBC between different countries such as UK and Spain may be the result of complex but significant differences of the immunogenetic background and exposure to distinct microbial mimics (such as MYCGO in Spain and *E. coli* in UK) (Vilagut *et al.*, 1997, Butler *et al.*, 1995).
These observations do not prove the role of mycobacteria as the cause of PBC but rather favour the concept that infectious agents can act as co-factors for the precipitation of an immune-mediated response (Baum, 1995, von Herrath, 2000, von Herrath *et al.*, 2003). They also provide the impetus for extensive investigation of PDC-$E2_{212-226}$- mimicking sequences of microbial origin that could serve as targets of PBC-specific cross-reactive responses, similar to what has been found for the mycobacterial hsp65 mimic.

CHAPTER 4

Lactobacilli and PBC

4.1 BACKGROUND

The findings of the previous Chapter (*see Chapter* 3) suggested that the motif SxGDL[IL]AE shared by the immunodominant mitochondrial autoepitope on PDC-E2 (PDC-$E2_{212\text{-}226}$) and mycobacterial hsps is the focus of cross-reactive responses in patients with PBC. It was also found that among other microbial proteins this same motif is present only in β galactosidase (lactase) of *Lactobacillus delbrueckii* (BGAL LACDE), subsp. *bulgaricus*. Of interest, the corresponding mimic of human PDC-$E2_{212\text{-}226}$ in *E. coli*, (ECOLI PDC-$E2_{231\text{-}245}$) which has been repeatedly suggested to be involved in the induction of human PDC-E2 autoimmunity, does not contain this motif (*see* Figure 4.1) (Shimoda *et al.*, 1998, Shimoda *et al.*, 2000, Van de Water *et al.*, 2001).

In an attempt to investigate whether the microbial BGAL LACDE and ECOLI PDC-E2 mimics are targets of antibody responses and cross-react with human PDC-E2, the homologous peptidyl sequences were constructed and tested by ELISA for reactivity and competitive cross-reactivity using sera from patients with PBC, - with or without PDC-E2 autoantibodies -, pathological and normal controls.

4.2 METHODS

4.2.1 Subjects

4.2.1.1 PBC patients

4.2.1.1.1 AMA positive PBC patients

Antibody responses to microbial/self mimics were tested in the group of 50 British patients with PBC enrolled in the study of mycobacterial/self cross-reactive responses (see Chapter 3). Histology was compatible with PBC in all 30 cases in whom a biopsy had been performed; 7 of these were at stage I, 5 at stage II, 9 at stage III and 9 at stage IV. In the remaining 20 patients biopsy was not performed due to the early clinical stage or conversely, to the end stage of the disease. At the time of serum collection, 41 of the 50 patients were symptomatic with a mean duration of disease of 47.2 months (range 1-179). Overall, 22 patients were classified as early stage or intermediate stage and 28 as advanced or end stage disease.

4.2.1.2 AMA negative PBC patients

Twenty-three patients with biopsy-proven PBC (20 female, 45.7 years, range 24-69) consistently negative for AMA by conventional IIFL, and for anti-PDC-E2 antibodies by ELISA and western blot, using as antigen purified PDC, were also studied. At the time of serum collection, 19 patients were symptomatic with a mean duration of disease of 32.1 months (range 17-97); 7 were at stage II, 9 at stage III and 7 at stage IV.

4.2.1.3 Pathological controls

As pathological controls, 157 sera (all AMA negative) from patients with other liver and non-liver related disorders were tested including 41 patients with chronic HBV infection (37 female, 50.2±13 years), all HBV DNA positive; 52 patients with chronic HCV infection (48 female, 53 years±11), all HCV RNA positive; 28 patients with autoimmune thyroid dysfunction (26 female, 47.2±15 years), all anti-thyroid peroxidase antibody positive; and 36 patients with Crohn's disease (34 female, 42±9).

4.2.1.4 Normal controls

Thirty-three healthy volunteer members of staff (30 female, 49.2±9.2 years), all AMA negative, were tested as normal controls.

4.2.2 Detection of AMA

AMA was retested by conventional IIFL (*see* 2.2.2.1), immunoblotting (*see* 2.2.2.2), and by ELISA (*see* 2.1.6.1).

4.2.3 Protein database search and analysis

Microbial mimics of human PDC-$E2_{212-226}$ (excluding those of mycobacterial hsp65), containing the SxGDL[ILV]AE motif were identified by scanning the SWISSPROT protein database using the *Advanced ProteinInfo* programme (*see* 2.2.1.1). The resulting similarities were further evaluated using the protein-protein sequence comparison '*BLASTp 2 sequences*' programme (*see* 2.2.1.2.2) and compared with the previously described similarity shared by human PDC-$E2_{212-226}$ and the corresponding ECOLI PDC-$E2_{231-245}$ mimic.

4.2.4 Peptide synthesis

Four 15-mer biotinylated peptides containing the homologous LACDE $BGAL_{266-280}$ (RDSEGDLVAEKLGPI), ECOLI PDC-$E2_{231-245}$ (KVAAEQSLITVEGDK), human PDC-$E2_{212-226}$ (KLSEGDLLAEIETDK) sequences and an irrelevant 15 aa control peptide were constructed (*see* 2.2.2.3).

4.2.5 ELISA

Antibody binding to the peptides was determined by ELISA (*see* 2.2.2.3). The final peptide concentration was 15μg/ml and the dilution of serum samples was 1/200 (*see* 2.2.2.3).

4.2.6 Inhibition studies

To investigate whether the simultaneous reactivity to LACDE $BGAL_{266-280}$ and human PDC-$E2_{212-226}$ was due to cross-reactivity, competition ELISA was performed (*see*

2.2.2.4.1), measuring residual anti-LACDE $BGAL_{266-280}$ or anti-PDC-$E2_{212-226}$ reactivity after pre-incubation respectively with LACDE $BGAL_{266-280}$, PDC-$E2_{212-226}$, PDC antigen, control peptide and control antigen (CYP2D6, *see* 2.1.6.1) as liquid phase competitors (final concentrations: 5, 10, 25, 50, 100, 250, 500, 1000 μg/ml). Inhibition of anti-ECOLI PDC-$E2_{231-245}$ reactivity was measured under conditions similar to those described for LACDE $BGAL_{266-280}$ (*see* 2.2.2.4).

4.2.7 Anti-peptide antibody affinity

The relative affinity of anti-peptide antibody binding (*see* 2.2.2.5) was tested in one serum reactive to LACDE $BGAL_{266-280}$/ECOLI PDC-$E2_{231-245}$/human PDC-$E2_{212-226}$ and in 3 LACDE $BGAL_{266-280}$/ human PDC-$E2_{212-226}$ double reactive sera .

4.3 RESULTS

4.3.1 Protein database search and analysis

LACDE $BGAL_{266-280}$ is the only non-hsp65 mycobacterial microbial sequence sharing the motif SxGDL[ILV]AE with human $PDC\text{-}E2_{212-226}$. LACDE $BGAL_{266-280}$ shares 8 aa in a row (7 identities) and has a total of 10/15 (67%) similarity with human $PDC\text{-}E2_{212-226}$ (Figure 4.1).

The corresponding $BGAL_{246-260}$ of *Lactococcus lactis* has 7/15 (47%) similarity (2 identities) with human $PDC\text{-}E2_{212-226}$; the corresponding $BGAL_{266-280}$ of *Lactococcus sakei* has 5/15 (33%) similarity (2 identities); and the *Lactococcus acidophilus* $BGAL_{268-282}$ (EDV*N*GS*AIL*EQ*D*VPL) has 7/15 (47%) similarity (2 identities) (Figure 4.1).

The corresponding ECOLI $PDC\text{-}E2_{231-245}$ sequence (**K***V**A***AE***Q***SL***I***T***V***E***G***DK**), shares 11/15 (73%) similarity (5 identities) with human $PDC\text{-}E2_{212-226}$ (**K***L**S***EG***D***LL***A***E***I***E***T***DK**) (identities in **bold**, conservative substitutions in *italic*) (Figure 4.1), while LACDE $BGAL_{266-280}$ (*R*D*S*EG*D*L*V*AEKLGPI) and ECOLI $PDC\text{-}E2_{231-245}$ sequence (*K*V*A*AE*Q*S*LI*TVEGDK) share 6/15 (40%) similarity (1 identity).

Species	Protein	Sequence															aa position	Identities	Similarity (%)
E. coli		**K**	*V*	*A*	**A**	**E**	*Q*	**S**	**L**	*I*	**T**	*V*	**E**	**G**	**D**	**K**	(aa 231-245)	5	11/15 (73%)
		:	.	.			.		:	.		.	:	.	:	:			
Human	PDC-E2	K	L	S	E	G	D	L	L	A	E	I	E	T	D	K	(aa 212-226)		
		.		:	:	:	:	:	.	:	:			.					
Lactobacillus delbrueckii subsp. *bulgaricus*	BGAL	*R*	D	**S**	**E**	**G**	**D**	**L**	*V*	**A**	**E**	K	L	*G*	P	I	(aa 266-280)	7	10/15 (67%)
Lactococcus lactis subsp. *lactis*	BGAL	L	Y	D	*D*	E	*Q*	**L**	*I*	*G*	**E**	C	H	*G*	F	D	(aa 246-260)	2	7/15 (47%)
Lactobacillus sakei	BGAL	*R*	D	Q	H	Q	I	*I*	**L**	*T*	A	D	L	**T**	S	G	(aa 266-280)	2	5/15 (33%)
Lactobacillus acidophilus	BGAL	E	D	V	*N*	**G**	S	*A*	*I*	*L*	**E**	Q	*D*	V	P	L	(aa 268-282)	2	7/15 (47%)

FIGURE 4.1 *Sequence alignment of the immunodominant autoepitope on human E2 subunit of pyruvate dehydrogenase complex (PDC-$E2_{212-226}$), and mimics of E. coli PDC-2 and Lactobacillus β-galactosidase (BGAL) mimics. Amino acids in standard single letter code; Colon (:) and bold letters, Identical residues; Full stop, (.) and italic, Conservative substitutions; aa, amino acid*

4.3.2 ELISA

Percentages of anti-peptide reactivity in PBC patients, pathological and healthy controls are given in Table 4.1.

4.3.2.1 Single anti-peptide antibody reactivity

4.3.2.1.1 IgG class anti-peptide reactivity

IgG class antibody reactivity to the human PDC-$E2_{212-226}$ was found in 48 (96%) of 50 AMA positive PBC patients (mean $OD^{test/control}$ 3.24±1.26), in 1 (4%) of 23 AMA negative PBC, 5 (3%) of 157 pathological controls, among which were 1 HBV infected woman, 3 HCV infected patients (2 female) and 1 woman with Crohn's disease ($p<0.05$ for all), all AMA negative by IIFL, ELISA or Western blot. None of the 33 healthy controls reacted with human PDC-$E2_{212-226}$.

IgG class antibody reactivity to LACDE $BGAL_{266-280}$ was found in 15 (30%) AMA positive PBC patients (mean $OD^{test/control}$ 3.13±1.18), in 1 (4%, p=0.03) AMA negative PBC, 19 (12 %, p=0.006) pathological controls (mean $OD^{test/control}$ 3.07±1.1) including 3 with HBV (p=0.01); 5 with HCV (p=0.09), 2 with autoimmune thyroid disease (p=0.04), 6 with Crohn's disease (17%, p=0.24), and in 3 of the normal controls (p=0.15).

IgG class antibody reactivity to ECOLI PDC-$E2_{231-245}$ was present in 2 (4%) AMA positive PBC patients, in none AMA negative PBC and in 1 (0.4%) of the controls - an AMA negative chronic HCV infected woman (p>0.05 for all).

4.3.2.1.2 IgG subclass anti-peptide reactivity

Among the 48 anti-human PDC-$E2_{212-226}$ reactive AMA positive PBC patients, 42 (87.5%) had IgG antibody reactivity predominantly of the IgG3 subclass including all 15 cases reacting with LACDE $BGAL_{266-280}$. Anti-PDC-$E2_{212-226}$ reactivity of the AMA negative PBC case was of the IgG1 and IgG3 subclass.

Among the 15 LACDE $BGAL_{266-280}$ reactive AMA positive PBC cases, all but one had IgG antibody reactivity predominantly of the IgG3 subclass; the single AMA negative PBC case with reactivity to LACDE $BGAL_{266-280}$ was of the IgG1,3,4 subclasses.

Among the 19 pathological controls and the 3 healthy subjects reactive with LACDE $BGAL_{266-280}$, antibody binding was of the IgG4 (19 cases) or the IgG2 subclass (2 cases) and the IgG1,2,3 in one case (an HCV infected woman with IgG3 anti-$PDC\text{-}E2_{212-226}$ reactivity).

Overall, 14 (28%) of the 50 AMA positive PBC cases had IgG3 anti-LACDE $BGAL_{266-280}$ antibody reactivity compared to none of the AMA negative, 1 (0.6%, $p<0.0001$) of the 157 pathological controls and none of the 33 healthy controls ($p<0.05$ for all) (Table 4.1).

Among the 2 ECOLI $PDC\text{-}E2_{231-246}$ reactive AMA positive PBC cases, anti-ECOLI $PDC\text{-}E2_{231-246}$ antibody reactivity was of the IgG1,3 isotype in one and IgG1,2,3,4 in the other.

4.3.2.2 Double anti-peptide antibody reactivity

IgG class antibody reactivity to the LACDE $BGAL_{266-280}$/ $PDC\text{-}E2_{212-226}$ pair was found in 15 (30%) PBC patients (7 at early or intermediate stage and 8 at advanced or end stage disease), none in anti-PDC-E2 negative PBC, 1 (1%) amongst pathological controls (a 53-year-old AMA negative HCV infected woman), and in none of the healthy controls ($p<0.01$ for all).

PBC patients with double LACDE $BGAL_{266-280}$/$PDC\text{-}E2_{212-226}$ tended to be younger compared to unreactive patients (50.6±2.8 *vs* 56.5±1.95, $p=0.065$).

There was no significant correlation between double reactivity and clinical stage or other clinical and experimental data.

Double ECOLI $PDC\text{-}E2_{231-245}$/ $PDC\text{-}E2_{212-226}$ reactivity was found in one AMA positive patient, reactive also with LACDE $BGAL_{266-280}$.

Table 4.1 Antibody reactivity to human pyruvate dehydrogenase complex E2 subunit $(PDC\text{-}E2)_{212\text{-}226}$, and its microbial mimics *E. coli* (ECOLI) $PDC\text{-}E2_{231\text{-}245}$, and β galactosidase $(BGAL)_{266\text{-}280}$ of *Lactobacillus delbrueckii* (LACDE) expressed in absolute numbers and percentages (%), in 50 anti-mitochondrial antibody (AMA) positive patients with PBC, 23 AMA negative PBC patients, 157 pathological controls including 41 with chronic hepatitis B virus (HBV) infection, 52 with chronic hepatitis C virus (HCV) infection, 28 with autoimmune thyroiditis, 36 patients with Crohn's disease and 33 healthy controls

	Single Reactivity				
	$PDC\text{-}E2_{212\text{-}226}$		ECOLI $PDC\text{-}E2_{231\text{-}245}$	LACDE $BGAL_{266\text{-}280}$	
	Total IgG	IgG3	Total IgG	Total IgG	IgG3
AMA positive PBC (n=50)	48/50 (96%)	42/50 (84%)	2†/50 (4%)	15/50 (30%)	14/50 (28%)
AMA negative PBC (n=23)	1/23 (4%)§§	0§§	0*	1/23(4%)¶	0¶
Pathological controls (n=157)	5/157 (3%)§§	1/157 (1%)§§	1 (1%)*	19/157(12%)¶¶	1/157(1%)§§
Chronic HBV	1/41 (2%)§§	0§§	0*	3/41 (7%)¶	0§
Chronic HCV	3/52 (6%)§§	1/52 (2%)§§	0*	5/52 (10%)*	1/52 (2%)§
Autoimmune thyroiditis	0§§	0§§	0*	2/28 (7%)¶	0¶¶
Crohn's disease	1/36 (3%)§§	0§§	0*	6/36 (17%)*	0¶¶
Healthy Controls (n=33)	0§§	0§§	0*	3/33 (9%)*	0¶¶

*Comparisons of anti-peptide reactivity are between AMA positive PBC and AMA negative PBC, pathological or healthy control groups; *, $p>0.05$; ¶, $p<0.05$; ¶¶, $p<0.01$; §, $p<0.01$; §§, $p<0.001$; †, one case had IgG1,3 and one IgG1,2,3,4*

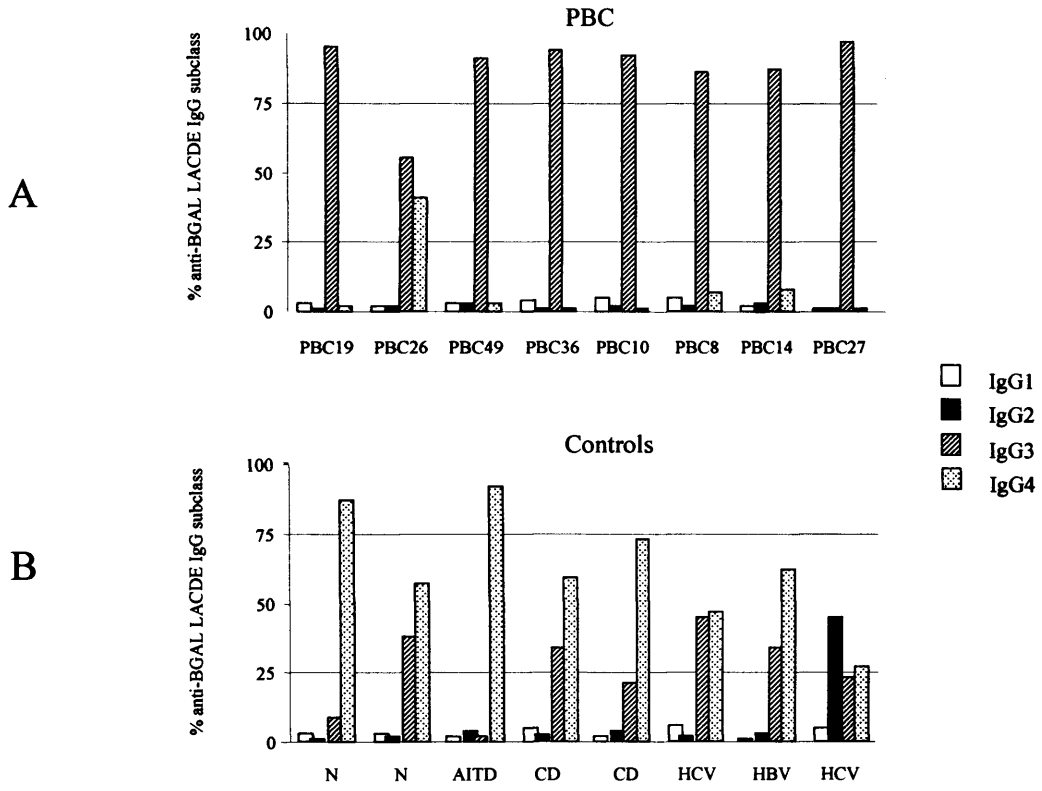


FIGURE 4.2 *Distribution of IgG subclass antibody binding against anti-β galactosidase $(BGAL)_{266\text{-}280}$ of Lactobacillus delbrueckii (LACDE) in 8 representative PBC cases (A) and 8 controls (B) including 2 normal (N) subjects, 1 with autoimmune thyroid disease (AITD), 2 with Crohn's disease (CD), 2 with hepatitis C virus infection (HCV) and one with hepatitis B virus (HBV) infection. Anti-LACDE $BGAL_{266\text{-}280}$ antibody reactivity is expressed as a percentage of IgG subclass (see 2.2.2.3)*

4.3.3 Comparison of antibody binding to LACDE and ECOLI mimics

Of the 15/15 AMA positive sera reacting with LACDE $BGAL_{266-280}$ at a dilution of 1/200, 13/15 (87%) reacted at 1/500 and 10/15 (66%) at $1/10^{-3}$ dilutions. At $1/10^{-4}$ dilution, there were still 3/15 (20%) sera which were reactive to LACDE $BGAL_{266-280}$ (Table 4.2). Of the 2 PBC sera reacting with ECOLI PDC-$E2_{231-245}$ at 1/200, none was reactive at a dilution of 1/500.

Table 4.2 Antibody reactivity against *Lactobacillus delbrueckii* (LACDE) β galactosidase $(BGAL)_{266-280}$ and *E. coli* (ECOLI) ECOLI pyruvate dehydrogenase complex E2 subunit (PDC-$E2_{231-245}$) in serial dilutions of serum samples of patients with primary biliary cirrhosis. At 1/200 dilution reactivity to LACDE $BGAL_{266-280}$ and ECOLI PDC-$E2_{231-245}$ was present in 15 and 2 cases, respectively

	Total number of reactive cases			
Serum sample dilution	*1/200*	*1/500 (%)*	*$1/10^3$ (%)*	*$1/10^4$ (%)*
LACDE $BGAL_{266-280}$	15	13/15 (87%)	10/15 (66%)	3/15 (20%)
ECOLI PDC-$E2_{231-245}$	2	0	0	0

4.3.4 Inhibition studies

Inhibition results of anti-peptide binding by pre-incubation with relevant and control peptides and antigens are given in detail in Table 4.3. A representative case is presented in Figure 4. 3.

IgG3 subclass antibody binding to LACDE $BGAL_{266\text{-}280}$ was inhibited by 67-79.6% after pre-incubation with the LACDE $BGAL_{266\text{-}280}$; by 62-75.7% after pre-incubation with human PDC-$E2_{212\text{-}226}$ and by 65-78.2% after pre-incubation with PDC, in all 4 cases tested (Table 4.3). Insignificant inhibition was observed by pre-incubation with the irrelevant control peptide and antigen (Figure 4. 3A) (*see* 2.2.2.4).

Antibody binding to human PDC-$E2_{212\text{-}226}$ was also significantly inhibited after pre-incubation with the PDC-$E2_{212\text{-}226}$, LACDE $BGAL_{266\text{-}280}$ and the PDC antigen (Table 4.3 and Figure 4.3B).

Inhibition studies of the human PDC-$E2_{212\text{-}226}$/ECOLI PDC-$E2_{231\text{-}245}$/LACDE $BGAL_{266\text{-}280}$ reactive serum revealed inability of ECOLI PDC-$E2_{231\text{-}245}$ to inhibit significantly the antibody binding to human PDC-$E2_{212\text{-}226}$ while the reverse was possible (Table 4.3 C-D). ECOLI PDC-$E2_{231\text{-}245}$ and LACDE $BGAL_{266\text{-}280}$ were unable to inhibit reactivity to each other (maximum inhibition <20%).

Table 4.3 A-B. Percentage inhibition of anti-LACDE $BGAL_{266-280}$ (A) and anti-human $PDC\text{-}E2_{212-226}$ (B) antibody reactivity by pre-incubation with relevant and control peptides and antigens in four LACDE $BGAL_{266-280}$ /$PDC\text{-}E2_{212-226}$ double reactive PBC cases; C-D. Inhibition of anti-ECOLI $PDC\text{-}E2_{231-245}$ (C) and anti-human $PDC\text{-}E2_{212-226}$ (D) antibody reactivity by pre-incubation with relevant mimics and control peptides and antigens

Case	Maximum Inhibition %					
A						
	LACDE $BGAL_{266-280}$	Human $PDC\text{-}E2_{212-226}$	PDC antigen	Control antigen	Control peptide	
1	75.8	75.7	73.1	11.7	9.3	
2	67	65.1	66.8	7.3	8.2	
3	77.7	62	65	10.4	6.2	
4*	79.6	73.5	78.2	7.3	5.2	
B						
	Human $PDC\text{-}E2_{212-226}$	LACDE $BGAL_{266-280}$	PDC antigen	Control antigen	Control peptide	
1	72.8	75	75.9	7.2	3.1	
2	81.5	83.2	81.2	8	7.1	
3	75	78.3	73.4	10.1	6.3	
4*	78.9	67	79.8	7.6	5.7	
C						
	ECOLI $PDC\text{-}E2_{231-245}$	Human $PDC\text{-}E2_{212-226}$	LACDE $BGAL_{266-280}$	PDC antigen	Control antigen	Control peptide
	63.2	81.1	34.2	86.3	10.1	9.2
D						
	Human $PDC\text{-}E2_{212-226}$	ECOLI $PDC\text{-}E2_{231-245}$	LACDE $BGAL_{266-280}$	PDC antigen	Control antigen	Control peptide
	73.1	23.4	79.2	78.4	12.3	11.4

**See Figure 4.3 A-B; LACDE, Lactobacillus delbrueckii; BGAL, β galactosidase; PDC-E2, pyruvate dehydrogenase complex E2; ECOLI, E. coli*

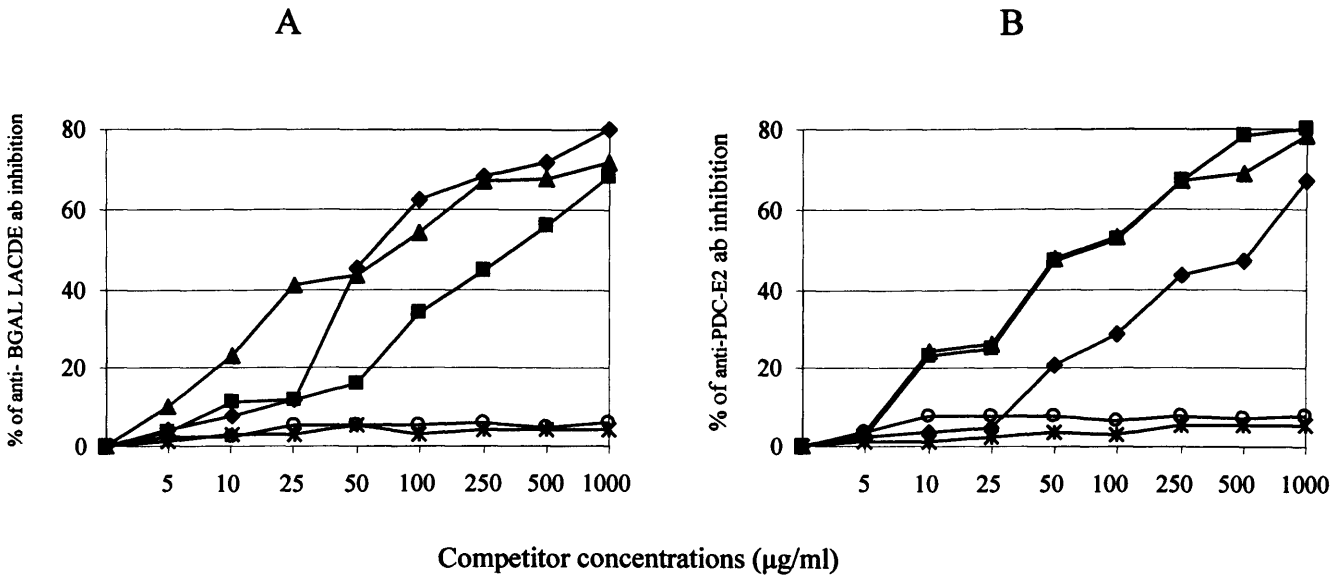


FIGURE 4. 3 *Inhibition of antibody (ab) binding against LACDE $BGAL_{266-280}$ (A) or human $PDC\text{-}E2_{212-226}$ (B) of a representative serum sample by pre-incubation with LACDE $BGAL_{266-280}$ (◆), human $PDC\text{-}E2_{212-226}$ (▲), PDC antigen (■), control peptide (✳) and control protein (○), in the presence of competitor peptide at different concentrations, 0; 5; 10; 15; 25; 50; 100; 250; 500; 1000 µg/ml. LACDE, Lactobacillus delbrueckii; BGAL, β galactosidase (lactase); PDC, pyruvate dehydrogenase complex; ab, antibody*

4.3.5 Affinity of anti-peptide antibody

The relative affinity index (rAI) representing the concentration of the chaotropic reagent (urea) that elutes 50% of the anti-peptide antibody binding (*see* 2.2.2.5) for the one case reactive with human PDC-E2$_{212-226}$, ECOLI PDC-E2$_{231-245}$,LACDE BGAL$_{266-280}$ mimics and three LACDE BGAL$_{266-280}$/human PDC-E2$_{212-226}$ double reactive cases is illustrated in Figure 4.4. In each case, higher antibody rAI was found for LACDE BGAL$_{266-280}$ compared to human PDC-E2$_{212-226}$. In the multiple-reactive case, the lowest antibody rAI was that to ECOLI PDC-E2$_{231-245}$ (Figure 4.4).

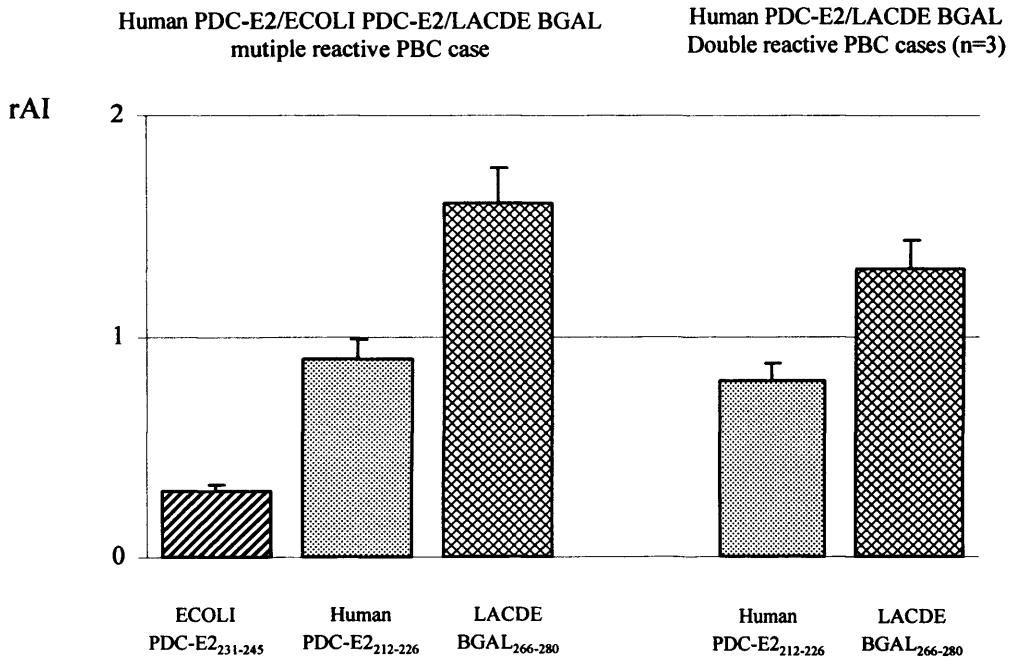


Figure 4.4 *Relative affinity indices (rAI) of anti-peptide antibody binding in a multiple human PDC-E2$_{212-226}$/ECOLI PDC-E2$_{231-245}$/ LACDE BGAL$_{266-280}$ reactive case (left panel) and three double human PDC-E2$_{212-226}$/LACDE BGAL$_{266-280}$ reactive cases (mean+SD). Each point is the average of 3 observations (see 2.2.2.5). ECOLI, E. coli; PDC-E2, pyruvate dehydrogenase complex E2 subunit; LACDE, Lactobacillus delbrueckii, BGAL, β galactosidase*

4.4 DISCUSSION

This section has found that almost one third of patients with primary biliary cirrhosis have an IgG3 subclass antibody response against a lactobacillus sequence that shares the motif SxGDL[IL]AE with the major mitochondrial PDC-$E2_{212-226}$ autoepitope. Reactivity is virtually absent to ECOLI PDC-$E2_{231-245}$, a human PDC-$E2_{212-226}$ mimic which does not contain the SxGDL[IL]AE motif (Burroughs *et al.*, 1992, Shimoda *et al.*, 1998). IgG3 anti-LACDE $BGAL_{266-280}$ antibody reactivity is highly PBC specific, being found only in one case of a large number of pathological and healthy controls.

Not only is the IgG3 anti-LACDE $BGAL_{266-280}$/PDC-$E2_{212-226}$ antibody reactivity specific for PBC but it is also cross-reactive, since its binding to the microbial sequence LACDE $BGAL_{266-280}$ is inhibited by pre-incubation with human PDC-$E2_{212-226}$ and *vice versa*. Importantly, the binding to the LACDE sequence is also inhibited by the purified PDC mitochondrial antigen, demonstrating that the cross-reactive microbial/self recognition targets the naturally occurring autoantigen (*see* Table 4.3 and Figure 4.3). These findings are in sharp contrast to those obtained for the ECOLI PDC-$E2_{231-245}$, which has proved a poor inhibitor of anti-human PDC-$E2_{212-226}$ reactivity (*see* Table 4.3).

Anti-LACDE $BGAL_{266-280}$ antibody reactivity was present, though significantly less frequently, in conditions other than PBC: remarkably this was always of the IgG4 and/or IgG2 subclasses and was never associated with reactivity to the self-epitope PDC-$E2_{212-226}$. This indicates that only in the case of PBC do the anti-BGAL antibodies also recognise a conformation of a relevant sequence that matches that of the PDC-E2 peptide.

It is unlikely that these findings reflect an epiphenomenon, restricted to IgG3 antibodies, arising from LACDE $BGAL_{266-280}$/PDC-$E2_{212-226}$ amino acid similarity. If this were the case then all anti-PDC-$E2_{212-226}$ positive cases would be expected to react with LACDE $BGAL_{266-280}$. Moreover, there would then be expected to be the same frequency of non-cross-reactive, non-IgG3, anti-LACDE $BGAL_{266-280}$ reactivity in PBC subjects as observed in controls. Conversely, the anti- LACDE $BGAL_{266-280}$ antibody present in controls would be expected to react with the PDC-$E2_{212-226}$ mimic. But this is not the case. Further support for the potential biological significance of the observed anti-LACDE $BGAL_{266-280}$ reactivity has been provided by the fact that anti-LACDE $BGAL_{266-280}$ reactivity was significantly

more frequent and tended to persist even in a $1/10^4$ dilution compared to that against ECOLI PDC-$E2_{231-245}$ which disappeared at 1/500 serum dilution (*see* Table 4.2). The fact that anti-PDC-E2 antibody negative patients with PBC do not have anti-LACDE $BGAL_{266-280}$ antibodies strengthens the significance of the observed reactivity and its potential relation to the appearance of anti-PDC-E2 antibodies. Notably, IgG3 anti-$BGAL_{266-280}$ LACDE reactivity was present in AMA positive patients with PBC regardless of disease severity, equally present at early as well as at late stages of its natural history.

What the link is between the PBC specificity of a microbe-self cross-reactive response and its belonging to the IgG3 isotype remains to be clarified. *Lactobacillus delbrueckii* subsp. *bulgaricus* is essential to starter cultures and for yoghurt production (Borchers *et al.*, 2002). Of potential relevance is the fact that whilst there are Lactobacillus strains able to promote peripheral tolerance to self proteins, there exist others that, depending on the immunological state of the host, can induce T-cells to overproduce IFN-γ (Solis Pereyra and Lemonnier, 1991, Solis-Pereyra *et al.*, 1997, Maassen *et al.*, 2000, Borchers *et al.*, 2002, Perdigon *et al.*, 2002, Christensen *et al.*, 2002, Aattour *et al.*, 2002, Maassen *et al.*, 2003) . This cytokine is known to promote production of antibodies of the IgG3 isotype and to limit that of IgG4 antibodies, through its inhibitory activity of Th2 cell programmes (Stevens *et al.*, 1988, Snapper and Mond, 1993, Snapper *et al.*, 1997). Of interest in terms of pathogenicity is the singular ability of IgG3 antibodies to recruit the major effectors of tissue damage, such as complement and Fc receptorbearing cells involved in antibody-dependent cell-mediated cytotoxicity (ADCC) (Anderson and Looney, 1987, Wiener *et al.*, 1988, Jefferis, 1990). Thus, in theory, IgG3 anti-$BGAL_{266-280}$ LACDE antibodies may engage effectors of damage against target cells over-expressing PDC-$E2_{212-226}$, - or an as-yet unidentified mimic thereof - the physically exposed peptidyl sequence representing the dominant AMA epitope. The fact that the antibody affinity to the LACDE BGAL sequence was higher than that to PDC-E2 of human or *E. coli* origin suggests that the response to the *lactobacillus* mimic may precede that to PDC-E2 (Figure 4.4).

In conclusion, the present findings indicate a clear, disease-specific occurrence of IgG3 antibodies cross-reacting against a peptide of BGAL LACDE and the dominant epitope of PDC-E2 in a significant sub-set of PBC patients. Since BGAL LACDE is a major enzyme of an organism widely used in food production, its possible role as an environmental trigger

in the pathogenesis of PBC must now seriously be considered. However, whether LACDE containing fermented products accelerate PBC specific autoimmunity in genetically susceptible individuals or in contrast induce tolerance and hence offer an approach to therapeutic interventions in patients with PBC, remains to be clarified (Faria and Weiner, 1999, Borchers *et al.*, 2002, Christensen *et al.*, 2002, Maassen *et al.*, 2003). Nevertheless, these findings provide the stimulus for continuous search for other microbial mimics originated from infectious agents, particularly those epidemiologically linked to PBC that could serve as targets of cross-reactive immune responses.

CHAPTER 5

E. coli and PBC

5.1 BACKGROUND

The mechanism by which PDC-$E2_{212-226}$, the immunodominant mitochondrial autoepitope, becomes the focus of PBC-specific anti-mitochondrial immune responses remains obscure (Baum, 1995, Gershwin *et al.*, 2000).

Several infectious agents have been postulated to trigger AMA production, and common bacteria such as mycobacteria and *Lactobacilli* may fall into this category, as has been indicated by the results obtained in Chapter 3 & 4 of the present Thesis (Van de Water *et al.*, 2001, Baum, 1995). There is some experimental and epidemiological evidence to suggest, however, that recurrent urinary tract infection (UTI) caused by *Escherichia coli* (ECOLI) may also be pathogenetically linked to PBC (Stemerowicz *et al.*, 1988, Hopf *et al.*, 1989, Burroughs *et al.*, 1992, Butler *et al.*, 1995a, Butler *et al.*, 1995b, Shimoda *et al.*, 1998, Shimoda *et al.*, 2000, Parikh-Patel *et al.*, 2001, Butler *et al.*, 1993). Hopf *et al.* (1989) reported a high incidence of rough form mutants of ECOLI, in faecal samples from patients with PBC. In 52% of patients with recurrent UTI, Butler *et al.* (1995a) found antibodies reacting with PDC-E2. Conversely, patients with PBC reportedly have a higher incidence of recurrent UTI (Butler *et al.*, 1993, Butler *et al.*, 1995b).

The fact that PDC-E2 proteins from a number of microbial OADC proteins and in particular those of ECOLI demonstrate PBC-specific antibody and T-cell cross-reactivity with human PDC-E2, lends support to ECOLI infection having a role in the induction of AMA, through a mechanism of molecular mimicry (Fussey *et al.*, 1991, Baum, 1995, Shimoda *et al.*, 1998, Shimoda *et al.*, 2000). Others have challenged this view arguing that cross-reactive immunity can readily be predicted, taking into account the highly conserved nature of PDC-E2 among species, and in particular of the inner lipoyl domain (Van de Water *et al.*, 2001).

If microbial mimicry is implicated in breaking immunological tolerance to PDC-E2, there is the alternative possibility that short microbial sequences totally unrelated to OADC but still good mimics of the immunodominant PDC-E2 epitope are involved. This has been proven to be the case for the mycobacterial $hsp65_{90-104}$ sequence (Chapter 3) and β galactosidase (lactase) of *Lactobacillus delbrueckii* (Chapter 4) and may well be true for other non-OADC ECOLI or other microbial mimics of the human PDC-$E2_{212-226}$.

Interrogating protein databases, sequences with high degree of similarity to PDC-$E2_{212-226}$ were found (see below) in proteins of ECOLI, *Helicobacter pylori* (HELPY), *Pseudomonas aerigunosa* (PSEAE), human CMV (HCMV), and *Haemophilus influenzae* (HAEIN). Intriguingly, these sequences had a greater similarity to human PDC-$E2_{212-226}$ than did ECOLI PDC-E2 itself (*see* below). The present study investigated whether these sequences are targets of antibody responses and PDC-$E2_{212-226}$ cross-reactive immunity both at peptide and full protein level in 55 AMA-positive PBC and 23 AMA-negative PBC patients and compared the results with those of a large number of normal and pathological controls including women with recurrent UTI and without liver disease.

5.2 METHODS

5.2.1 Subjects

5.2.1.1 PBC patients

Serum samples were obtained from 55 patients with PBC (median age 56±13 years; 46 female), all AMA-positive (1/40 – 1/10480), median duration of disease 38 months (range 1-184) attending the out-patient clinic of Liver Transplantation and Hepatobiliary Medicine Unit, Royal Free Hospital. Demographic, biochemical, immunological and clinical characteristics of these patients, are given in detail in Chapters 3 & 4. Twenty-nine of them underwent screening tests for significant bacteruria (*see* below), and recorded data were available, 19 being positive and 10 negative for recurrent UTI. In the remaining 26 patients urine tests were not performed.

Twenty-three women with PBC consistently negative for AMA by conventional IIFL, and for anti-PDC-E2 antibodies by ELISA and western blot were also studied (*see* 4.2.1.2).

5.2.1.2 Pathological and normal controls

Pathological controls included 170 patients with other liver (27 chronic HBV infection; 42 chronic HCV infection; 17 alcoholic liver disease; 15 AIH-1 and 12 with AIH-2) and non-liver disorders (38 autoimmune thyroid disease; 10 polyomyositis; 9 systemic lupus erythematosus). Twenty women (median age 53 years, range 24-85) attending a UTI out-patient clinic were also tested; these subjects were part of a larger group of 50 women and were included in the present study on the basis that unthawed aliquots of serum samples were available from them (Burroughs *et al.*, 1984, Butler *et al.*, 1993, Butler *et al.*, 1995a, Butler *et al.*, 1995b). All had a history of long-standing recurrent episodes of significant bacteruria and all were receiving prophylactic antibiotics at the time the serum was taken. Significant bacteruria was defined as the presence of 10^5 organisms per ml or more growing in pure culture, regardless of the presence of symptoms or pyuria (Burroughs *et al.*, 1984, Butler *et al.*, 1993, Butler *et al.*, 1995a, Butler *et al.*, 1995b). All but two women were infected by ECOLI (Burroughs *et al.*, 1984). None of the patients had clinical or biochemical evidence of liver disease. Ten of these were diagnosed as AMA-positive by immunoblot, showing OADC (M2) specific

bands, and/or ELISA, though not by IIFL (*see*, 2.1.2, 2.1.6 and 2.2.2.2). The prevalence of AMA seropositivity in this group of women was similar to that (52%) previously reported by Butler *et al.* (1993, 1995a).

Twenty-eight healthy, volunteer members of staff (median age 33 years, range 22-68, 23 female), were tested as normal controls.

5.2.2 Detection of AMA

AMA was retested by conventional IIFL (*see* 2.2.2.1), immunoblotting (*see* 2.2.2.2), and by ELISA (*see* 2.1.6.1).

5.2.3 Protein database search and analysis

Microbial homologues to human PDC-$E2_{212-226}$ were identified by scanning the SwissProt protein database using the *ProteinInfo* programme (*see* 2.2.1.1). The resulting sequences were further evaluated using the *PSI-BLAST* programme (*see* 2.2.1.2.1). Ten non-PDC-E2 related microbial sequences giving the highest percentage of homology and the best score were synthesized.

5.2.4 Peptide synthesis

Eleven 15-mer biotinylated peptides containing the relevant human PDC-E2 and microbial sequences and an irrelevant 15 aa control peptide (*see* 2.2.2.3) were constructed.

5.2.5 ELISA

Antibody binding to the peptides was determined by ELISA (*see* 2.2.2.2). The final peptide concentration was 15μg/ml and the dilution of serum samples was 1/200.

5.2.6 Inhibition studies

To investigate whether the simultaneous reactivity to ECOLI maltose E $(MALE)_{95-109}$ and human PDC-$E2_{212-226}$ was due to cross-reactivity, competition ELISA were performed (*see* 2.2.2.4.1), measuring residual anti-ECOLI $MALE_{95-109}$ or anti-PDC-$E2_{212-226}$ reactivity after incubation respectively with ECOLI $MALE_{95-109}$, PDC-$E2_{212-226}$, purified MALE ECOLI protein (*see* 2.1.4), PDC antigen (*see* 2.1.2), control peptide and antigen (CYP2D6, *see* 2.1.2) as liquid phase competitors. Inhibition of anti-ECOLI

ATP-dependent helicase hrpA $(HRPA)_{153\text{-}167}$ reactivity was measured under conditions similar to those described for ECOLI $MALE_{95\text{-}109}$.

5.2.7 Anti-peptide antibody affinity

The relative affinity of antibody binding (*see* 2.2.2.5) to ECOLI $MALE_{95\text{-}109}$ and PDC-$E2_{212\text{-}226}$ was carried out in 2 ECOLI $MALE_{95\text{-}109}$/PDC-$E2_{212\text{-}226}$ double reactive cases.

5.2.8 Three-dimensional modelling

Three-dimensional modelling of the ECOLI $MALE_{95\text{-}109}$ was carried out by analysing the structure of MALE ECOLI with the Cn3D visualization tool (*see* 2.1.2.3).

5.3 RESULTS

5.3. 1 Protein database search and analysis

The 10 non-OADC-related microbial sequences sharing extensive homology with human PDC-$E2_{212\text{-}226}$ are shown in Figure 5.1. Six are from ECOLI proteins, and one each from HELPY, PSEAE, HCMV, and HAEIN. ClpX is the ATP-binding subunit of the caseinolytic protease (Clp) complex, the ClpP, proteolytic, subunit of which is autoantibody target in PBC (Wang *et al.*, 1997, Singh *et al.*, 2000, Mayo *et al.*, 2000). ECOLI MALE is a soluble periplasmic protein and therefore potentially accessible to antibody (Boos and Shuman, 1998). HELPY UREB is a known antibody target in HELPY infection and PSEAE $DCDA_{355\text{-}363}$ is a CD8 epitope cross-recognised with PDC-$E2_{212\text{-}220}$ (Futagami *et al.*, 1998, Kita *et al.*, 2002). Human PDC-$E2_{212\text{-}226}$ (**K***L*S**E**G*D***LL***A***E***I***E***T*DK**) shares 11/15 (73%) similarity (5 identities) with the corresponding ECOLI PDC-$E2_{231\text{-}245}$ sequence (**K***V***A**AE*Q***SL***I***T***V***E***G*DK**, identities in **bold**, conservative substitutions in *italic*). According to the *PSI-BLAST* scoring system (*see* 2.2.1.2.1), ECOLI $CLPX_{280\text{-}294}$ ranks first in homology with human PDC-$E2_{212\text{-}226}$, among the non-OADC-related sequences of all species in the protein database, while all other 9 microbial mimics rank in a better position than does the ECOLI PDC-$E2_{231\text{-}245}$.

To investigate whether this over-representation of ECOLI homologues is accidental, due to the large number of ECOLI proteins in the database, or is biologically meaningful, a search for ECOLI mimics of $CYP2D6_{257\text{-}271}$, the immunodominant epitope

of LKM1 in AIH-2 has been performed (Alvarez *et al.*, 1999, Manns *et al.*, 1991). $CYP2D6_{257-271}$ was selected in view of its analogy with PDC-E2 in relation to PBC: 1) it is the major autoantigen of an autoimmune liver disease (Alvarez *et al.*, 1999, Manns *et al.*, 1991); 2) an intracellular enzyme exposed on a cell membrane (Robin *et al.*, 1997, Muratori *et al.*, 2000, Sugimura *et al.*, 2002); 3) LKM-1 antibodies are able to inhibit the enzymatic activity of CYP2D6 (Manns *et al.*, 1990, Manns and Obermayer-Straub, 1997, Kerkar *et al.*, 2003); 4) molecular mimicry has been proposed to account for immunological features of AIH-2 (Manns *et al.*, 1991).

In the database no ECOLI proteins were found to share 6-mers in common with $CYP2D6_{257-271}$; a 15-mer from maltodextrin phosphorylase shared 40% identity.

Sequence	aa position	ID	Protein	species	Identities	Similarity (%)
K *V* *A* A E *Q* S **L** *I* T *V* **E** *G* **D K**	(231-245)	PDC-E2	Pyruvate dehydrogenase complex-E2	*E.coli*	5	11/15 (73)
K L S E G D L L A E I E T D K	(212-226)	PDC-E2	Pyruvate dehydrogenase complex-E2	Human		
K *A* **S E G** *E* **L L A** *Q* *V* **E** *P* *E* D	(280-294)	CLPX	ATP-dependent clp X	*E.coli*	8	14/15 (93)
L *M* *T* *D* **G** I **L L A E I** *Q* Q **D** *R*	(153-167)	HRPA	ATP-dependent helicase hrpA	*E.coli*	7	12/15 (80)
G Y *A* *Q* *S* G **L L A E I** T *P* **D K**	(95-109)	MALE	Periplasmic maltose-binding protein	*E.coli*	7	11/15 (73)
D *A* *A* V E **D L L A E** *V* S Q P **K**	(605-619)	FADP	Fatty acid oxidation complex alpha	*E.coli*	6	9/15 (60)
L *A* *T* L D **D L L A E I** G L G *N*	(517-531)	SPOT	(P)ppGpp synthetase II	*E.coli*	6	9/15 (60)
G Q *A* M *V* **D L L A E** Y **E** K V G	(81-95)	NARW	Nitrate reductase 2	*E.coli*	6	8/15 (53)
R **L** *G* *D* *T* **D L** *I* **A E** *V* **E** H **D** Y	(22-36)	UREB	Urease beta subunit	*Helicobacter pylori*	7	13/15 (87)
A **L** *A* **E G D L L** A V R S *A* G A	(355-369)	DCDA	Diaminopimelate decarboxylase	*Pseudomonas aeruginosa*	7	9/15 (60)
V T *P* *N* *V* **D L L A E** *L* M *A* R S	(663-677)	V120	Capsid assembly protein UL47	Human cytomegalovirus	5	10/15 (67)
Q N **S E G D L L** E L Y C *G* *N* G	(205-219)	TRMA	tRNA (uracil-5-)-methyltranferase	*Haemophilus influenzae*	6	8/15 (53)

Figure 5. 1. *Sequence alignment between human E2 subunit of human pyruvate dehydrogenase complex (PDC-E2)$_{212\text{-}226}$, and microbial mimics. Amino acids in standard single-letter code. Identities in* ***bold****; Conservative substitutions in italic; aa, amino acid; ID, identification code*

5.3.2 ELISA

Anti-peptide antibody binding details for patients with PBC, pathological and healthy controls are given in Table 5.1 A-B, 5. 2 and 5.3.

5.3.2.1 Single anti-peptide antibody reactivity

Reactivity to human PDC-$E2_{212-226}$ was found in 52 (94.5%) of 55 AMA-positive PBC patients, in 1 (4%) of 23 AMA-negative PBC, in 16 (8.4%) of 190 pathological controls, among whom there were 10 women with recurrent UTI, all but one positive for AMA by ELISA and/or immunoblot; 1 HBV infected, 52 year old, male subject (AMA-negative); 2 HCV infected patients (a 67 year old female, anti-PDC-E2 positive by immunoblotting with a liver histology not compatible with PBC and a 37 year old male subject AMA-negative); 2 women (42 and 76 year old) with autoimmune thyroiditis (both AMA-negative); and a 36 year old female with SLE (high-titre AMA-positive by IFL with normal liver function tests) without history of liver disease. Among the 28 healthy controls, 1 (3.6%) reacted with PDC-$E2_{212-226}$ [a 62 year old male, AMA-positive at low-titre (1/40) by IFL with repeatedly normal liver function tests and without clinical signs of liver disease].

Reactivity to at least one of the 10 PDC-$E2_{212-226}$ microbial mimics was found in 39 (70.9%) AMA-positive PBC patients (among whom 3 did not react with any of the 6 ECOLI peptides); in 2 (9%) AMA-negative PBC patients ($p<0.0001$) (one reacted with ECOLI $SPOT_{517-531}$ and one with ECOLI $HRPA_{153-167}$), in 28 (14.7%) of the pathological controls including 11 (55%, $p=0.2$) with recurrent UTI; 2 with HBV; 5 with HCV; 5 with autoimmune thyroiditis; 1 with polyomyositis, 1 with SLE, 2 with AIH-1; and 1 with AIH-2, and 2 normal controls ($p<0.05$, for all).

Among the 3 PBC patients non reactive to PDC-$E2_{212-226}$, one reacted with ECOLI $SPOT_{517-531}$ alone, one with ECOLI $SPOT_{517-531}$ and ECOLI $HRPA_{153-167}$, and one with ECOLI $MALE_{95-109}$ and ECOLI $FADP_{605-619}$.

5.3.2.2 Double anti-peptide antibody reactivity

Double reactivity to at least one PDC-$E2_{212-226}$/microbial pair was found in 36 (65.4%) AMA-positive PBC patients, 1 (4%) AMA-negative PBC ($p<0.0001$) and in 13 (6.8%,

$p<0.01$) pathological controls, 9 with recurrent UTI, 1 with HCV, 2 with autoimmune thyroiditis, 1 with SLE, and in one healthy control ($p<0.01$).

Among the 36 PBC cases reacting with at least one PDC-E2/microbial pair, 33 (91.7%) were reactive with at least one ECOLI peptide among which 3 (9.1 %) were reactive with $ClpX_{280-294}$; 15 (45.5%) with $HRPA_{153-167}$; 16 (48.5%) with $MALE_{95-109}$; 11 (33.3%) with $FADP_{605-619}$; 10 (30.3%) with $SPOT_{517-531}$ and 6 (18.2%) with $NARW_{81-95}$. Other reactivities jointly with PDC-$E2_{212-226}$ included 1 (3%) with HEPLY $UREB_{22-36}$; 2 (6%) with PSEAE $DCDA_{355-369}$; 2 (6%) with HCMV $V120_{663-677}$; and 3 (9.1%) with HAEIN $TRMA_{205-219}$.

Double reactivity to at least one PDC-$E2_{212-226}$/ECOLI pair was found in 17 of 19 (89.5%) PBC patients with recurrent UTI, in 2 of 10 (20%) PBC patients with no history of recurrent UTI ($p<0.01$ *vs* PBC with recurrent UTI) and in 14 of 26 (53.8%) PBC patients with no urine tests ($p=0.01$ *vs* PBC with recurrent UTI). Of the 9 UTI patients without PBC who reacted with human PDC-$E2_{212-226}$, all reacted with at least one ECOLI peptide, 8 were AMA-positive by ELISA and/or immunoblot. There was no significant correlation between double reactivity and other experimental and clinical data.

Table 5.1A Antibody binding to human PDC-$E2_{212-226}$ and ECOLI mimics in 55 AMA-positive PBC patients

	PDC-$E2_{212-226}$ human	CLPX	HRPA	MALE ECOLI	FADP	SPOT	NARW	*	Recurrent UTI
1	3.7	neg	3.4	2.7	neg	2.1	neg	3	pos
2	7.1	neg	3.4	6.8	2.7	neg	neg	3	pos
3	4.3	neg	neg	5.3	neg	neg	neg	1	pos
4	2	neg	2.3	3.2	2.3	neg	neg	3	pos
5	2.3	neg	neg	2.3	neg	neg	neg	1	pos
6	4.3	neg	2	neg	neg	3.4	neg	2	pos
7	4	neg	neg	neg	2.7	neg	2	2	pos
8	3	neg	2	2.1	neg	neg	2.1	3	pos
9	2	neg	3.4	neg	neg	neg	2.5	2	pos
10	5.6	neg	neg	2	neg	3.9	neg	2	pos
11	2.3	neg	neg	neg	neg	neg	neg	0	pos
12	3	neg	3.4	neg	neg	2.3	neg	2	pos
13	2.1	3.6	2	2.5	2.6	neg	neg	4	pos
14	2	neg	neg	neg	2.3	4.3	neg	2	pos
15	2.7	2.3	neg	3.2	neg	neg	3.4	3	pos
16	neg	neg	neg	neg	neg	2.5	neg	1	pos
17	4.6	neg	neg	2.3	neg	neg	neg	1	pos
18	3.4	neg	3.4	neg	2.9	2.7	2.3	4	pos
19	6.7	neg	5.6	neg	3.5	6	neg	3	pos
20	4.9	neg	neg	neg	neg	neg	neg	0	neg
21	2.8	neg	neg	neg	neg	neg	neg	0	neg
22	2.2	neg	neg	2.2	neg	neg	neg	1	neg
23	2	neg	neg	neg	neg	neg	neg	0	neg
24	2	neg	neg	neg	neg	neg	neg	0	neg
25	2.9	neg	neg	neg	neg	neg	neg	0	neg
26	4	neg	neg	neg	neg	neg	neg	0	neg
27	5.6	neg	neg	neg	2.3	neg	neg	1	neg
28	2.3	neg	neg	neg	neg	neg	neg	0	neg
29	7.1	neg	neg	neg	neg	neg	neg	0	neg
30	2.1	neg	neg	neg	neg	2	neg	1	nt
31	3.8	neg	neg	4.5	neg	neg	neg	1	nt
32	3.4	neg	neg	neg	neg	neg	neg	0	nt
33	2.3	neg	neg	neg	neg	neg	neg	0	nt
34	2.9	neg	neg	neg	neg	neg	neg	0	nt
35	3.4	neg	2.3	neg	neg	neg	neg	1	nt
36	2	neg	neg	neg	neg	neg	neg	0	nt
37	2.6	neg	neg	neg	neg	neg	neg	0	nt
38	neg	neg	6.7	neg	neg	2.9	neg	2	nt
39	2.3	neg	neg	neg	neg	neg	neg	0	nt
40	5	neg	neg	neg	neg	neg	neg	0	nt
41	2.3	neg	3	3.2	neg	neg	neg	2	nt
42	3.3	neg	4.6	neg	neg	neg	neg	1	nt
43	3.8	neg	neg	neg	neg	neg	neg	0	nt
44	2	neg	2.5	neg	neg	neg	neg	1	nt
45	2.7	neg	neg	neg	2.6	neg	2	2	nt
46	5	neg	neg	neg	neg	neg	neg	0	nt
47	2.7	2.3	neg	neg	neg	neg	neg	1	nt
48	2.9	neg	neg	2.7	neg	neg	neg	1	nt
49	4	neg	neg	neg	2	2	neg	2	nt
50	2.4	neg	neg	neg	3.6	neg	neg	1	nt
51	3	neg	2.3	neg	neg	2.9	neg	2	nt
52	3	neg	neg	2.1	neg	neg	neg	1	nt
53	2.7	neg	neg	3.2	neg	neg	neg	1	nt
54	2.9	neg	neg	neg	neg	neg	neg	0	nt
55	neg	neg	neg	2.3	2.9	neg	neg	2	nt
***	52	3	16	17	12	12	6	36	
%	(94.5)	(5.4)	(29.1)	(30.9)	(21.8)	(21.8)	(10.9)	(65.4)	

Table 5.1B Antibody binding to human PDC-$E2_{212-226}$ and non-ECOLI microbial mimics in 55 AMA-positive PBC patients

	PDC-$E2_{212-226}$ human	UREB HELPY	DCDA PSEU	V120 HCMV	TRMA HAEIN	**
1	3.7	neg	neg	neg	3.2	4
2	7.1	neg	neg	neg	neg	3
3	4.3	neg	neg	neg	neg	1
4	2	neg	neg	neg	neg	3
5	2.3	neg	neg	neg	neg	1
6	4.3	neg	neg	neg	neg	2
7	4	neg	neg	neg	4.9	3
8	3	neg	2.3	2.3	neg	5
9	2	neg	neg	neg	neg	2
10	5.6	neg	neg	neg	neg	2
11	2.3	neg	neg	neg	neg	0
12	3	neg	neg	neg	neg	2
13	2.1	neg	neg	neg	neg	4
14	2	neg	neg	neg	neg	2
15	2.7	neg	neg	neg	neg	3
16	neg	neg	neg	neg	neg	1
17	4.6	neg	neg	neg	neg	1
18	3.4	neg	neg	neg	neg	4
19	6.7	neg	neg	neg	neg	3
20	4.9	neg	neg	neg	neg	0
21	2.8	neg	neg	neg	neg	0
22	2.2	neg	neg	neg	neg	1
23	2	2.3	neg	neg	neg	1
24	2	neg	neg	neg	neg	0
25	2.9	neg	neg	neg	neg	0
26	4	neg	neg	neg	neg	0
27	5.6	neg	neg	neg	neg	1
28	2.3	neg	neg	3.2	neg	1
29	7.1	neg	neg	neg	neg	0
30	2.1	neg	neg	neg	neg	1
31	3.8	neg	neg	neg	2.5	2
32	3.4	neg	neg	neg	neg	0
33	2.3	neg	2.7	neg	neg	1
34	2.9	neg	neg	neg	neg	0
35	3.4	neg	neg	neg	neg	1
36	2	neg	neg	neg	neg	0
37	2.6	neg	neg	neg	neg	0
38	neg	neg	neg	neg	neg	2
39	2.3	neg	neg	neg	neg	0
40	5	neg	neg	neg	neg	0
41	2.3	neg	neg	neg	neg	2
42	3.3	neg	neg	neg	neg	1
43	3.8	neg	neg	neg	neg	0
44	2	neg	neg	neg	neg	1
45	2.7	neg	neg	neg	neg	2
46	5	neg	neg	neg	neg	0
47	2.7	neg	neg	neg	neg	1
48	2.9	neg	neg	neg	neg	1
49	4	neg	neg	neg	neg	2
50	2.4	neg	neg	neg	neg	1
51	3	neg	neg	neg	neg	2
52	3	neg	neg	neg	neg	1
53	2.7	neg	neg	neg	neg	1
54	2.9	neg	neg	neg	neg	0
55	neg	neg	neg	neg	neg	2
***	52	1	2	2	3	39
%	(94.5)	(1.8)	(3.6)	(3.6)	(5.4)	(70.9)

Footnote to Table 5.1A-B

*Absorbance values are expressed as $OD^{test}/OD^{control}$ peptide and considered negative (neg) when <2; pos, positive; nt, no urine test; total number of * ECOLI or ** microbial peptides recognized by an individual patient; ***, total number (%) of patients recognizing a peptide; UTI, urinary tract infection; AMA, anti-mitochondrial antibody; PDC-E2, pyruvate dehydrogenase complex-E2; ECOLI, Escherichia coli; ClpX, caseinolytic protease X; HRPA, ATP-dependent helicase hrpA; MALE, maltose E; FADP, fatty acid oxidation complex alpha subunit; SPOT, (P) ppGpp synthetase II; NARW, nitrate reductase 2; HELPY, Helicobacter pylori; UREB, urease beta subunit; PSEU, Pseudomonas aeruginosa; DCDA, diaminopimelate decarboxylase; HCMV, human cytomegalovirus; V120, capsid assembly protein UL47; HAEIN, Haemophilus influenzae; TRMA, tRNA (uracil-5-)-methyltranferase*

Table 5.2 Single and double reactivity to self and microbial peptides expressed in absolute numbers and percentages (%), in 55 anti-mitochondrial antibody (AMA) positive patients with primary biliary cirrhosis (PBC), including 19 with recurrent urinary tract infection (UTI), 10 without UTI and 26 with no urine test, and 23 AMA-negative PBC patients

	AMA-positive PBC	AMA-positive PBC			AMA-negative PBC
	Total	with UTI	without UTI	no urine test	
	n=55	n=19	n=10	n=26	n=23
PDC-E2$_{212-226}$	52 (94.5)	18(95)	10 (100)	24 (92.3)	1 (4)
ECOLI CLPX$_{280-294}$	3 (5.4)	2 (10.5)	0	1 (3.8)	0
ECOLI HRPA$_{153-167}$	16 (29.1)	10 (52.6)	0	6 (23.1)	1 (4)
ECOLI MALE$_{95-109}$	17 (30.9)	10 (52.6)	1 (10)	6 (23.1)	0
ECOLI FADP$_{605-619}$	12 (21.8)	7 (36.8)	1 (10)	4 (15.3)	0
ECOLI SPOT$_{517-531}$	12 (21.8)	8 (42.1)	0	4 (15.3)	1 (4)
ECOLI NARW$_{81-95}$	6 (10.9)	5 (26.3)	0	1 (3.8)	0
ECOLI (at least 1 peptide)	36 (65.5)	18 (94.7)	2 (20)	16 (61.5)	2 (8)
HELPY UREB$_{22-36}$	1 (1.8)	0	1 (10)	0	0
PSEAE DCDA$_{355-369}$	2 (3.6)	1 (5.3)	0	1 (3.8)	0
HCMV V120$_{663-677}$	2 (3.6)	1 (5.3)	1 (10)	0	0
HAEIN TRMA$_{205-219}$	2 (3.6)	2 (10.5)	0	1 (3.8)	0
PDC-E2$_{212-226}$/ ECOLI CLPX$_{280-294}$	3 (5.4)	2 (10.5)	0	1 (3.8)	0
PDC-E2$_{212-226}$/ ECOLI HRPA$_{153-167}$	15 (27.3)	10 (52.6)	0	5 (19.2)	1 (4)
PDC-E2$_{212-226}$/ ECOLI MALE$_{95-109}$	16 (29.1)	10 (52.6)	1 (10)	5 (19.2)	0
PDC-E2$_{212-226}$/ ECOLI FADP$_{605-619}$	11 (20)	7 (36.8)	1 (10)	3 (11.5)	0
PDC-E2$_{212-226}$/ ECOLI SPOT$_{517-531}$	10 (18.2)	7 (36.8)	0	3 (11.5)	0
PDC-E2$_{212-226}$/ ECOLI NARW$_{81-95}$	6 (10.9)	5 (26.3)	0	1 (3.8)	0
PDC-E2$_{212-226}$/ HELPY UREB$_{22-36}$	1 (1.8)	0	1 (10)	0	0
PDC-E2$_{212-226}$/ PSEAE DCDA$_{355-369}$	2 (3.6)	1 (5.3)	0	1 (10)	0
PDC-E2$_{212-226}$/ V120 HCMV$_{663-677}$	2 (3.6)	1 (5.3)	1 (10)	0	0
PDC-E2$_{212-226}$/ HAEIN TRMA$_{205-219}$	3 (5.4)	2 (10.5)	0	1 (10)	0
PDC-E2$_{212-226}$/ ECOLI (at least 1pair)	33 (60)	17 (89.5)	2 (20)	14 (53.8)	1 (4)

Table 5.3 Single and double reactivity to self and microbial peptides expressed in absolute numbers and percentages (%), in 190 pathological controls and 28 healthy subjects

	UTI n=20	HBV n=27	HCV n=42	ALD n=17	AITD n=38	PM n=10	SLE n=9	AIH-1 n=15	AIH-2 n=12	Healthy n=28
PDC-$E2_{212-226}$	10 (50)	1 (3.7)	2 (4.8)	0	2 (5.2)	0	1 (11)	0	0	1 (3.6)
ECOLI $CLPX_{280-294}$	0	0	1 (2.4)	0	0	1 (10)	0	0	1 (8.3)	0
ECOLI $HRPA_{153-167}$	3 (15)	1 (3.7)	1 (2.4)	0	1 (2.6)	0	0	0	0	1 (3.6)
ECOLI $MALE_{95-109}$	4 (20)	0	2 (4.8)	0	4 (10.5)	0	1 (11)	1 (6.6)	0	1 (3.6)
ECOLI $FADP_{605-619}$	0	0	0	0	0	0	0	0	0	0
ECOLI $SPOT_{517-531}$	5 (25)	1 (3.7)	0	0	1 (2.6)	0	0	0	1 (8.3)	0
ECOLI $NARW_{81-95}$	2 (10)	0	0	0	0	1 (10)	0	1 (6.6)	0	0
ECOLI (at least 1 peptide)	11 (55)	2 (7.4)	4 (9.5)	0	5 (13.2)	1 (10)	1 (11)	2 (13.2)	1 (8.3)	2 (7.2)
HELPY $UREB_{22-36}$	3 (15)	0	1 (2.4)	0	1 (2.6)	0	0	2 (13.2)	0	0
PSEAE $DCDA_{355-369}$	1 (5)	0	0	0	1 (2.6)	0	0	0	0	0
HCMV $V120_{663-677}$	1 (5)	0	1 (2.4)	0	0	0	0	0	0	0
HAEIN $TRMA_{205-219}$	4 (20)	1 (3.7)	1 (2.4)	0	0	0	1 (11)	0	0	0
PDC-$E2_{212-226}$/ ECOLI $CLPX_{280-294}$	0	0	0	0	0	0	0	0	0	0
PDC-$E2_{212-226}$/ ECOLI $HRPA_{153-167}$	1 (5)	0	0	0	0	0	0	0	0	0
PDC-$E2_{212-226}$/ ECOLI $MALE_{95-109}$	4 (20)	1	0	0	2 (5.2)	0	1 (11)	0	0	1 (3.6)
PDC-$E2_{212-226}$/ ECOLI $FADP_{605-619}$	0	0	0	0	0	0	0	0	0	0
PDC-$E2_{212-226}$/ ECOLI $SPOT_{517-531}$	3 (15)	0	0	0	0	0	0	0	0	0
PDC-$E2_{212-226}$/ ECOLI $NARW_{81-95}$	2 (10)	0	0	0	0	0	0	0	0	0
PDC-$E2_{212-226}$/ HELPY $UREB_{22-36}$	2 (10)	0	1 (2.4)	0	0	0	0	0	0	0
PDC-$E2_{212-226}$/ PSEAE $DCDA_{355-369}$	1 (5)	0	1 (2.4)	0	0	0	0	0	0	0
PDC-$E2_{212-226}$/ V120 $HCMV_{663-677}$	1 (5)		0	0	0	0	0	0	0	0
PDC-$E2_{212-226}$/ HAEIN $TRMA_{205-219}$	3 (15)		0	0	0	0	1 (11)	0	0	0
PDC-$E2_{212-226}$/ ECOLI (at least 1pair)	9 (45)	0	0	0	2 (5.2)	0	1 (11)	0	0	1 (3.6)

Footnote to Tables 5.2 & 5.3

PBC, primary biliary cirrhosis; UTI, urinary tract infection; HBV, hepatitis B virus; HCV, hepatitis C virus; ALD, alcoholic liver disease; AITD, autoimmune thyroid disease; PM, polyomyositis; SLE, systemic lupus erythematosus; AIH, autoimmune hepatitis; n, number; PDC-E2; pyruvate dehydrogenase complex-E2; ECOLI, Escherichia coli; HELPY, Helicobacter pylori; PSEAE, Pseudomonas aeruginosa; HCMV, Human cytomegalovirus; HAEIN, Haemophilus influenzae

5.3.3 Inhibition studies

Inhibition of reactivity to microbial/self mimics by pre-incubation with relevant and control antigens is detailed in Table 5.4 and Figure 5.2.

Antibody binding to ECOLI $MALE_{95-109}$ (Table 5.4A) was inhibited by 66.7-79.6% after pre-incubation with the ECOLI $MALE_{95-109}$ in the seven cases tested. Among these, in cases 31, 53 (see Figure 5.2A-B), 3, 5 and 48 antibody binding was inhibited by 68.3-70.1% after pre-incubation with human PDC-$E2_{212-226}$ and by 67-73.2% after pre-incubation with PDC antigen (Table 5.4A). Inhibition ranged from 52-78% after pre-incubation with the full MALE protein in the 3 cases tested.

In cases 31 and 53 (Figure 5.2C-D), antibody binding to human PDC-$E2_{212-226}$ was significantly inhibited after pre-incubation with the PDC-$E2_{212-226}$, ECOLI $MALE_{95-109}$, PDC antigen and with the full ECOLI MALE protein.

As can be seen in Figure 5.2 and Table 5.4 the ECOLI $MALE_{95-109}$ was a better inhibitor of binding to both itself and PDC-$E2_{212-226}$ than PDC-$E2_{212-226}$.

Similar sets of results were obtained when competition experiments were performed measuring anti-ECOLI $HRPA_{153-167}$ antibody binding (Table 5.4C).

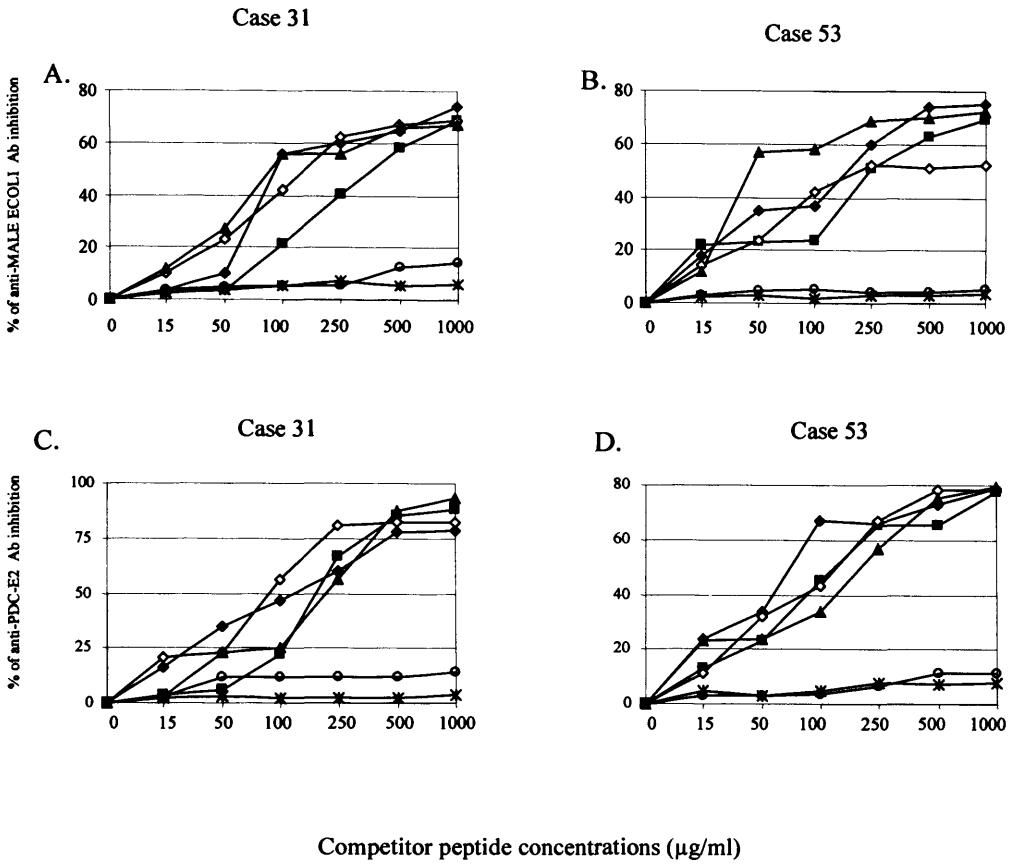


Figure 5. 2. *A-D: Inhibition of antibody (ab) binding against ECOLI MALE$_{95\text{-}109}$ (A,B) and human PDC-E2$_{212\text{-}226}$ (D,E). Pre-incubation with ECOLI MALE$_{95\text{-}109}$ (◆), human PDC-E2$_{212\text{-}226}$ (■), full ECOLI MALE protein (◊), PDC antigen (▲), control antigen (○) and control peptide (✳) of serum 31 (A,C) and 53 (B, D). On the vertical axis is the percentage of inhibition of antibody binding to the peptide in the presence of competitor peptide or antigen at different concentrations: 0; 15; 50; 100; 250; 500; 1000 µg/ml. ECOLI, E. coli; MALE, maltose E; PDC, pyruvate dehydrogenase complex*

Table 5.4 Inhibition of anti-ECOLI $MALE_{95-109}$ (A) or anti-PDC-$E2_{212-226}$ (B) or anti-ECOLI $HRPA_{153-167}$ (C) antibody reactivity by pre-incubation with relevant peptides and antigens.

	Case	Maximum Inhibition %					
A.		ECOLI $MALE_{95-109}$	ECOLI MALE protein	PDC-$E2_{212-226}$	PDC antigen	Control antigen	Control peptide
	31[a]	73.8	68.5	69.2	67	12	9.1
	53b	75.5	52	69.7	72.3	5.2	3.4
	15	79.6	78	22	18	13.8	8.3
	3	69.1		69.7	73.2	11.3	5.4
	5	71.2		70.1	68.2	8.8	8.9
	41	66.7		12.1	14.7	11.8	5.4
	48	72.2		68.3	68.1	12.9	11.1
B.		PDC-$E2_{212-226}$	ECOLI $MALE_{95-109}$	ECOLI MALE protein	PDC antigen	Control antigen	Control peptide
	31[c]	87.9	78.8	82.2	93.4	14	3.4
	53[d]	77.5	79	78.1	79.2	11.2	7.9
C.		ECOLI $HRPA_{153-167}$		PDC-$E2_{212-226}$	PDC antigen	Control antigen	Control peptide
	42	72.1		62.7	60.4	13.9	9.3
	18	77.6		76.3	75	5.2	1.3
	12	79.6		18.5	22.6	4.5	8.3
	1	72.8		73.2	77	9.2	9.1
	2	82.1		12.7	11.1	14.2	11.2
	9	65		20.7	19.5	18.9	6.9
	19	69.9		66.8	66	13.8	6.2
	41	69.3		67.2	65.6	12.1	7.8

[a], See Fig 5.2A ; [b], See Fig 5.2B; [c], See Fig 5.2C; [d], See Fig 5. 2D, ECOLI, E. coli; MALE, maltose E ; PDC-E2, pyruvate dehydrogenase complex E2 subunit ; HRPA, ATP-dependent helicase hrpA

5.3.4 Affinity of anti-peptide antibody

The relative affinity of antibody binding to ECOLI $MALE_{95\text{-}109}$ and PDC-$E2_{212\text{-}226}$ for cases 31 and 53 is illustrated in Figure 5.4 demonstrating higher relative antibody affinity to ECOLI $MALE_{95\text{-}109}$ compared to PDC-$E2_{212\text{-}226}$, the avidity indices being 1.23 *vs* 0.83 and 1.3 *vs* 0.47 mol/l, respectively.

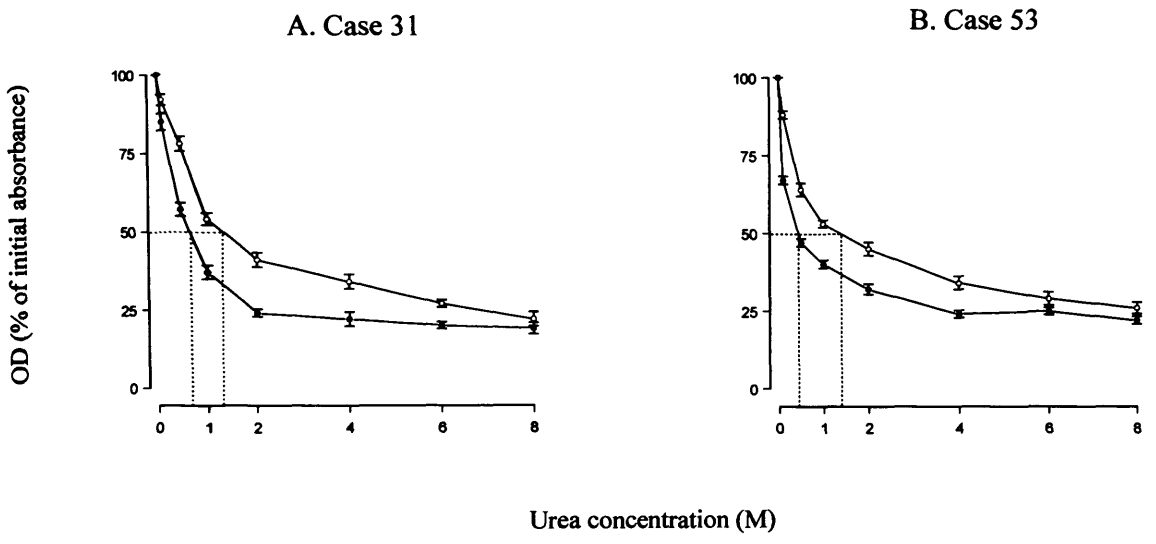


Figure 5.3 *Affinity profile of anti-peptide antibody. ELISA microplate wells were coated with ECOLI* $MALE_{95\text{-}109}$ *and human* PDC-$E2_{212\text{-}226}$. *After incubation with individual serum samples of cases 31 (A) and 53 (B) at a dilution of 1/1000, urea was added at increasing concentrations and allowed to inhibit antibody-antigen interaction for 15 minutes. Absorbance values were plotted as the percentage of initial absorbance against urea concentration (mean ± standard deviation). Each point is the average of 5 observations. From this the relative affinity index, defined as the concentration of urea that elutes 50% of the antibody reactivity, for both ECOLI* $MALE_{95\text{-}109}$ (○) *and human* PDC-$E2_{212\text{-}226}$ (●) *was extrapolated (-----). ECOLI, E. coli; MALE, maltose E; PDC-E2, pyruvate dehydrogenase complex-E2; OD, optical density*

5.4 DISCUSSION

The present Chapter has looked for microbial proteins containing peptidyl sequences mimicking human PDC-$E2_{212-226}$ and among a number of hits the best 10 mimics were selected for further analysis. Six of these were from *E. coli* while 4 were, one each, from *Helicobacter pylori*, *Pseudomonas aerigunosa*, human CMV, and *Haemophilus influenzae*.

The potential biological significance of these homologies was emphasized by the fact that their sequences were better mimics to human PDC-$E2_{212-226}$ than the ECOLI PDC or other microbial PDC corresponding sequences. The extent of similarity and the large number of non OADC ECOLI mimics of the human PDC-$E2_{212-226}$ epitope seems more than coincidental as indicated by the lack of microbial mimics for the control $CYP2D6_{257-271}$ peptide, representing the immunodominant epitope targeted by LKM-1 autoantibody in patients with AIH-2 (Alvarez *et al.*, 1999, Manns *et al.*, 1991, Yamamoto *et al.*, 1993). So a possibility arises that, if there is a microbial involvement in the generation of the characteristic AMA and/or in the pathogenesis of PBC, the considerable number of microbial mimics contributes to the selection of this particular sequence on PDC-E2 as a dominant epitope.

Sequence similarity does not necessarily lead to structural/conformational similarity and, hence, needs not equate with actual cross-reactivity (antigenic mimicry), particularly in the case of B-cell epitopes (Oldstone, 1987, Dyrberg *et al.*, 1990, Quaratino *et al.*, 1995, Bogdanos *et al.*, 2000). In the present work such seems to be the case with the ECOLI ClpX peptide. This bears the strongest sequence similarity with the human PDC-$E2_{212-226}$, but antibodies to it were only seen in 5.4% of PBC cases. Nevertheless, two-thirds of the PBC patients react with at least one of the ECOLI peptides, up to 1/3 of these 55 patients specifically reacting with ECOLI $MALE_{95-109}$ and/or ECOLI $HRPA_{153-167}$.

Within PBC, reactivity to ECOLI peptides was strongly associated with a history of recurrent UTI, reactivity to at least one ECOLI peptide being present in virtually all recurrent UTI positive PBC patients and in only a minority of those without recurrent UTI. Outside PBC, approximately half of the patients with recurrent UTI had PBC specific anti-PDC-E2 antibodies and this was always in association with reactivity to ECOLI mimics. Four of these AMA-positive microbial/self reactive patients were

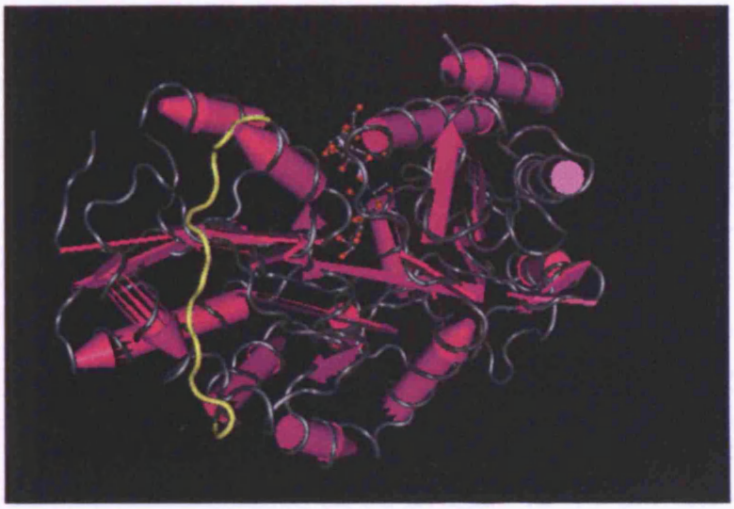
followed-up for 5 years, remaining positive for AMA and ECOLI/PDC-E2 reactivity in the absence of biochemical and clinical signs of PBC. The anti-ECOLI reactivity seen in virtually all the patients with PBC and recurrent UTI was shown here to be cross reactive with the homologous PDC-E2 sequence in a synthetic peptide, and, importantly, in a full length purified ECOLI protein, as shown for the maltose binding protein. This latter periplasmic ECOLI gene product was able to abrogate reactivity to both ECOLI $MALE_{95\text{-}109}$ and PDC-E2 $_{212\text{-}226}$. Notably, three-dimensional modelling of MALE ECOLI predicts $MALE_{95\text{-}109}$ to be located in a solvent-accessible surface region of the protein, compatible with antibody recognition on the whole protein (*see* Figure 5. 4) (Laver *et al.*, 1990, Howard *et al.*, 1998).

The findings of the present work strengthen the notion that a microbial exposure, especially through ECOLI-urinary tract infection, may be instrumental to the appearance and/or maintenance of AMA responses by a cross-reactive mechanism (Stemerowicz *et al.*, 1988, Hopf *et al.*, 1989, Burroughs *et al.*, 1992, Butler *et al.*, 1995a, Butler *et al.*, 1995b, Shimoda *et al.*, 1998, Shimoda *et al.*, 2000, Parikh-Patel *et al.*, 2001, Butler *et al.*, 1993). In view of the remarkable sequence similarities of PDC-E2 across species, Burroughs *et al.* (1992) originally suggested that molecular mimicry between PDC-E2 of microorganisms, especially of ECOLI, and PDC-E2 of man could lead to a cross-reactive immune response manifesting itself as AMA. This suggestion arose in the light of early epidemiological considerations, such as an association of AMA with UTI and the fact that sera and T-cell clones from patients with PBC are capable of human/microbial PDC-E2 cross-recognition (Stemerowicz *et al.*, 1988, Hopf *et al.*, 1989, Burroughs *et al.*, 1992, Butler *et al.*, 1995a, Butler *et al.*, 1995b, Shimoda *et al.*, 1998, Shimoda *et al.*, 2000, Parikh-Patel *et al.*, 2001, Butler *et al.*, 1993). A significant addition to the present debate is the evidence recently provided by Parikh-Patel *et al.*, who, in the first controlled epidemiological analysis, showed a positive association of PBC with UTI (Parikh-Patel *et al.*, 2001).

The role of molecular mimicry and UTI in PBC needs, however, to be revisited, especially since the present study has been able to demonstrate cross-reactive immunity between human $PDC\text{-}E2_{212\text{-}226}$ and microbial sequences, particularly from *E. coli*, which do not belong to microbial OADC. Furthermore as shown in Figure 5.2 and 5.3 and Table 5.4 there appears to be greater antibody affinity for the ECOLI mimics than for $PDC\text{-}E2_{212\text{-}226}$. This, and the observation that reactivity to non-PDC-E2 mimics in the

control population is more common than that to PDC-$E2_{212-226}$, both indicate that reactivity to the bacterial sequences may precede that against self.

A



B

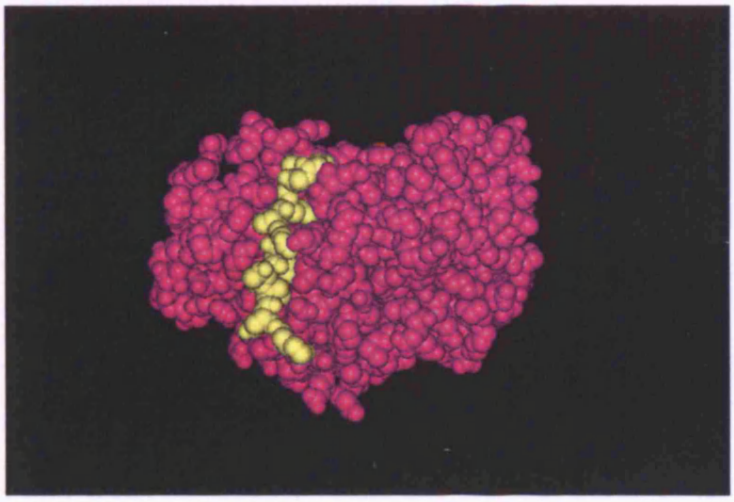


***Figure 5.4**. Three dimensional structure of the periplasmic maltose binding protein of E. coli (ECOLI MALE) with a wire frame (A) backbone (grey) coloured by secondary structure (purple) and a space fill (B) backbone. The relevant ECOLI MALE$_{95\text{-}109}$ sequence is displayed in yellow. The structure was analyzed with the Cn3D visualization tool (see 2.1.2.3)*

CHAPTER 6

Helicobacter pylori and PBC

6.1 BACKGROUND

In the previous Chapter (*see* Chapter 5), it was shown that in spite the universal antibody recognition of the PDC-$E2_{212-226}$ autoepitope, its close *Helicobacter pylori* (HELPY) mimic was rarely recognised in serum samples from PBC patients (1/55, 1.8%). This finding was surprising considering the degree and the nature of the microbial/self amino acid homology, on top of experimental evidence linking HELPY infection to PBC (Lin *et al.*, 1995, Fox *et al.*, 1995, Fox *et al.*, 1998, Figura *et al.*, 1998, Nilsson *et al.*, 1999, Nilsson *et al.*, 2000a, Nilsson *et al.*, 2000b, Monstein *et al.*, 2002, Fallone *et al.*, 2003). Thus, HELPY and *Helicobacter hepaticus* (HELHEP) have recently been implicated in the pathogenesis of PBC since their DNA has been found in liver tissue of patients whose sera contained antibodies to the microbe (Lin *et al.*, 1995, Fox *et al.*, 1995, Fox *et al.*, 1998, Figura *et al.*, 1998, Nilsson *et al.*, 1999, Nilsson *et al.*, 2000a, Nilsson *et al.*, 2000b, Monstein *et al.*, 2002, Fallone *et al.*, 2003). Moreover, the PDC-$E2_{212-226}$-mimicking HELPY sequence originates from the urease beta (UREB) subunit, a protein that with urease A (UREA) represents a major focus of anti-HELPY immunity (Pappo *et al.*, 1995, Suerbaum and Josenhans, 1999, Zevering *et al.*, 1999, Kimmel *et al.*, 2000, Suerbaum and Michetti, 2002).

In gastric pathology, HELPY has been shown to be cross-reactively targeted at the T-cell level with the gastric H^+K^+-ATPase proton pump, the major autoantigen in autoimmune gastritis, with which it shares extensive similarities (Amedei *et al.*, 2003).

In the present study the nature of antibody unresponsiveness to the HELPY UREB mimic was investigated. Since B- and T-cell epitopes frequently do not co-localise, the possibility that the HELPY UREB mimic and PDC-$E2_{212-226}$ are targets of cross-reactive response at the CD4 T-cell level has also been explored. Finally, the disease-specificity of antibody reactivity to HELPY antigens in patients with PBC was compared to those of a large number of controls including patients with viral hepatitides, autoimmune thyroiditis, systemic lupus erythematosus and healthy subjects.

6.2 METHODS

6.2.1 Subjects

Serum samples were obtained from 112 patients with PBC (mean age 53±14.2 years; 99 female), all but 15 AMA-positive by IIF, (median titre: 1/1280, range 1/40 – 1/10480), median duration of disease 59 months (range 1-227) attending the out-patient clinic of Liver Transplantation and Hepatobiliary Medicine Unit, Royal Free Hospital.

Pathological controls included 98 AMA-negative patients (mean age 54±11.9 years, 89 female), all positive for anti-HELPY antibodies by ELISA and immunoblotting including 25 with chronic HBV infection; 31 with chronic HCV infection; 23 with autoimmune thyroid disease and 19 with systemic lupus erythematosus.

Sixteen healthy, AMA-negative, volunteer members of staff (mean age 38±8 years, 13 female), all anti-HELPY antibody positive, were tested as normal controls.

6.2.2 Antibody detection

AMA was detected by conventional IIFL, immunoblotting and ELISA (*see* 2.2.2). Antibodies to HELPY were tested by commercially available ELISA (*see* 2.1.6.1) and immunoblotting using as antigen a purified HELPY antigen extract containing UREB (*see* 2.1.6.2).

6.2.3 Protein database search and analysis

The UREB HELPY homologue of human PDC-$E2_{212-226}$ was identified by scanning the SwissProt protein database using the *ProteinInfo* programme (*see* 2.2.2.1). Amino acid comparison of full-length human PDC-E2 and HELPY UREB was carried out using the *BLASTp* 2 sequences protein-protein comparison programme (*see* 2.2.1.2.2). The observed similarities were analysed in relation to those between human PDC-E2 and UREA HELPY, UREB HELHEP or UREA HELHEP.

6.2.4 Peptide synthesis

Three 15-mer peptides containing the relevant human inner PDC-$E2_{212-226}$, PDC-$E2_{91-105}$, and HELPY $UREB_{22-36}$ mimics and an irrelevant 15 aa control peptide were constructed (see 2.2.2.3).

6.2.5 ELISA

Antibody binding to the peptides was determined by ELISA (*see* 2.2.2.3). The final peptide concentration was 15μg/ml and the dilution of serum samples was 1/200.

6.2.6 Inhibition studies

6.2.6.1 Peptides

To investigate whether the simultaneous reactivity to HELPY $UREB_{22-36}$ and human PDC-$E2_{212-226}$ was due to cross-reactivity, competition ELISA was performed, measuring residual anti-HELPY $UREB_{22-36}$ or anti-PDC-$E2_{212-226}$ reactivity after incubation respectively with HELPY $UREB_{22-36}$, PDC-$E2_{212-226}$ and a control peptide as liquid phase competitors (*see* 2.4.2.2.1).

6.2.6.2 Antigens

The possibility that PDC-E2 and HELPY UREB are targets of cross-reactive antibody responses at the level of full proteins was addressed through inhibition studies investigating inhibition of antibody reactivity to human liver PDC-E2, on immunoblot using as solid phase competitor a purified HELPY extract containing the UREB subunit (*see* 2.1.6.2); conversely, antibody recognition of UREB was tested before and after solid phase incubation of serum samples with purified PDC from porcine heart mitochondria (*see* 2.1.2).

6.2.7 Three-dimensional modelling

Three-dimensional modelling of the relevant HELPY $UREB_{22-36}$ was carried out by analysing the structure of UREA/UREB HELPY complex with the Cn3D visualization tool (*see* 2.1.2.3).

6.2.8 Proliferation assay of PBMC

Proliferative responses (*see* 2.2.3.3) to the HELPY $UREB_{22-36}$, PDC-$E2_{212-226}$ peptides and PDC antigen were investigated in 7 PBC patients and 5 pathological controls (4 with HCV, 1 with alcoholic hepatitis), all female (median age 54, range 37-68), all positive for anti-HELPY antibodies.

6.3 RESULTS

6.3.1 Protein database search and analysis

BLASTp 2 sequences comparison revealed the best similarity between the 569 aa long UREB protein and the inner lipoyl domain of PDC-E2 to involve the immunodominant PDC-$E2_{212-226}$ epitope. HELPY $UREB_{22-36}$ shares 13/15 (87%) similarity (7 identities) with the human PDC-$E2_{212-226}$ (*see* Figure 6.1).

Two other HELPY UREB sequences had similarity with PDC-E2; but the extent of similarity was lower; 47% (6 identities) and 53% (5 identities) and the PDC-E2 mimics correspond to sequences which are not PDC-E2 epitopes (*see* Figure 6.1).

The best homology UREA HELPY shares with PDC-E2 is a 8/15 (53%) similarity (5 identities) between HELPY $UREA_{1-14}$ and PDC-$E2_{424-438}$ (*see* Figure 1); no similarity exists between the UREA HELPY and the PDC-$E2_{212-226}$.

The corresponding sequence on UREB of HELHEP shares also homology with human PDC-$E2_{212-226}$, though to a lesser extent compared to that of HELPY (*see* Figure 6.1).

Antigen	aa	Sequence	Similarity (%)	Identities
human PDC-E2 (ILD)	(212-226)	K L S E G D L L A E I E T D K	13/15 (87%)	7
		. : . . . : : . : : . : :		
HELPY UREB	(22-36)	R L G D T D L I A E V E H D Y		
HELPY UREB	(463-477)	A N A S I P T P Q P V Y Y R E	7/15 (47%)	6
		: : : : : .		
human PDC-E2	(293-307)	V A A V P P T P Q P L A P T P		
HELPY UREB	(463-477)	A N A S I P T P Q P V Y Y R E	8/15 (53%)	5
		. . . : : : : :		
human PDC-E2	(283-297)	P Q V P P P T P P P V A A V P		
HELPY UREA	(1-14)	M K L T P K E L D K L M L H Y	9/15 (60%)	5
		. : : : : . : . .		
human PDC-E2	(424-438)	V L L V R K E L N K I L E G R		
HELHEP UREB	(20-34)	R L G D T N L F A E I E K D Y	13/15 (87%)	6
		. : . . . . : . : : . : :		
human PDC-E2 (ILD)	(212-226)	K L S E G D L L A E I E T D K		

Figure 6.1 *Sequence alignment between human E2 subunit of human pyruvate dehydrogenase complex (PDC-E2), and urease B (UREB) and urease A (UREA) of Helicobacter pylori (HELPY) and Helicobacter hepaticus (HELHEP). Amino acids in standard single-letter code. Identities in* ***bold****; Conservative substitutions in italic; ILD, inner lipoyl domain*

6.3.2 Reactivity to peptides and antigens

Reactivity to human PDC-$E2_{212-226}$ was found in a total of 104 PBC patients: 94 (97%) of 97 AMA-positive PBC cases, and 10 (67%) of 15 AMA-negative PBC cases.

Among the 104 anti-PDC-$E2_{212-226}$ positive patients, reactivity to HELPY $UREB_{22-36}$ was found in 2 (2%) and reactivity to the HELPY UREB antigen in 72 (69%) including the 2 anti-HELPY $UREB_{22-36}$ positive cases. Among the 10 anti-PDC-$E2_{212-226}$ negative patients, there was no reactivity to HELPY $UREB_{22-36}$, 7 (70%) reacting with the HELPY UREB antigen.

Among the 98 pathological controls, reactivity to human PDC-$E2_{212-226}$ was found in 3 (3%) including 2 patients with HCV and one with SLE; and to HELPY $UREB_{22-36}$ in 2 (one with HBV and one with HCV) not reacting to human PDC-$E2_{212-226}$. There was no correlation of antibody reactivity to PDC-$E2_{212-226}$ or PDC-E2 antigen and individual UREB or UREA (or other HELPY antigens) (*see* Figure 6.2A). The pattern of reactivity to HELPY antigens by immunoblot was similar between anti-PDC-E2 positive and negative PBC cases, as well as between PBC and controls (Figure 6.2B).

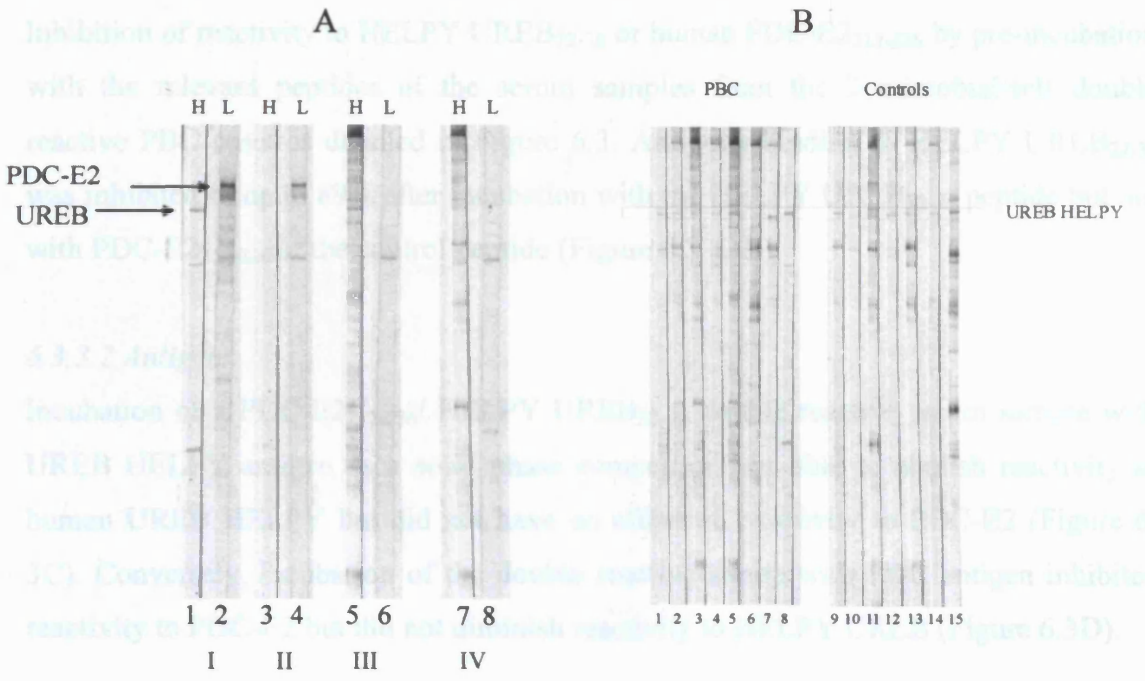


Figure 6.2 *Patterns of reactivity to HELPY and PDC antigens*

(A). Antibody reactivity to HELPY (H) and human liver (L) PDC antigens in representative PBC cases. Note the presence of reactivity to both UREB (lane 1) and PDC-E2 (lane 2) in the serum sample (I); absence of reactivity to UREB (lane 3) in the presence of reactivity to PDC-E2 (lane 4) in another PBC case (II); and presence of reactivity to UREB (lane 5 and 7) in the absence of reactivity to PDC-E2 (lanes 6 and 8) in two other PBC cases (III and IV)

(B). Patterns of reactivity to HELPY antigens in PBC cases – anti-PDC-E2 positive (lanes 1-4) or negative (5–8) and controls (9-15)

6.3.3 Inhibition studies

6.3.3.1 Peptides

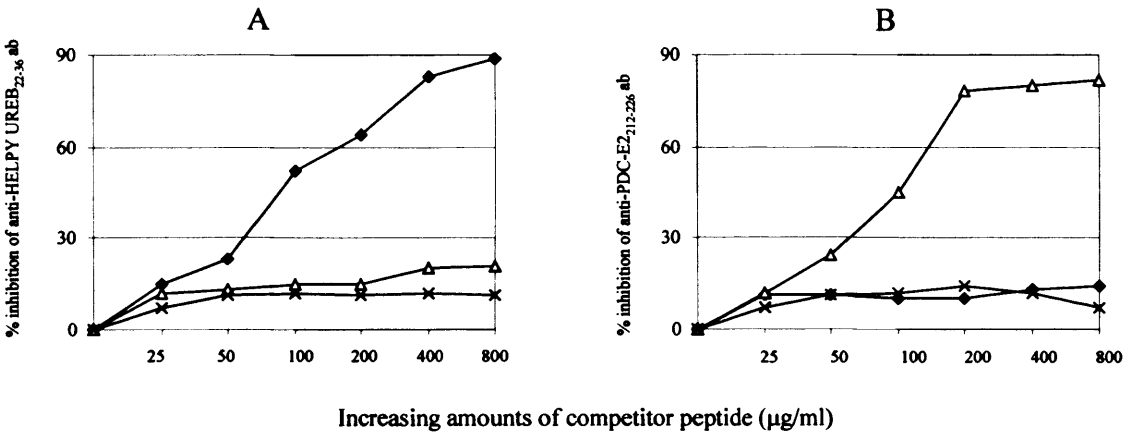
Inhibition of reactivity to HELPY $UREB_{22-36}$ or human PDC-$E2_{212-226}$ by pre-incubation with the relevant peptides of the serum samples from the 2 microbial/self double reactive PBC cases is detailed in Figure 6.3. Antibody binding to HELPY $UREB_{22-36}$ was inhibited in up to 89% after incubation with the HELPY $UREB_{22-36}$ peptide but not with PDC-$E2_{212-226}$ or the control peptide (Figure 6.3 A-B).

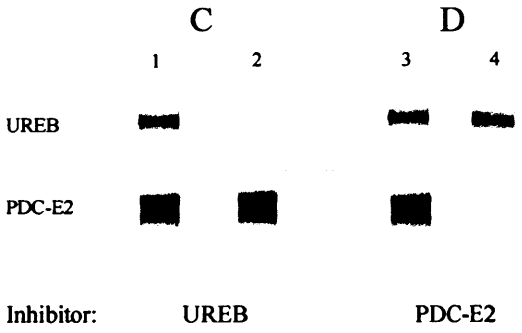
6.3.3.2 Antigens

Incubation of a PDC-$E2_{212-226}$/ HELPY $UREB_{22-36}$ double reactive serum sample with UREB HELPY antigen as a solid phase competitor was able to abolish reactivity to human UREB HELPY but did not have an effect on reactivity to PDC-E2 (Figure 6. 3C). Conversely, incubation of the double reactive serum with PDC antigen inhibited reactivity to PDC-E2 but did not diminish reactivity to HELPY UREB (Figure 6.3D).

6.3.4 Three-dimensional modelling

Three dimensional modelling predicts the core of HELPY $UREB_{22-36}$ not to be exposed on the surface of the UREA/UREB complex (*see* Figure 6.4).


A
B
% inhibition of anti-HELPY UREB22-36 ab
% inhibition of anti-PDC-E2212-226 ab
90
60
30
0
25
50
100
200
400
800
Increasing amounts of competitor peptide (µg/ml)


C
D
1
2
3
4
UREB
PDC-E2
Inhibitor:
UREB
PDC-E2

Legend to Figure 6. 3

A-B: Inhibition of antibody binding against HELPY UREB$_{22-36}$ (A) and human PDC-E2$_{212-226}$ (B). Pre-incubation with HELPY UREB$_{22-36}$ (◆), human PDC-E2$_{212-226}$ (△), and control peptide (×) of a HELPY UREB$_{22-36}$/PDC-E2$_{212-226}$ double reactive serum sample. On the vertical axis is the percentage of inhibition of antibody binding to the peptide in the presence of competitor peptide or antigen at different concentrations: 0; 25; 50; 100; 200; 400; 800 μg/ml.

C-D: Antibody binding against HELPY UREB (top band) and human PDC-E2 (bottom band) before (lanes 1 and 3) and after pre-incubation (2 and 4) with the HELPY extract antigen (C) or PDC antigen (D) of a HELPY UREB$_{22-36}$/PDC-E2$_{212-226}$ double reactive serum. Note that HELPY antigen as inhibitor is able to abolish reactivity to human UREB HELPY (lane 2, top) but does not have an effect on reactivity to PDC-E2 (lane 2, bottom). Conversely, incubation of double reactive sera with PDC antigen inhibits reactivity to PDC-E2 (lane 4, bottom) but does not diminish reactivity to HELPY UREB (lane 4, top). HELPY, Helicobacter pylori; PDC, pyruvate dehydrogenase complex; UREB, urease B

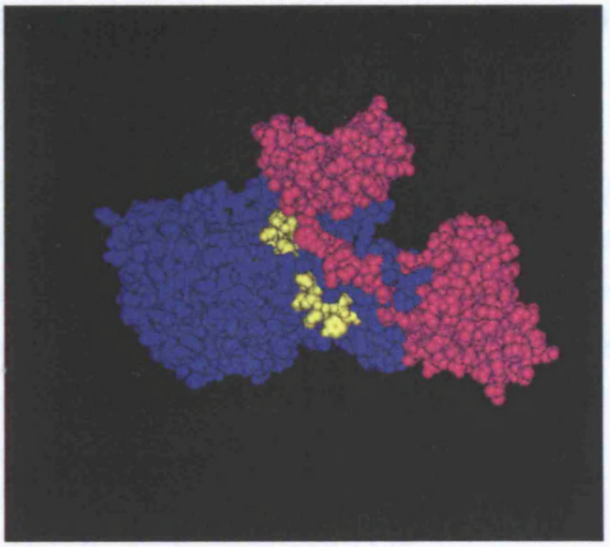


Figure 6.4 *Three-dimensional modelling structure of the urease B (UREB; blue colour) and urease A (UREA; pink colour) complex of Helicobacter pylori (HELPY) with a space fill. The PDC-E2$_{212-226}$ mimicking HELPY UREB$_{22-36}$ sequence is shown in yellow. The structure was analysed with the Cn3D visualisation tool. Note the lack of exposure of the core of the peptidyl sequence to the surface of the molecule in contrast to its corners*

6.3.5 Proliferation assay of PBMC

Proliferative responses to peptides and antigens is summarised in Figure 6.5. Stimulation of PBMC with the non-specific stimulator PHA resulted in strong proliferative responses in PBC patients (mean SI ± SD: 22.3 ± 4.64) and pathological controls (13.6 ± 2.25).

Proliferative responses to the PDC-$E2_{212-226}$ peptide were found in 6 of the 7 PBC patients (4.1 ± 0.8) and 1 (2.9 ± 1.2) of 5 pathological controls (2 ± 0.3) - a 53-year old woman with chronic HCV infection negative for AMA. All seven PBC patients (7.67 ± 2) and one control (the 53-year HCV infected woman) recognised also the PDC antigen. No reactivity was found to the HELPY $UREB_{22-36}$ in PBC patients (1.44 ± 0.1) or pathological controls (1.24 ± 0.3) (Figure 6.5).

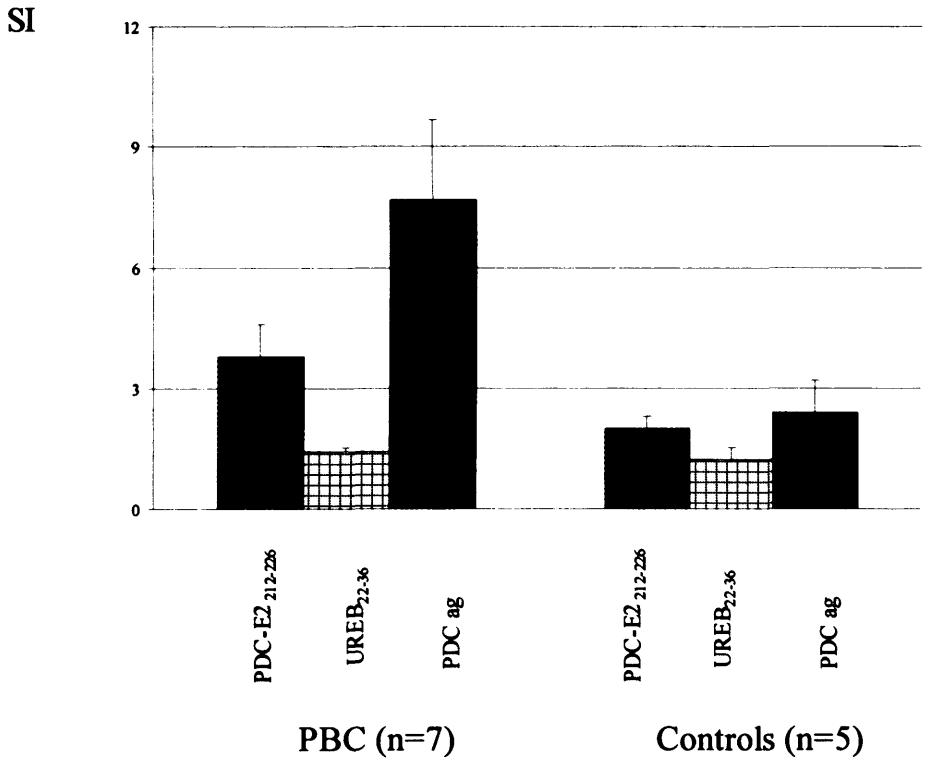


Figure 6. 5 *Proliferative responses of peripheral blood mononuclear cells from 7 PBC patients and 5 pathological controls to the PDC-$E2_{212-226}$, HELPY $UREB_{22-36}$, PDC antigen (ag). A stimulation index (SI) ≥ 3 was considered positive; PBC, primary biliary cirrhosis; PDC, pyruvate dehydrogenase complex; UREB, urease B*

6.4 DISCUSSION

The present study has investigated the role of molecular mimicry between *helicobacter pylori* and PDC-E2, the major mitochondrial autoantigen in PBC and found no evidence of cross-reactive immunity in PBC patients.

This finding was unexpected in view of the fact that not only do HELPY and PDC-E2 share a striking similarity in molecular mimicry terms, but also the microbial sequence originates from UREB, an antigen which, in complex with urease A (UREA), is a key target of anti-HELPY immunity (Pappo *et al.*, 1995, Suerbaum and Josenhans, 1999, Zevering *et al.*, 1999, Kimmel *et al.*, 2000, Suerbaum and Michetti, 2002). Moreover, the best similarity between microbe and self involves the immunodominant autoepitope on PDC-E2 (PDC-$E2_{212-226}$). Of pathogenic relevance is the fact that *helicobacter* DNA has been found in bile and liver tissue from patients with PBC, their sera containing antibodies to HELPY, a microbe able to induce cross-reactive immune responses against gastric antigens, and hepatobiliary pathology in animals (Lin *et al.*, 1995, Fox *et al.*, 1995, Fox *et al.*, 1998, Figura *et al.*, 1998, Nilsson *et al.*, 1999, Nilsson *et al.*, 2000a, Nilsson *et al.*, 2000b, Monstein *et al.*, 2002, Fallone *et al.*, 2003, Amedei *et al.*, 2003).

In spite of the universal antibody recognition of the PDC-$E2_{212-226}$ autoepitope, its close HELPY UREB mimic was rarely recognised in serum samples from PBC patients, unlike other cases where microbial mimics do exhibit double- and frequently cross-reactivity (*see* Chapter 3, 4 and 5).

A possible explanation for this finding is provided by the molecular modelling study which predicts that the native microbial sequence would be inaccessible to B lymphocyte receptors (Figure 6.4). Furthermore, the sequence would not be seen by antibody to intact PDC-E2 or its dominant epitope in the mimicking PDC-E2 sequence. However the synthetic peptide itself would not be in the restricted conformation existing in the native protein, and yet it does not react with antibodies recognising the PDC-E2 peptide that it so strongly mimics. It seems, therefore, that the similarity UREB HELPY shares with PDC-E2, is not enough for cross-recognition in spite of great amino acid homology (Dyrberg *et al.*, 1990, Wucherpfennig *et al.*, 1997). Compared to the human PDC-$E2_{212-226}$ autoepitope (KLSEGDLLAEIETDK), the HELPY $UREB_{22-36}$ mimic (RLGDTLIAEVEHDY) has a positively-charged and bulky histidine (H_{34}) which would not be a conservative substitution for T_{224} of PDC-E2 or any of the residues in the same relative position in any of the other microbial mimics that have

shown antibody cross-reactivity to PDC-$E2_{212-226}$ (*see* Chapter 5), and this is evident also for the bulky and aromatic tyrosine (Y_{36}), replacing the K_{226} that has been identified as an important residue in the PDC-E2 epitope. These differences could lead to a non-recognised conformation in a sequence that is otherwise so similar.

The above considerations would account for the absence of a distinct pattern of PBC-specific reactivity to HELPY antigens in PBC patients compared to pathological and healthy controls (*see* Figure 6.2).

In the case of T-cell epitopes there is no conformational restriction as to which peptide may be cleaved for presentation by MHC on B-lymphocytes (Delves and Roitt, 2000b, Delves and Roitt, 2000a). However, binding for presentation may well be significantly impaired by the substitutions referred to (Wucherpfennig *et al.*, 1997). So it is not surprising that, in the present case, there is no evidence that the mimicking urease peptide is a CD4 epitope either. It seems therefore unlikely that HELPY is involved in the induction of AMA antibody responses, either directly or indirectly, by a mechanism of molecular mimicry.

These clear-cut negative findings illustrate that sequence similarity does not necessarily lead to structural/conformational similarity and, hence, need not equate with actual cross-reactivity (antigenic mimicry) (Oldstone, 1987, Quaratino *et al.*, 1995). Conversely, they suggest that cross-reactivities between autopitopes and mimicking peptides of infecting organisms, when they are observed, are probably not trivial, and may well have a bearing on the mechanism of disease.

CHAPTER 7

Caseinolytic protease complex and PBC

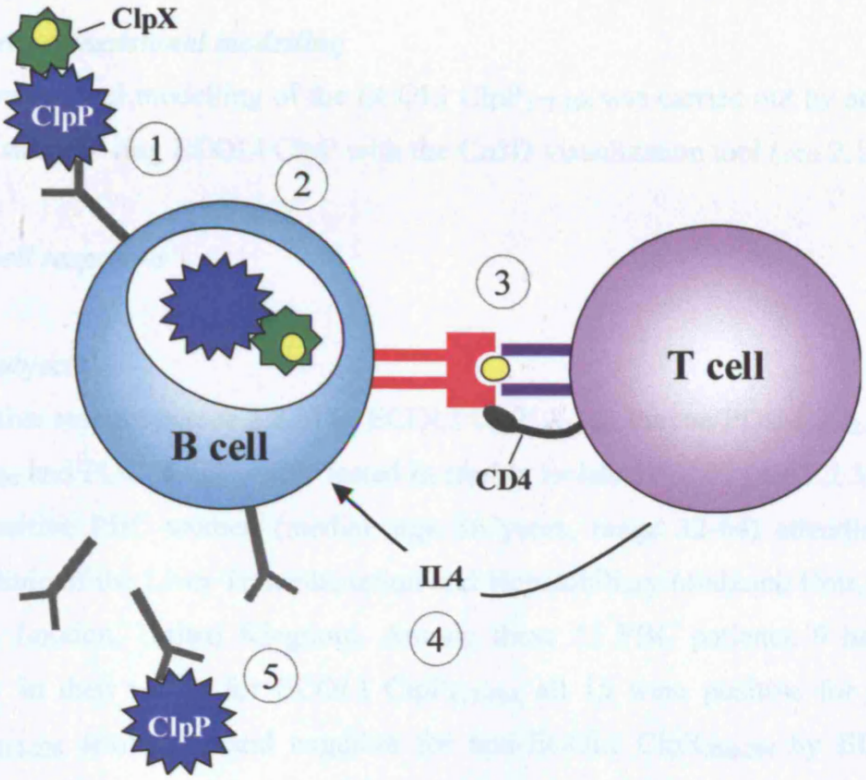
7.1 BACKGROUND

During the completion period of this Thesis, a study from a Spanish group of Investigators reported that antibodies directed to ECOLI $ClpP_{177-194}$ are strongly associated with PBC being present in one third of patients with PBC but in less than 1% of a large number of pathological and normal controls (Mayo *et al.*, 2000). This finding was of relevance to the present study because of the following: 1) there are no sequence similarities between the ECOLI ClpP and human PDC-E2 antigens that would account for association between anti-ClpP and anti-PDC-E2 reactivity; 2) the anti-ClpP and anti-PDC-E2 antibodies are not cross-reactive; 3) ECOLI ClpP protein forms an important proteolytic complex with ClpX, a peptidyl sequence of which (ECOLI $ClpX_{280-294}$) bears the strongest sequence similarity to human $PDC\text{-}E2_{212-226}$ but is virtually unrecognised by antibodies in patients with PBC as shown in Chapter 5 (Wang *et al.*, 1997, Mayo *et al.*, 2000, Singh *et al.*, 2000).

These various observations led to the hypothesis (*see* Figure 1) that during ECOLI infection, B-cells with receptors recognising a repetitive, exposed sequence of ClpP, internalise the entire ClpP/ClpX complex, and present the human $PDC\text{-}E2_{212-226}$-mimicking peptide of ClpX on HLA class II. PBC-specific CD4 T-cells, cross-recognising through molecular mimicry the human $PDC\text{-}E2_{212-226}$ epitope and the ClpX peptide would provide help in the production of disease specific antibodies against ClpP.

This hypothesis leads to three testable predictions that became the focus of the present section:

1. The ECOLI $ClpP_{177-194}$ sequence must be located in a solvent-accessible surface region of the protein, compatible with B-cell receptor recognition (Laver *et al.*, 1990).
2. The ECOLI $ClpX_{280-294}$ mimic –but not $ClpP_{177-194}$ - must be recognised by CD4 T-cells from PBC patients.
3. Finally, PDC-specific T-cells must be able to provide help for the production of anti-ECOLI $ClpP_{177-194}$ by virtue of cross-recognition by ECOLI $ClpX_{280-294}$ (Stevens *et al.*, 1988, Mosmann and Coffman, 1989, Mahon *et al.*, 1995).



PDC-E2	K	L	S	E	G	D	L	L	A	E	I	E	T	D	K
	:	.	:	:	:	.	:	:	:	.	.	:	.	.	
ECOLI CLPX	K	A	S	E	G	E	L	L	A	Q	V	E	P	E	D

:, identity; ., conservative substitution

Figure 7. 1
Hypothesis: The P subunit of the Clp complex of E. coli is recognised by a specific antibody present on the surface of a B lymphocyte (1). The complex is internalised (2), processed and a peptide of the X subunit is inserted in an HLA class II molecule and presented to a CD4 helper T lymphocyte possessing the appropriate T-cell receptor (TCR) (3). The helper T lymphocyte releases soluble mediators such as IL4 (4) stimulating B lymphocyte proliferation and release of anti-P specific antibodies (5). Of note is the fact that the peptide potentially recognised by the helper T lymphocyte shares striking similarity with the primary biliary cirrhosis (PBC) specific PDC-E2 immunodominant epitope (inset). It can be hypothesised that the cross-reactivity between these two antigenic determinants at the helper T-cell level may account for the PBC-specific antimicrobial response (P subunit of Clp complex of ECOLI) described in the paper by Mayo et al. (2000)

7.2 METHODS

7.2.1 Three-dimensional modelling

Three-dimensional modelling of the ECOLI $ClpP_{177-194}$ was carried out by analysing the structure of the 7-ring ECOLI ClpP with the Cn3D visualization tool (*see* 2.1.2.3).

7.2.2 T-cell responses

7.2.2.1 Subjects

Proliferative responses (*see* 2.2.3) to ECOLI $ClpX_{280-294}$, human PDC-$E2_{212-226}$, ECOLI $ClpP_{177-194}$ and PDC antigen were tested in freshly isolated PBMC (*see* 2.2.3.1) from 15 AMA positive PBC women (median age 56 years, range 32-64) attending the out-patient clinic of the Liver Transplantation and Hepatobiliary Medicine Unit, Royal Free Hospital, London, United Kingdom. Among these 15 PBC patients, 9 had antibody reactivity in their serum for ECOLI $ClpP_{177-194}$; all 15 were positive for anti-human PDC-$E2_{212-226}$ antibodies and negative for anti-ECOLI $ClpX_{280-294}$ by ELISA. Nine demographically matched women were also tested as pathological controls including 4 women with chronic HCV infection, three with alcoholic hepatitis and 2 with AIH-1. Three age-matched healthy female subjects were tested as normal controls. All twelve controls were negative for AMA, ECOLI $ClpX_{280-294}$, human PDC-$E2_{212-226}$, and ECOLI $ClpP_{177-194}$ reactivity.

7.2.2.2 Standard proliferation assay

A standard proliferation assay (*see* 2.2.3.3) was performed to investigate cellular responses of PBC patients and controls in the presence of individual ECOLI $ClpX_{280-294}$, human PDC-$E2_{212-226}$, ECOLI $ClpP_{177-194}$ and PDC antigen.

7.2.2.3 Intracellular cytokine staining

The antigen-specific cellular response was further evaluated by intracellular cytokine staining (*see* 2.2.3.4). Briefly, PBMC from 5 anti-ECOLI $ClpP_{177-194}$ antibody positive PBC women resuspended in complete medium, were cultured for 2 weeks with purified PDC antigen in the presence of IL-2 and irradiated autologous PBMC. At day 14, 1 x 10^6 cells/ml were cultured for 72 hours with individual ECOLI $ClpX_{280-294}$, human PDC-$E2_{212-226}$, and ECOLI $ClpP_{177-194}$ peptides in complete medium, without IL-2, and

in the presence of irradiated autologous PBMC. At day 4, cells were washed and subjected to intracellular cytokine staining with QRed-conjugated anti-CD4, and PE-conjugated anti-IFN-γ.

7.2.3 T-cell 'help' for B-cell antibody production

To test whether ECOLI $ClpX_{280-294}$-specific T-cell responses have the ability to provide 'help' for the production of anti-ECOLI $ClpP_{177-194}$ antibodies, PBMC from 5 PBC patients with proliferative responses to ECOLI $ClpX_{280-294}$ and 5 without were cultured for 2 weeks in the presence of purified PDC antigen and IL-2 (*see* 2.2.4). At day 14, cells were washed and 1 x 10^6/ml PBMC were cultured for 3 days with ECOLI $ClpX_{280-294}$, in the presence of 0.5 x 10^6/ml irradiated autologous PBMC but without IL-2. At the end of the culture, the cell-free supernatant was collected and tested by ELISA for antibody reactivity to ECOLI $ClpX_{280-294}$, ECOLI $ClpP_{177-194}$, and an irrelevant control peptide (*see* 2.2.2.3).

7.3 RESULTS

7.3.1 Three-dimensional modelling

A three-dimensional model of ECOLI ClpP predicts $ClpP_{177\text{-}194}$ to be repetitively exposed on the surface of the molecule (Figure 7.2).

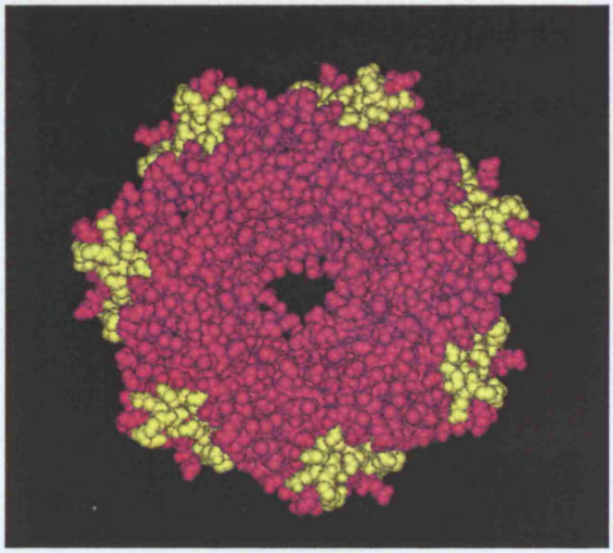


Figure 7.2 *Three dimensional modelling of the hepta-symmetric caseinolytic protease P (ClpP) ring-like structure of Escherichia coli with a space fill backbone (purple). The structure is viewed face-on. The immunodominant ECOLI* $ClpP_{177\text{-}194}$ *B-cell epitope is exposed on the surface of the 7-mer molecule (displayed in yellow). The structure was analysed with the Cn3D visualisation tool*

7.3.2 T-cell responses

7.3.2.1 Standard proliferation assay

Eight of the nine anti-ECOLI $ClpP_{177-194}$ antibody positive PBC women showed strong T-cell responses to ECOLI $ClpX_{280-294}$ (Figure 7.3). These responses were absent in the 6 anti-ECOLI $ClpP_{177-194}$ antibody negative PBC women and in the 12 demographically matched controls. Proliferative responses to ECOLI $ClpX_{280-294}$ were stronger than those to human PDC-$E2_{212-226}$ in all but one ECOLI $ClpX_{280-294}$ reactive PBC cases. There were no T-cell responses to ECOLI $ClpP_{177-194}$.

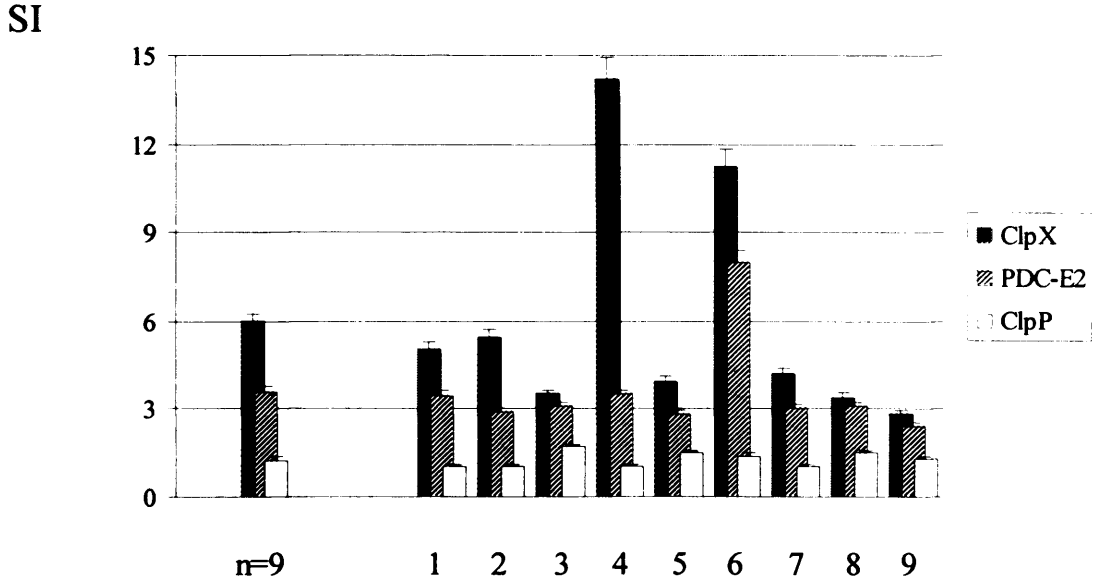


Figure 7.3 *Proliferative responses of peripheral blood mononuclear cells (PBMC) in 9 PBC patients seropositive for anti-Escherichia coli (ECOLI) caseinolytic protease (Clp)$P_{177-194}$ antibodies to ECOLI $ClpX_{280-294}$, the human pyruvate dehydrogenase complex-E2 (PDC-E2)$_{212-226}$ mimic and the ECOLI $ClpP_{177-194}$ epitope. Results are expressed as the stimulation index (SI) which is the ratio of the mean counts per min (cpm) from triplicate determinations in the presence of antigen to the mean cpm obtained in the absence of antigen. The wells exhibiting a mean SI >3 were considered positive. Results are presented as mean+SD for the total number of patients (9 cases) and for individual cases*

7.3.2.2 Intracellular cytokine staining

A mean of 43,000 lymphocyte gated events per sample were collected (minimum 20,000) by flow cytometry. All experiments were done in triplicate. In all five cases, similar or higher numbers of IFN-γ producing CD4 cells were seen following stimulation with ECOLI $ClpX_{280-294}$, mean % of IFN-γ producing CD4 cells (12.7%) ± SD (3.8) than with human $PDC\text{-}E2_{212-226}$ (10.2± 3.1) (*see* Figure 7.4). Stimulation with ECOLI $ClpP_{177-194}$ induced IFN-γ-producing CD4 cells comparable in numbers to those of control wells (without peptide) (5.6 ±1.3 *vs* 4.9 ±1.7).

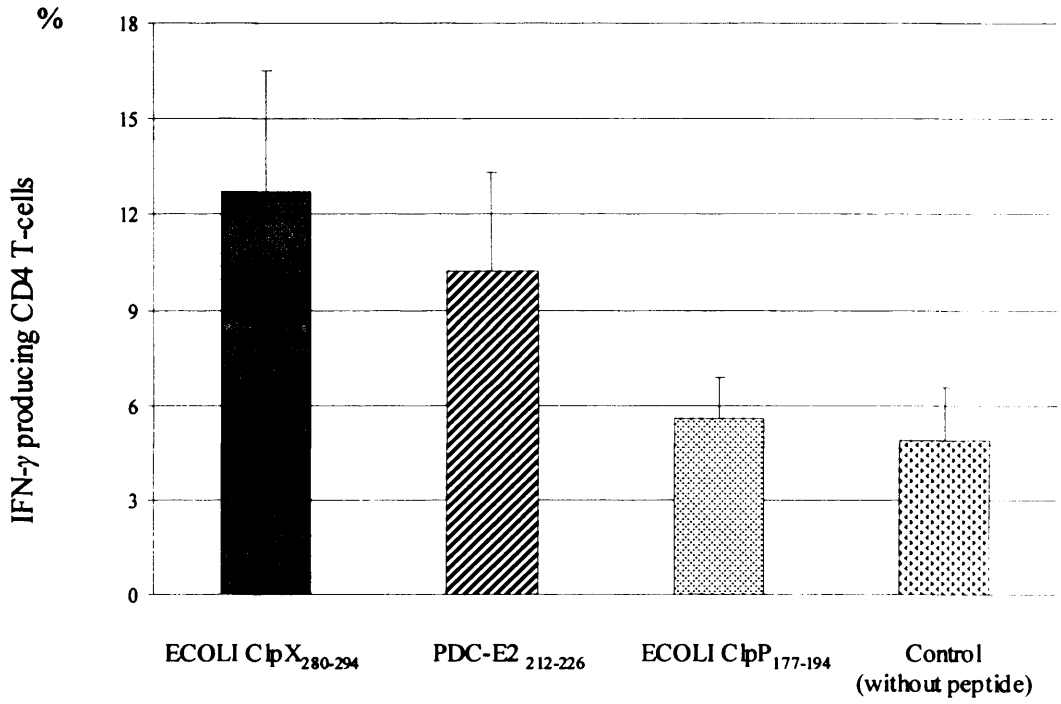


Figure 7.4 *Percentage of interferon (IFN)-γ producing CD4 T-cells in the presence of individual peptides determined by intracellular cytokine staining. PBMC from 5 anti-ECOLI $ClpP_{177-194}$ antibody positive PBC women resuspended in complete medium, were cultured for 2 weeks with purified PDC antigen in the presence of IL-2 and irradiated autologous PBMC. At day 14, cells were washed and cultured for 72 hours with individual ECOLI $ClpX_{280-294}$, human $PDC\text{-}E2_{212-226}$, ECOLI $ClpP_{177-194}$ and control peptides in complete medium, without interleukin-2. At day 4, cells were washed and subjected to intracellular cytokine staining with QRed-conjugated anti-CD4, and PE-conjugated anti-IFN-γ. The frequency of interferon (IFN)-γ producing CD4 T-cells is similar or higher after stimulation with ECOLI $ClpX_{280-294}$ than with $PDC\text{-}E2_{212-226}$, decreasing in the presence of ECOLI $CLPP_{177-194}$, or control (without peptide). ECOLI, Escherichia coli; Clp, caseinolytic protease X; PDC, pyruvate dehydrogenase complex*

7.3.3 T-cell 'help' for B-cell antibody production

Anti-ECOLI $ClpP_{177-194}$ antibody reactivity was found only in the supernatant of patients who had shown proliferative responses to ECOLI $ClpX_{280-294}$ (Figure 7.5).

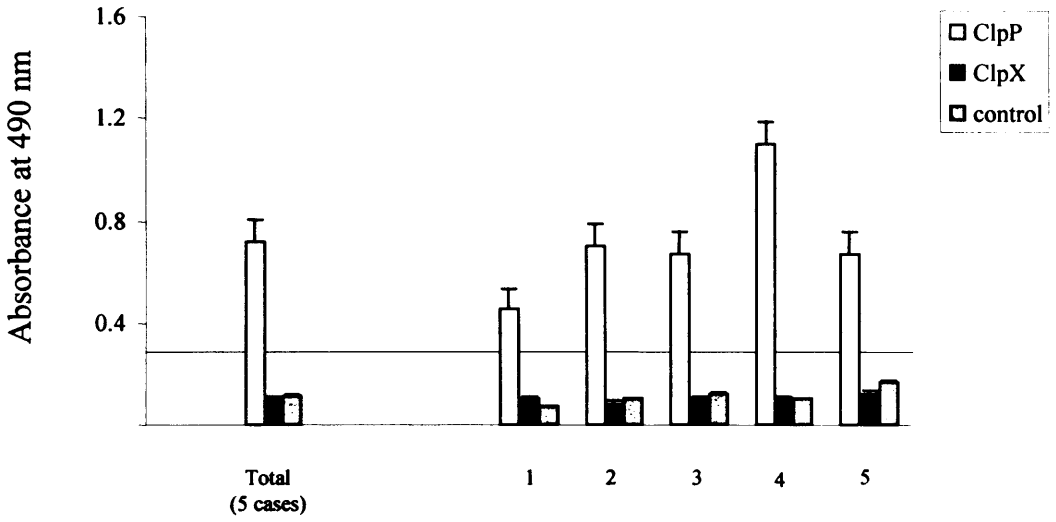


Figure 7.5 *Detection by ELISA of anti-peptide antibody in five patients with primary biliary cirrhosis (PBC) positive for proliferative responses to E. coli caseinolytic protease X (ClpX)$_{280-294}$. Cells were cultured with E. coli $ClpX_{280-294}$ and cell-free supernatant was used to determine antibody reactivity against E. coli $ClpX_{280-294}$, Escherichia coli $ClpP_{177-194}$ and an irrelevant control peptide. A cut off for positive reaction was determined as absorbance-optical density (OD) = 0.29, this cut off representing OD values exceeding mean+5SD of 36 readings using serum from 12 healthy subjects against E. coli $ClpP_{177-194}$ and is represented as a horizontal line*

7.4 DISCUSSION

The aim of the present section of the Thesis was to examine the hypothesis that anti-ECOLI ClpP antibodies specifically found in patients with PBC may arise through 'help' given by auto-reactive T-cells that recognise a peptide of ClpX, the regulatory sub-unit of the Clp protease complex, that is a structural mimic of a dominant T-cell epitope of PDC-E2 (Figure 7.1) (Stevens *et al.*, 1988, Mosmann and Coffman, 1989, Mahon *et al.*, 1995, Mayo *et al.*, 2000, Singh *et al.*, 2000, Wang *et al.*, 1997). This would account for the facts that anti-ClpP and PDC-E2 antibodies are not cross-reactive, (ursurprisingly, since the respective antigens do not share amino acid homologies), and that there is no antibody reactivity to ClpX in patients with PBC, notwithstanding the strong sequence similarity with the dominant PDC-E2 autoepitope (*see* Chapter 5).

This hypothesis generated three predictions which have been tested experimentally: Firstly, the ECOLI $ClpP_{177-194}$ sequence must be located in a solvent-accessible surface region of the protein, compatible with B-cell receptor recognition (Laver *et al.*, 1990). A three-dimensional model of ECOLI ClpP predicts $ClpP_{177-194}$ to be repetitively exposed on the surface of the molecule (Figure 7.2). Furthermore, the proposed ClpX T-cell epitope is the core of a segment that is essential for the formation of the ClpP/ClpX complex (Kim *et al.*, 2001). Secondly, the ECOLI $ClpX_{280-294}$ mimic - but not $ClpP_{177-194}$ - must be recognised by CD4 T-cells from PBC patients. Both, a standard proliferation assay and intracellular cytokine staining revealed that ECOLI $ClpX_{280-294}$ is a target of CD4-specific T-cell responses. Intriguingly, the ECOLI $ClpX_{280-294}$ mimic proved to be a better stimulus for the production of IFN-γ not only than the ECOLI $ClpP_{177-194}$ peptide but also of the homologous human $PDC\text{-}E2_{212-226}$. Finally, PDC-specific T-cells should be able to provide 'help' for the production of anti-ECOLI $ClpP_{177-194}$ by virtue of cross-recognition by ECOLI$ClpX_{280-294}$ (Stevens *et al.*, 1988, Mosmann and Coffman, 1989, Mahon *et al.*, 1995, Mayo *et al.*, 2000). This seems to be the case as anti-ECOLI $ClpP_{177-194}$ antibody reactivity was found only in the cell-free supernatant of cultures from PBC patients who have been cultured in the presence of ECOLI $ClpX_{280-294}$.

The findings of the present section strengthen the contention that a microbial exposure, such as that of recurrent *E. coli*-urinary tract infection, may be instrumental to the appearance and/or the maintenance of anti-mitochondrial responses by complex

molecular mimicry mechanisms operating at the T-cell level (Stemerowicz *et al.*, 1988, Hopf *et al.*, 1989, Burroughs *et al.*, 1992, Butler *et al.*, 1995a, Butler *et al.*, 1995b, Shimoda *et al.*, 1998, Shimoda *et al.*, 2000, Parikh-Patel *et al.*, 2001, Butler *et al.*, 1993). The kind of scenario proposed in this case is analogous to what is commonly seen in immune reactions to viral infections (Stavnezer, 1996, Liang and Mamula, 2000, Snapper *et al.*, 1997, Welsh *et al.*, 2004). If correct in the case of antibodies to ECOLI ClpP, it may also give an insight into other findings in PBC, such as the occurrence of various, disease-specific anti-nuclear antibodies, where again the antigens have no obvious relationship to PDC-E2 (Courvalin and Worman, 1997, Szostecki *et al.*, 1997).

CHAPTER 8

Hepatitis C and liver kidney microsomal type 1 antibody

8.1 BACKGROUND

In the previous chapters (Chapters 3-7) the concept of molecular mimicry has been used as a tool for the identification of microbial agents sharing homologies with the PDC-$E2_{212-226}$, the major mitochondrial autoepitope in patients with PBC. These studies have been based on the assumption that the knowledge of the self target in the form of a peptidyl sequence may indirectly provide useful information as to the identity of microbial agents responsible for the initiation and/or the maintenance of the autoimmune response through a mechanism of immunological cross-reactivity. Molecular mimicry has been also suggested as a mechanism accountable for viral triggered autoimmunity. LKM1 antibody, for example, which is the serological hallmark of AIH-2, is also present in up to 10% of chronic hepatitis C virus (HCV) infected patients.

The only defined target of LKM1, both in AIH2 and HCV infection, is CYP2D6, a 50 kDa microsomal enzyme which is also present on the hepatocyte plasma membrane (Alvarez *et al.*, 1985, Gueguen *et al.*, 1989), (Robin *et al.*, 1997, Muratori *et al.*, 2000, Sugimura *et al.*, 2002). The presence of B-cell epitopes on CYP2D6 has been investigated by several groups and there is consensus that the region spanning amino acids 252-271 represents the immunodominant epitope in LKM1 positive AIH-2, being less frequently recognised by LKM1 positive HCV infected patients (Manns *et al.*, 1991, Yamamoto *et al.*, 1993, Muratori *et al.*, 1995, Dalekos *et al.*, 1999).

The mechanism by which this particular short cytochrome sequence becomes the target of LKM1 antibody responses remains speculative (Manns and Obermayer-Straub, 1997, Bogdanos and McFarlane, 2003). Having noted that $CYP2D6_{252-271}$ shares amino acid homology with sequences of the NS5B and the E1 proteins of the HCV polyprotein and the ICP4 of HSV1, Manns *et al.* (1991) proposed that molecular mimicry and immunological cross-reactivity could explain reactivity to these short sequences and ultimately result in the production of LKM1. This hypothesis has never been addressed experimentally.

The present study investigates whether $CYP2D6_{252-271}$ and its viral homologues are targets of a humoral immune response and whether this immune response is cross-reactive. Peptides spanning the relevant sequences were constructed to test antibody binding by

ELISA in sera from 23 LKM1 seropositive chronically HCV infected patients, and, as control, 23 LKM1 seronegative chronically HCV infected patients.

8.2 METHODS

8.2.1 Subjects

Twenty-three patients with chronic hepatitis C (median age 47 years, range 22-79; 17 female), seropositive for LKM1 (LKM^{pos}/HCV^{pos}) by IIFL at a median titre of 1/320 (range 1/40 - 1/2,560) attending the out-patient clinic of the Department of Internal Medicine, Cardioangiology, Hepatology, University of Bologna, Italy, were studied. In 19 the HCV genotype was known, being 1a in seven, 1b in eight and 2a in four. Twelve patients were under IFNα treatment, with a mean 6.13±3.3SD months duration of therapy. All patients were HLA class I and class II typed (*see* below).

Twenty-three patients with chronic hepatitis C, seronegative for LKM1 (HCV^{pos}/LKM^{neg}) and matched for age, sex, HCV genotype, and IFNα treatment (type/dose/duration) were studied as controls (Table 8.1). All patients attended the out-patient clinic of the Department of Internal Medicine, Sismanoglion Hospital, Athens. Greece.

All of the patients with chronic hepatitis C were HCV RNA positive and anti-HCV antibody positive by a third generation enzyme-linked immunosorbent assay. All patients were negative for HBsAg, anti-HBsAg antibody, anti-Hepatitis B core (anti-HBc) antibody, and anti-human immunodeficiency virus.

8.2.2 Antibody detection

LKM1 was detected by IIFL (*see* 2.2.2.1). A commercially available ELISA was used to detect IgG anti-HSV1 specific antibodies (*see* 2.1.6.1).

8.2.3 Protein database search and analysis

The '*BLASTp 2 sequences*' programme was used (*see* 2.2.1.2.2) to search for aa sequence similarity between human $CYP2D6_{252\text{-}271}$ and HCV polyprotein, and the ICP4 HSV1 protein, formerly known as 175 kDa immediate early protein (IE175), as they were provided by the protein sequence databank SWISS-PROT. The SYFPEITHI programme was used for HLA B51 epitope prediction (www.uni-tuebingen.de/uni/kxi/).

8.2.4 Peptide synthesis

The following five 15-mer biotinylated peptides, NS5B $HCV_{2769-2783}$ /ICP4 $HSV1_{156-170}$/$CYP2D6_{257-271}$; E1 $HCV_{310-324}$/$CYP2D6_{252-266}$ containing the relevant homologous sequences (*see* Figure 8.1) and an irrelevant control peptide were constructed (*see* 2.2.2.3).

8.2.5 ELISA

Antibody binding to the peptides was determined by ELISA (*see* 2.2.2.3). The final peptide concentration was of 5μg/ml and the dilution of serum samples was of 1/200.

8.2.6 Inhibition studies

To investigate whether the simultaneous reactivity to E1 $HCV_{310-324}$ and homologous $CYP2D6_{252-266}$ was due to cross-reactivity, competition ELISAs were performed (*see* 2.2.2.4.1), measuring residual anti-E1 $HCV_{310-324}$ antibody reactivity after incubation with E1 $HCV_{310-324}$, $CYP2D6_{252-266}$, $CYP2D6_{257-271}$, recombinant CYP2D6 protein, ICP4 $HSV1_{156-170}$, the irrelevant control peptide and the control protein (porcine heart mitochondrial PDC antigen) as liquid phase competitors (final concentrations: 5, 10, 25, 50, 100, 250, 500, 1000 μg/ml). Anti-ICP4 $HSV1_{156-170}$ reactivity after pre-incubation with ICP4 $HSV1_{156-170}$, $CYP2D6_{257-271}$, $CYP2D6_{252-266}$, recombinant CYP2D6 protein, E1 $HCV_{310-324}$, the irrelevant control peptide and the control protein was measured under conditions identical to those used for E1 $HCV_{310-324}$.

8.3 RESULTS

8.3.1 Protein database search and analysis

Sequence similarities between published NS5B and E1 proteins of the HCV genome polyprotein and $CYP2D6_{252-271}$ (peptide $CYP2D6_{252-266}$ and peptide $CYP2D6_{257-271}$) are shown in Figure 8.1. The NS5B HCV sequence has 8 similarities (6 identities) with $CYP2D6_{257-271}$. The E1 HCV sequence has 8 similarities (6 identities) with $CYP2D6_{252-266}$. Previous studies have reported 5 aa identities not taking into account that Isoleucine $(I)_{313}$, Threonine $(T)_{314}$ and Alanine $(A)_{319}$ of E1 HCV polyprotein are similar to Leucine $(L)_{255}$ (conserved substitution), Threonine $(T)_{256}$ and Threonine $(T)_{261}$ (conserved substitution) of $CYP2D6_{252-266}$ (*see* Figure 8.1).

8.3.2 ELISA

ELISA results of the LKM1 seropositive and LKM1 seronegative patients with hepatitis C and absorbance values for individual sera reactive with peptides are detailed in Table 8.1.

8.3.2.1 Reactivity to peptides

Reactivity to NS5B $HCV_{2769-2783}$ was found in 1 (4%) of $LKM1^{pos}/HCV^{pos}$ and 1 (4%) of $LKM1^{neg}/HCV^{pos}$ patients. Reactivity to the E1 $HCV_{310-324}$ was present in 14 (61%) of $LKM1^{pos}/HCV^{pos}$ and 14 (61%) $LKM1^{neg}/HCV^{pos}$ patients. There was no significant difference in absorbance values expressed as $OD^{test}/OD^{control}$ peptide between $LKM1^{pos}/HCV^{pos}$ (mean OD 4.28 ± 1.46SD) and $LKM1^{neg}/HCV^{pos}$ (mean OD 4.50 ± 2.50SD) patients.

Reactivity to ICP4 $HSV1_{156-170}$ was present in 16 (70%) $LKM1^{pos}/HCV^{pos}$ (mean OD 2.5 ± 0.44SD) and in 14 (61%) $LKM1^{neg}/HCV^{pos}$ (mean OD 3 ± 0.67SD).

Reactivity to both E1 $HCV_{310-324}$ /ICP4 $HSV1_{156-170}$ was present in 10 (43%) $LKM1^{pos}/HCV^{pos}$ and 11 (48%) $LKM1^{neg}/HCV^{pos}$.

Reactivity to $CYP2D6_{252-271}$ was present in 2 (8%) $LKM1^{pos}/HCV^{pos}$ patients reacting also with E1 $HCV_{310-324}$ and ICP4 $HSV1_{156-170}$ and in none of the $LKM1^{neg}/HCV^{pos}$ patients (Table 1).

8.3.2.2 Anti-HSV1 detection

Antibodies against HSV1 (negative <20 RU/ml) were detected in 18 (78%, mean 71.07 ± 38.72 SD RU/ml) $LKM1^{pos}/HCV^{pos}$, including all the 16 reacting against ICP4 $HSV1_{156-170}$, and in 17 (74%, mean 68.9 RU/ml ± 34SD) $LKM1^{neg}/HCV^{pos}$ patients including all the 14 reactive with ICP4 $HSV1_{156-170}$.

Antigen	sequence																						aa position
ICP4 HSV1								L	S	P	R	P	P	A	Q	P	P	R	R	R	R	H	156-170
NS5B HCV									P	P	G	D	P	P	Q	P	E	Y	D	L	E	L	I	2769-2783
												:	:		:	:			:	:				
CYP2D6						E	H	R	M	T	W	D	P	A	Q	P	P	R	D	L				257-271
CYP2D6	D	E	L	L	T	E	H	R	M	T	W	D	P	A	Q									252-266
				.	:		:	:	:	.	:	:												
E1 HCV	P	G	H	I	T	G	H	R	M	A	W	D	M	M	M									310-324

FIGURE 8.1 *Amino acid sequence homologies between CYP2D6, E1 HCV and protein and ICP4 HSV1. The hexamer -PAQPPR- is shared by ICP4 HSV1 and CYP2D6 (underlined). Amino acids in standard single letter code; Colon (:), Identical residues; full stop, (.), Conservative substitution; CYP2D6, cytochrome P4502D6; E1 HCV, envelope 1 Hepatitis C Virus; ICP4 HSV1, infected cell protein 4 of herpes simplex virus type 1*

Table 8.1. Demographic details and peptide binding (absorbance) values of the 23 LKM1^{pos}/HCV^{pos} and 23 LKM1^{neg}/HCV^{pos} patients

HCV^{pos}/LKM1^{pos} group										HCV^{pos}/LKM1^{neg} group									
PATIENT NUMBER	SEX	AGE	HCV GENOTYPE	IFN	HCV NS5B 2769-83	HCV E1 310-24	HSV1 ICP4 156-170	CYP2D6 252-266	CYP2D6 257-271	PATIENT NUMBER	SEX	AGE	HCV GENOTYPE	IFN	HCV NS5B 2769-83	HCV E1 310-24	HSV1 ICP4 156-170	CYP2D6 252-266	CYP2D6 257-271
1	F	27	1a	Y	neg	4.2	2.3	neg	neg	24	F	22	1a	Y	neg	7	4	neg	neg
2	F	37	1a	N	neg	neg	neg	neg	neg	25	F	42	1a	N	neg	neg	neg	neg	neg
3	F	59	1a	Y	2	2.7	neg	neg	neg	26	F	64	1a	Y	neg	2.7	neg	neg	neg
4	M	22	1a	Y	neg	3.3	2.3	6.9	5.7	27	M	27	1a	Y	neg	2.4	5.5	neg	neg
5	F	44	1a	Y	neg	5.1	2.1	neg	neg	28	F	45	1a	Y	neg	neg	2.6	neg	neg
6	M	44	1a	N	neg	3.1	neg	neg	neg	29	M	45	1a	N	neg	neg	neg	neg	neg
7	F	79	1a	N	neg	2	neg	neg	neg	30	F	72	1a	N	neg	3.5	5.7	neg	neg
8	F	61	1b	Y	neg	5.7	2	neg	neg	31	F	57	1b	Y	neg	10.6	4.6	neg	neg
9	F	42	1b	N	neg	5.4	3.1	neg	neg	32	F	40	1b	N	neg	2.4	neg	neg	neg
10	F	34	1b	N	neg	2	neg	neg	neg	33	F	37	1b	N	neg	neg	2.2	neg	neg
11	F	39	1b	Y	neg	neg	3.1	neg	neg	34	F	36	1b	Y	2.7	neg	3	neg	neg
12	M	53	1b	Y	neg	neg	2	neg	neg	35	M	55	1b	Y	neg	2.9	2.2	neg	neg
13	F	63	1b	N	neg	3.8	2.7	4.4	3.8	36	F	63	1b	N	neg	4.6	3.1	neg	neg
14	M	34	1b	N	neg	neg	neg	neg	neg	37	M	38	1b	N	neg	4.9	2.1	neg	neg
15	M	36	1b	N	neg	neg	2	neg	neg	38	M	39	1b	N	neg	neg	neg	neg	neg
16	M	47	2a	Y	neg	neg	2.2	neg	neg	39	M	56	2a	Y	neg	2.8	3.8	neg	neg
17	F	65	2a	Y	neg	3.9	2.8	neg	neg	40	F	62	2a	Y	neg	neg	neg	neg	neg
18	F	47	2a	Y	neg	neg	2.1	neg	neg	41	F	51	2a	Y	neg	neg	neg	neg	neg
19	F	62	2a	N	neg	neg	2.3	neg	neg	42	F	62	2a	N	neg	neg	neg	neg	neg
20	F	59	nt	Y	neg	6.1	3.9	neg	neg	43	F	63	nt	Y	neg	8.3	3.4	neg	neg
21	F	45	nt	N	neg	9.3	2.9	neg	neg	44	F	45	nt	N	neg	2	neg	neg	neg
22	F	52	nt	N	neg	3.3	2.1	neg	neg	45	F	49	nt	N	neg	2.7	5.4	neg	neg
23	F	55	nt	N	neg	neg	neg	neg	neg	46	F	55	nt	N	neg	2.7	2	neg	neg
*					1	14	16	2	2						1	14	14	0	0
(%)					(4)	(61)	(70)	(9)	(9)						(4)	(61)	(61)		

Footnote to Table 8.1

*Absorbance values are expressed as $OD^{test}/OD^{control}$ peptide, when >2 is considered positive (see 2.2.2.3); *, total number and (%) percentage of patients recognising an individual peptide; HCV, hepatitis C virus; LKM1, liver kidney microsomal antibody type 1; NS5B, non structural protein 5B; E1, envelope 1 protein; HSV1, herpes simplex virus type 1; ICP4, infected cell protein 4; CYP2D6, cytochrome P450IID6; F, female; M, male; IFN, interferon; Y, treated with IFN; N, without IFN*

8.3.3 Inhibition studies

The degree of binding inhibition to E1 $HCV_{310-324}$ in the presence of competitor was up to 94% (case 4) and 95% (case 13) with E1 $HCV_{310-324}$; 81% (case 4) and 79% (case 13) with $CYP2D6_{252-266}$; 62% (case 4) and 48% (case 13) with $CYP2D6_{257-271}$; 77% (case 4) and 52% (case 13) with recombinant CYP2D6 protein; 9% (case 4) and 9% (case 13) with ICP4 $HSV1_{156-170}$; 9% (case 4) and 7% (case 13) with control peptide and 13% (case 4) and 14% (case 13) with control protein. In both patients, E1 $HCV_{310-324}$ reactivity was better pre-absorbed by $CYP2D6_{252-266}$ than by $CYP2D6_{257-271}$, 81% (case 4) and 79% (case 13) *vs* 62% (case 4) and 48% (case 13), respectively.

The degree of binding inhibition to ICP4 $HSV1_{156-170}$ in the presence of competitor was 87% (case 4) and 78% (case 13) with ICP4 $HSV1_{156-170}$; 44% (case 4) and 49% (case 13) with $CYP2D6_{252-266}$; 87% (case 4) and 72% (case 13) with $CYP2D6_{257-271}$; 77% (case 4) and 72% (case 13) with recombinant CYP2D6 protein; 12% (case 4) and 13% (case 13) with E1 $HCV_{310-324}$; 11% (case 4) and 15% (case 13) with control peptide and 11% (case 4) and 11% (case 13) with control protein. In both cases, liquid-phase ICP4 $HSV1_{156-170}$ reactivity was better pre-absorbed by pre-incubation of the serum with the $CYP2D6_{257-271}$ than with $CYP2D6_{252-266}$, 87% (case 4) and 72% (case 13) *vs* 44% (case 4) and 49% (13case), respectively. Pre-incubation of the two polyreactive sera with E1 $HCV_{310-324}$ or ICP4 $HSV1_{156-170}$ was not able to absorb reactivity against ICP4 $HSV1_{156-170}$ or E1 $HCV_{310-324}$, respectively.

8.3.4 HLA typing

8.3.4.1 Class I typing

A1, A2, and A3 antigens were the most common A antigens, being present in 7 (30%), 7 (30%), and 6 (26%) patients respectively (2 of them were A1/A2 positive). B14, B35, B44 were the most common B antigens being present in 6 (26%), 9 (39%), 6 (26%), respectively. HLA B51 was found in two (8%) patients (case 4, case 13): this allele was part of the haplotype A1, A30, B13, B51, Cw6, DR2, DR7, DR53, DQ2 in patient 4 and

A1, B18, B51, DR7, DR11, DR52, DR53, DQ2, DQ3 in patient 13. CW4 and CW7 were found in 7 (30%) and 5 (22%) patients.

8.3.4.2 Class II typing

DR7 was present in 14 (61%) patients, DR52 in 10 (43%) and DR53 in 12 (52%) patients; DQ2 was present in 17 (74%).

8.4 DISCUSSION

The results of the present section demonstrate that sera reacting with $CYP2D6_{252-266}$, a key epitope of CYP2D6, also react with E1 $HCV_{310-324}$, a hepatitis C virus sequence sharing a 8 aa homology with $CYP2D6_{252-266}$. Within a molecular mimicry pathogenic scenario, double reactivity is relevant if it is cross-reactive (Oldstone, 1987). The current findings unambiguously show that the double reactivity to E1 $HCV_{310-324}$/$CYP2D6_{252-266}$ is cross-reactive since antibody binding to the viral sequence E1 $HCV_{310-324}$ is inhibited by pre-incubation with $CYP2D6_{252-266}$, the viral-mimicking cytochrome sequence, but not by a scrambled control peptide. Importantly, the binding to the viral sequence is also inhibited by the recombinant, eukaryotically expressed, enzymatically active CYP2D6, demonstrating that the cross-reactive virus/self recognition targets the naturally occurring autoantigen (Oldstone, 1987). These findings give experimental support to two points of Manns' *et al.* (1991) proposal, namely that LKM1 arises through a mechanism of molecular mimicry and that this process is centred on the homologous sequences they originally described.

E1 $HCV_{310-324}$/$CYP2D6_{252-266}$ cross-reactivity, however, was present in only 2 of the 23 $LKM1^{pos}$/HCV^{pos} patients studied raising the questions as to why cross-reactivity is infrequent and as to how LKM1 is produced in the 21 non cross-reactive patients. Within the scope of molecular mimicry, an infrequent reactivity to the self sequence could simply reflect an infrequent reactivity to the corresponding microbial sequence (Oldstone, 1987). This possibility was advanced by Michitaka *et al.* (1994) who speculated that the hepatitis C viral sequence investigated in the present study rarely acts as a B cell epitope. Their hypothesis (Michitaka *et al.*, 1994) is proven unfounded by the present data showing that over 60% of $LKM1^{pos}$/HCV^{pos} positive patients, and indeed a similar proportion of the $LKM1^{neg}$/HCV^{pos} patients, react with E1 $HCV_{310-324}$. Similar to the present findings are those of other workers showing that the E1 $HCV_{307-330}$ is a frequently recognised HCV epitope being targeted by 39-92% of the sera from HCV infected patients, irrespective of their LKM1 status (Ray *et al.*, 1994, Pirisi *et al.*, 1995, Wang *et al.*, 1996, Zibert *et al.*, 1999). An alternative possibility to explain the infrequent reactivity is that the autoantigenic sequence itself is either cryptic or physically unavailable to the antibody (*see* 1.3.6)

(Sercarz *et al.*, 1993). This is also not the case, since CYP2D6$_{252\text{-}271}$ has been shown to be exposed on the surface of the molecule and is almost universally recognised by the LKM1 antibody produced by patients with type 2 AIH (Sugimura *et al.*, 2002). A third possibility is the 'loss of the original antigenic sin' (Steinman, 1999). According to this theory, reactivity to the epitope initiating the autoimmune process is lost while the response spreads to new autoepitopes (Steinman, 1999). This mechanism has been clearly documented in animal models where it is possible to follow the ontogenesis of the immune response, and cannot be excluded in the patients of the present study (Steinman, 1999, Tuohy *et al.*, 1999).

Is there anything setting apart these two cross-reactive patients from the others? The possibility that anti-CYP2D6$_{257\text{-}271}$ reactivity results from exposure to more than one self-mimicking virus was also considered (Oldstone, 1998, von Herrath *et al.*, 2003). The six aa sequence –PAQPPR- contained within the CYP2D6$_{257\text{-}271}$ epitope is completely shared by a hexameric sequence of the herpes simplex virus type 1 ICP4 protein (ICP4 HSV1$_{161\text{-}166}$), an antigen involved in the induction of autoimmunity by molecular mimicry (Manns *et al.*, 1991, Douvas and Sobelman, 1991, Misaki *et al.*, 1993, Boulware and Weber, 2000), known to elicit a humoral immune response in recurrently HSV1 infected individuals, though less frequently than other herpes antigens (Kahlon *et al.*, 1986, Kuhn *et al.*, 1987). In this study, sera from the two E1 HCV$_{310\text{-}324}$/ CYP2D6$_{257\text{-}271}$ reactive patients also reacted with the ICP4 HSV1$_{156\text{-}170}$ peptide, the reactivity to the herpes viral sequence being itself cross-reactive with CYP2D6$_{257\text{-}271}$. There still remain 8 LKM1^{pos}/HCV^{pos} patients who react with the E1 HCV$_{310\text{-}324}$ and ICP4 HSV1$_{156\text{-}170}$, have evidence of exposure to hepatitis C and herpes virus type 1 but do not react against CYP2D6$_{252\text{-}271}$, suggesting that reactivity to more than one mimicking micro-organism may be important in the generation of the autoantibody, but that other factors must be involved (Oldstone, 1998, von Herrath *et al.*, 2003).

In relation to the immunogenetic make up, the present study found that unique to the two patients cross-reactively recognising the cytochrome sequence was their possession of the allotype B51. Moreover, the sequence CYP2D6$_{263\text{-}271}$, contained within the B cell epitope CYP2D6$_{252\text{-}271}$, was found to have the fourth highest score (score: 22, range 1-28) amongst the 478 nonameric CYP2D6 sequences predicted to bind to B51. Consequently,

$CYP2D6_{263-271}$ almost certainly represents a naturally occurring B51-restricted CD8 epitope. How possession of this HLA class I allele can predispose to a cross-reactive tolerance breakdown and to autoimmune manifestations remains to be established, though the following three points may be of relevance. Firstly, B51 is known to confer predisposition to autoimmunity and autoimmune diseases, its possession, for example, imparting a high relative risk to the development of Behçet's disease (Ohno *et al.*, 1982, Mizuki *et al.*, 1992, Mizuki *et al.*, 2000). Secondly, in another autoimmune liver disease such as PBC, where B- and CD8 T-cell epitopes co-localise, a functional association has been shown between these two types of cells, the suggested link being autoantigen uptake by the B cell with cross-presentation to the CD8 T-cell (Kita *et al.*, 2002). Thirdly, the predisposing role of B51 to immune-mediated liver damage has been emphasised by the report that 3 $LKM1^{pos}/HCV^{pos}$ patients undergoing an anti-viral interferon-alpha treatment had to stop their medication following an abrupt increase in transaminases: all 3 patients were B51 positive (Muratori *et al.*, 1994). Moreover, none of the 16 $LKM1^{neg}/HCV^{pos}$ Greek patients investigated in the study presented in this Chapter with a known HLA were HLA B51 positive.

Recently we have gained an insight on how LKM1 may be produced in the majority of our $LKM1^{pos}/HCV^{pos}$ patients through the discovery that $CYP2D6_{193-212}$ represents a new and critical epitope (Kerkar *et al.*, 2003). Not only does this sequence act as a target to 100% of the AIH-2 patients, but also to half of the $LKM1^{pos}/HCV^{pos}$ patients, far exceeding, in frequency terms, $CYP2D6_{252-271}$ recognition in this latter patient group (Kerkar *et al.*, 2003). The newly described autoepitope is target of humoral cross-recognition for antibodies directed to HCV and cytomegalovirus (Kerkar *et al.*, 2003).

In summary, the results of this section offer experimental support to the notion that molecular similarity between CYP2D6, hepatitis C and herpes simplex virus can result in the production of a cross-reactive response, ultimately leading to the production of LKM1 autoantibody in patients with the appropriate immunogenetic background.

CHAPTER 9

Virus-self cross-reactivity leading to *de novo* autoimmune hepatitis

9.1 BACKGROUND

The mechanisms leading to the breakdown of self-tolerance and to the development of AIH are unknown (Vergani and Mieli-Vergani, 2003). A role for viruses as triggers of liver autoimmunity has been proposed, but never proven because the infections responsible for the induction of autoimmune responses are likely to occur years before the onset of autoimmune disease (Vento *et al.*, 1990, Manns *et al.*, 1991, Vento *et al.*, 1991, Mackie *et al.*, 1994, Vento *et al.*, 1995, Vento *et al.*, 1996, Vento *et al.*, 1997, Salcedo *et al.*, 2002, Vento and Cainelli, 2004). Moreover, the virus responsible for the breakdown of tolerance can be cleared by the host's immune system and may not be available for recognition by molecular probes or isolation by culture techniques, when the autoimmune process is taking place (von Herrath, 2000, Vento and Cainelli, 2004). In relation to AIH, studies of viral/self cross-reactive immunity have been focused at the time a full-blown clinical disease is present and are unable to provide information as to the pathogenic process taking place long before the onset of the disease (Oldstone, 1998, von Herrath, 2000, von Herrath *et al.*, 2003).

In the present Chapter immunological cross-reactivity was studied in a girl with primary and secondary LKM1 response following HCV infection acquired at the time of liver transplantation for whom serial retrospective serum samples were available allowing to investigate the evolution of cross-reactive immune responses from the day she acquired HCV-triggered LKM1 autoimmunity to overt AIH-2, nine years later (Mackie *et al.*, 1994).

9.2 CASE PRESENTATION

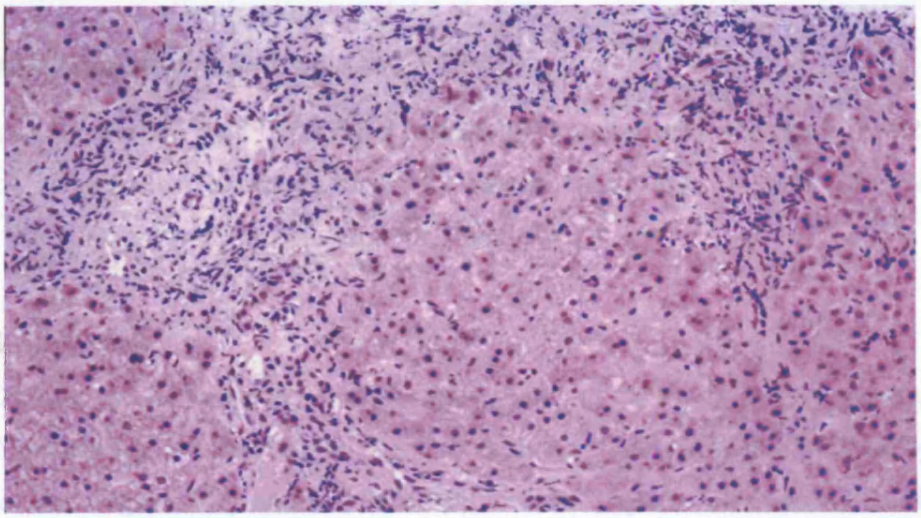
A 10 year-old girl with alpha-1 anti-trypsin deficiency, who received three orthotopic liver transplants (OLT) between 1989-1990 [the first, from a cytomegalovirus (CMV) positive donor, for end-stage cirrhosis; the following two for chronic rejection], developed a primary IgM and secondary IgG LKM1 autoantibody response two weeks after HCV infection, acquired at the time of the first transplant (Mackie *et al.*, 1994). Since two short amino-acid sequences are shared between the HCV and CYP2D6, at that time it was postulated that HCV infection was causally related to the development of LKM1. The patient, however, had no evidence of autoimmune liver disease and by 1994 she was negative for anti-LKM1 (Manns *et al.*, 1991). A routine liver biopsy in 1996 showed moderate portal and perivenular fibrosis with mild non-specific inflammation.

She remained well with normal blood tests until 1998, taking tacrolimus (blood levels 5-9 ng/ml) and prednisolone (2.5 mg/day). In 1998, 6 months after her steroids were stopped by her attending physician because of long-term normal liver function, blood tests showed: bilirubin 23 μmol/l (nv <20), aspartate aminotransferase 780 IU/l (nv <50), gamma glutamyl transpeptidase 159 IU/ (nv <50), tacrolimus level 7.4 ng/ml. LKM1 antibody was positive again by IFL at a titre of 1/2560. Serum HCV RNA by PCR was negative. A liver biopsy suggested *de-novo* post-OLT AIH (Figure 9.1). Prednisolone 30 mg/day, gradually decreased to 5 mg/day, led to persistent normalization of transaminases within four months. Anti-LKM1 decreased to 1/320 and 1/40 five and 12 months after restarting steroids. A liver biopsy during biochemical remission, showed severe porto-septal and bridging fibrosis with architectural distortion, but only minimal residual inflammation. Because of *de-novo* AIH and moderate renal dysfunction, tacrolimus was decreased (levels 3-5 ng/ml) and mycophenolate mofetil (500 mg twice daily) added. At 23 years of age, the patient is well, with normal liver function, negative anti-LKM1 and no signs of chronic liver disease, apart from mild splenomegaly.

To characterise the evolving LKM1 autoimmunity and investigate the role of virus/self molecular mimicry in its development, as part of this Thesis, 10 serum samples, collected between 1989-2000, and stored at –70°C were tested by ELISA, for fine specificity and immunoglobulin isotype of the evolving LKM1 autoantibody response using 34

biotinylated peptides spanning the entire CYP2D6 sequence, and for possible cross-reactive responses between $CYP2D6_{252\text{-}271}$, the major epitope of LKM1 reactivity and its HCV homologues. Moreover, having found through database searches that common viruses such as Herpes simplex virus type 1 (HSV1), CMV, Epstein-Barr virus (EBV) and adenovirus (ADV) contain sequences highly homologous to CYP2D6 (Figure 9.2), evidence of infection with these viruses and of cross-reactivity between virus and self mimics were also investigated.

A



B

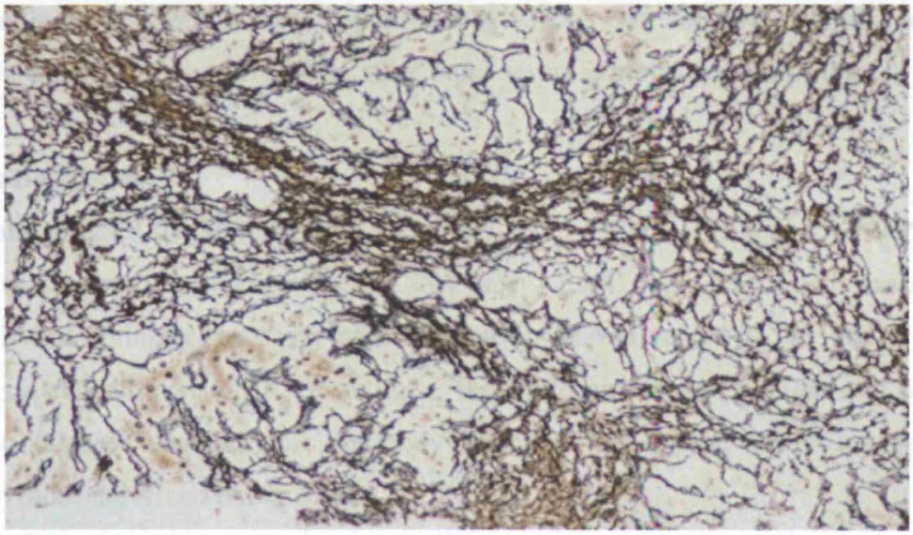


Figure 9. 1. *Liver biopsy at the time of diagnosis of de novo autoimmune hepatitis showing chronic hepatitis with moderate portal tract infiltration by lymphocytes and plasma cells (A), and porto-septal and bridging fibrosis (B)*

9.3 METHODS

9.3.1 Protein database search and analysis

The '*Advanced Proteininfo*' and '*BLASTp 2 sequences*' programmes were used (*see* 2..2.1) to search for aa sequence similarity between human CYP2D6$_{252\text{-}271}$ and viral mimics deposited in the SWISS-PROT protein sequence databank.

9.3.2 Peptide synthesis

Thirty-four 20-mer synthetic biotinylated peptides spanning the entire 517 aa sequence of the CYP2D6 protein and overlapping by 5 amino acids (*see* Table 9.1), 8 CYP2D625$_{254\text{-}271}$ viral mimics (*see* Figure 9.2) and an irrelevant control peptide were constructed (*see* 2.2.2.3).

9.3.3 ELISA

Antibody binding to the peptides was determined by ELISA (*see* 2.2.2.3). The final peptide concentration was that of 5μg/ml and the dilution of serum samples was of 1/200.

9.3.4 Inhibition studies

To investigate whether the simultaneous reactivity to E1 HCV1$_{310\text{-}324}$ and CYP2D6$_{252\text{-}266}$ was due to cross-reactivity, competition ELISA (*see* 2.2.2.4) were performed, measuring residual anti-viral antibody reactivity after incubation respectively with E1 HCV1$_{310\text{-}324}$, CYP2D6$_{252\text{-}266}$, recombinant CYP2D6 antigen, the irrelevant control peptide and control protein as solid phase competitors. Antibody detection was carried out under identical conditions to those described under *ELISA*.

9.3.5 Anti-LKM1 and anti-viral antibody detection

LKM1 was detected by IIFL (*see* 2.2.2.1, immunoblotting (*see* 2.2.2.2), and ELISA (*see* 2.2.2.3). Commercially available ELISA were used to detect IgG antibodies against HSV1 and human CMV (*see* 2.1.6.1). Reactivity against the full ICP4 HSV1 protein was tested by immunoblotting using as antigen infected-cell extract enriched for HSV1 proteins (*see* 2.1.6.1). Detection of the characteristic band of 175 kDa was carried out using an affinity

purified ICP4 HSV1 specific monoclonal antibody (mouse IgG1(k), as positive control. A commercially available immunoblot was used to detect antibodies against specific EBV antigens including Epstein-Barr nuclear antigen (EBNA).

9.3.6 Proliferation assay

A standard proliferation assay (*see* 2.2.3.3) was performed to investigated T-cell responses of PBMC from the patient (obtained in 2000) in response to stimulation with individual CYP2D6 peptides, and viral mimics.

9.3.7 HLA typing

The patient's HLA type originally determined by cytotoxicity (A3,24 B5,22 Bw4,6 DR7,16 DQ2,5), was retested using molecular techniques. Class I genotyping for HLA A and B was performed using sequence specific polymerisation (SSP)/PCR kits; class II genotyping for HLA DRB & DQB was performed by PCR/sequence specific oligonucleotide (SSO) probing using probes obtained from the British Society for Histocompatibility and Immunogenetics (BSHI). HLA typing was performed by the HLA typing Service at the Institute of Hepatology.

Table 9.1 Amino acid (aa) sequences of 34 synthetic peptides in standard single letter code, spanning the full length of the cytochrome P450 2D6 (CYP2D6) molecule. Each peptide overlaps by 5 aa with the preceding and subsequent peptide.

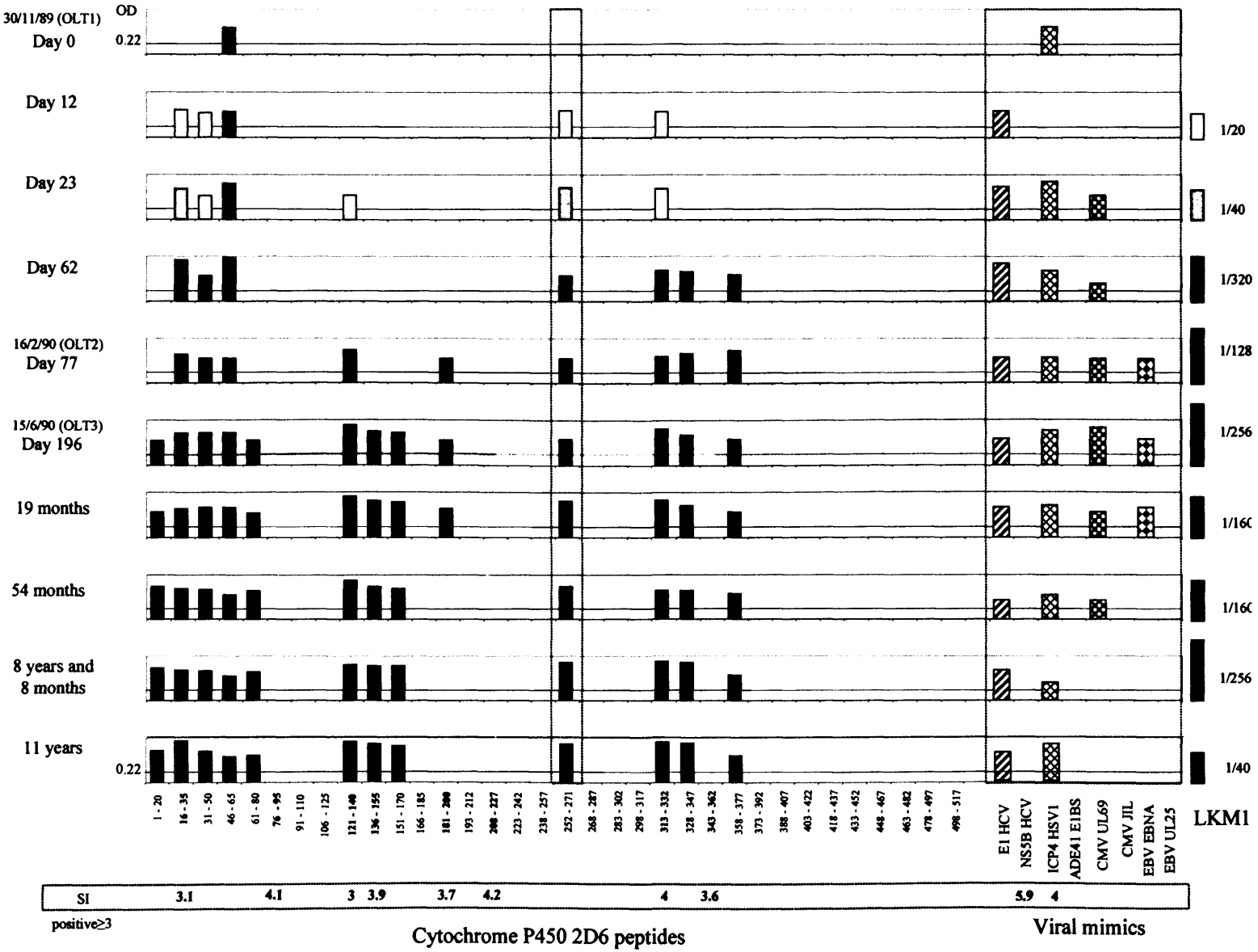
Peptide	Antigen	Aa	Sequence
1	CYP2D6	Iαv-20	MGLEALVPLAVIVAIFLLLV
2	CYP2D6	16-35	FLLLVDLMHRRQRWAARYPP
3	CYP2D6	31-50	ARYPPGPLPLPGLGNLLHVD
4	CYP2D6	46-65	LLHVDFQNTPYCFDQLRRRF
5	CYP2D6	61-80	LRRRFGDVFSLQLAWTPVVV
6	CYP2D6	76-95	TPVVVLNGLAAVREALVTHG
7	CYP2D6	91-110	LVTHGEDTADRPPVPITQIL
8	CYP2D6	106-125	ITQILGFGPRSQGVFLARYG
9	CYP2D6	121-140	LARYGPAWREQRRFSVSTLR
10	CYP2D6	136-155	VSTLRNLGLGKKSLEQWVTE
11	CYP2D6	151-170	QWVTEEAACLCAAFANHSGR
12	CYP2D6	166-185	NHSGRPFRPNGLLDKAVSNV
13	CYP2D6	181-200	AVSNVIASLTCGRRFEYDDP
14	CYP2D6	193-212	RRFEYDDPRFLRLLDLAQEG
15	CYP2D6	208-227	LAQEGLKEESGFLREVLNAV
16	CYP2D6	223-242	VLNAVPVLLHIPALAGKVLR
17	CYP2D6	238-257	GKVLRFQKAFLTQLDELLTE
18	CYP2D6	252-271	DELLTEHRMTWDPAQPPRDL
19	CYP2D6	268-287	PRDLTEAFLAEMEKAKGNPE
20	CYP2D6	283-302	KGNPESSFNDENLRIVVADL
21	CYP2D6	298-317	VVADLFSAGMVTTSTTLAWG
22	CYP2D6	313-332	TLAWGLLLMILHPDVQRRVQ
23	CYP2D6	328-347	QRRVQQEIDDVIGQVRRPEM
24	CYP2D6	343-362	RRPEMGDQAHMPYTTAVIHE
25	CYP2D6	358-377	AVIHEVQRFGDIVPLGMTHM
26	CYP2D6	373-392	GMTHMTSRDIEVQGFRIPKG
27	CYP2D6	388-407	RIPKGTTLITNLSSVLKDEA
28	CYP2D6	403-422	LKDEAVWEKPFRFHPEHFLD
29	CYP2D6	418-437	EHFLDAQGHFVKPEAFLPFS
30	CYP2D6	433-452	FLPFSAGRRACLGEPLARME
31	CYP2D6	448-467	LARMELFLFFTSLLQHFSFS
32	CYP2D6	463-482	HFSFSVPTGQPRPSHHGVFA
33	CYP2D6	478-497	HGVFAFLVSPSPYELCAVPR
34	CYP2D6	498-517	NGVPSPQPAPSPEALMYNKA

Hepatitis C virus (HCV)	Non structural protein 5B	NS5B						P	*P*	G	**D**	**P**	*P*	Q	**P**	E	Y	**D**	**L**
Hepatitis C virus (HCV)	Envelope 1	E1	*I*	**T**	**G**	**H**	**R**	**M**	*A*	**W**	**D**	M	M	M					
Human	Cytochrome P450IID6	$CYP2D6_{254-270}$	L	T	E	H	R	M	T	W	D	P	A	Q	P	P	R	D	L
Herpes simplex virus-11 (HSV-1)	Infected cell protein 4	ICP4					L	S	*P*	R	P	**P**	**A**	**Q**	**P**	**P**	**R**	*R*	R
Human adenovirus-41 (ADE41)	E1B small T-antigen	E1BS					V	*L*	*G*	C	S	**P**	**A**	**Q**	**P**	**P**	*H*	G	**L**
Human cytomegalovirus (HCMV)	UL69-protein	UL69					I	A	*A*	A	P	**P**	**A**	**Q**	**P**	**P**	S	*Q*	P
Human cytomegalovirus (HCMV)	Hypothetical protein J1L	J1L					**R**	T	*A*	P	A	**P**	**A**	**Q**	**P**	**P**	S	P	A
Epstein-Barr virus (EBV)	Nuclear protein EBNA	EBNA					P	P	*P*	A	A	**P**	**A**	**Q**	**P**	**P**	P	G	*V*
Epstein-Barr virus (EBV)	Virion protein BVRF1	UL25					*H*	*V*	L	*I*	**D**	**P**	**A**	*R*	L	**P**	**R**	**D**	T

Figure 9.2 *Local amino acid homology between $CYP2D6_{254-270}$ and viral mimics from hepatitis C virus (HCV), herpes simplex virus-1 (HSV1), human cytomegalovirus (HCMV), human adenonovirus-41 (ADE41) and Epstein-Barr virus (EBV). Amino acids in single letter code; identities in* ***bold****; conservative substitutions in italic*

9.4 RESULTS

Results are summarised in Figure 9.3. Before the first liver transplant, LKM1 was undetectable by IFL, immunoblotting (human liver antigen), and ELISA (recombinant CYP2D6). There was an IgG antibody response to $CYP2D6_{46-65}$ and HSV1 $ICP4_{156-170}$. IgG, but not IgM, to HSV1 were detected by ELISA. CMV and ADV reactivities were negative by complement fixation test. Serum HCV RNA, negative before transplantation, became positive on day 1 after surgery. On day 12, IgM anti-$CYP2D6_{1-35}$, - $CYP2D6_{252-271}$ -$CYP2D6_{313-332}$, and -$E1HCV_{310-324}$ were detected. At the same time LKM1 antibody belonging exclusively to the IgM isotype. was found. On day 23, there was an additional IgM $CYP2D6_{121-140}$ response and both IgG and IgM responses to $CYP2D6_{16-35}$ and $CYP2D6_{252-271}$. Both IgM and IgG LKM1 were detectable. Reactivities to the $CYP2D6_{252-271}$ mimicking peptides $E1HCV_{310-324}$, HSV1 $ICP4_{156-170}$ and CMV $UL69_{594-608}$ were also present; serum HCV RNA was positive and IgG antibodies directed to HSV1 ICP4 protein were detected by immunoblot. $E1HCV_{310-324}$/$CYP2D6_{252-271}$ double reactivity was cross-reactive by competition ELISA (Figure 9.4). On day 62, IgG reactivity to 7 CYP2D6 peptides was detected. LKM1 titre was 1/320. Viral serology was unchanged. On day 77 (second liver transplant), 9 CYP2D6 peptides were recognised by LKM1 antibody (titre 1/1280). A new reactivity to the mimic $EBNA_{296-310}$ and to full-length EBNA EBV was demonstrated by immunoblot. On day 196, (third liver transplant), 13 CYP2D6 peptides were recognised by LKM1 (1/2560). Similar reactivities were detected 19 and 54 months after the first transplant. When *de novo* AIH was diagnosed, 8 years and 8 months after the first transplant, anti-CYP2D6 reactivity involved a total of 12 CYP2D6 peptides defining 5 epitopes: $CYP2D6_{252-271}$, $CYP2D6_{313-347}$, $CYP2D6_{358-377}$, $CYP2D6_{1-80}$, and $CYP2D6_{121-170}$, the first three being LKM1 targets in both AIH-2 and HCV infection. HCV polymerase chain reaction was then negative and antibody responses to viral sequences were limited to E1HCV and ICP4 HSV1. Identical LKM1 reactivity was detected 11 years after the first liver transplant, when a standard proliferation assay revealed 8 T-cell epitopes (Figure 9.3), 4 of which overlapped with with corresponding B-cell epitopes. Repeating by molecular techniques the HLA typing, the patient was found to be HLA B51.


30/11/89 (OLT1)
Day 0
OD
0.22
Day 12
Day 23
Day 62
16/2/90 (OLT2)
Day 77
15/6/90 (OLT3)
Day 196
19 months
54 months
8 years and
8 months
11 years
0.22
1/20
1/40
1/320
1/128
1/256
1/16(
1/16(
1/256
1/40
LKM1
1 - 20
16 - 35
31 - 50
46 - 65
61 - 80
76 - 95
91 - 110
106 - 125
121 - 140
136 - 155
151 - 170
166 - 185
181 - 200
193 - 212
208 - 227
223 - 242
238 - 257
252 - 271
268 - 287
283 - 302
298 - 317
313 - 332
328 - 347
343 - 362
358 - 377
373 - 392
388 - 407
403 - 422
418 - 437
433 - 452
448 - 467
463 - 482
478 - 497
498 - 517
E1 HCV
NS5B HCV
ICP4 HSV1
ADE41 E1BS
CMV UL69
CMV JIL
EBV EBNA
EBV UL25
SI
positive≥3
3.1
4.1
3
3.9
3.7
4.2
4
3.6
5.9
4
Cytochrome P450 2D6 peptides
Viral mimics

Legend to Figure 9.3

Reactivity against 34 overlapping 20-mer peptides spanning residues 1-517 of cytochrome P450 2D6 (CYP2D6) and to microbial mimics of the epitope CYP2D6$_{252-271}$: envelope 1 (E1) ( *) and non-structural 5B (NS5B) of hepatitis C virus (HCV) polyprotein, infected cell protein 4 (ICP4) of herpes simplex virus type 1 (HSV1) (*  *), E1B small antigen (E1BS) of human adenovirus 41 (ADV41), UL69 of human cytomegalovirus (HCMV) (*  *), hypothetical protein J1L of HCMV, Epstein-Barr nuclear antigen (EBNA) (*  *) of Epstein-Barr virus (EBV) and the UL25 virion protein of EBV. Absorbance values (optical density, OD) on the y-axis (cut-off 0·22) are plotted against the 34 individual peptides boxed in white for IgM, black for IgG and grey for both IgM and IgG anti-peptide responses. Peptides inducing a T-cell stimulation index (SI) exceeding 3 are given in* **bold** *in the box at the bottom of the picture. Anti liver-kidney-microsomal 1 (LKM1) titres by immunofluorescence (IFL) are given on the right.*

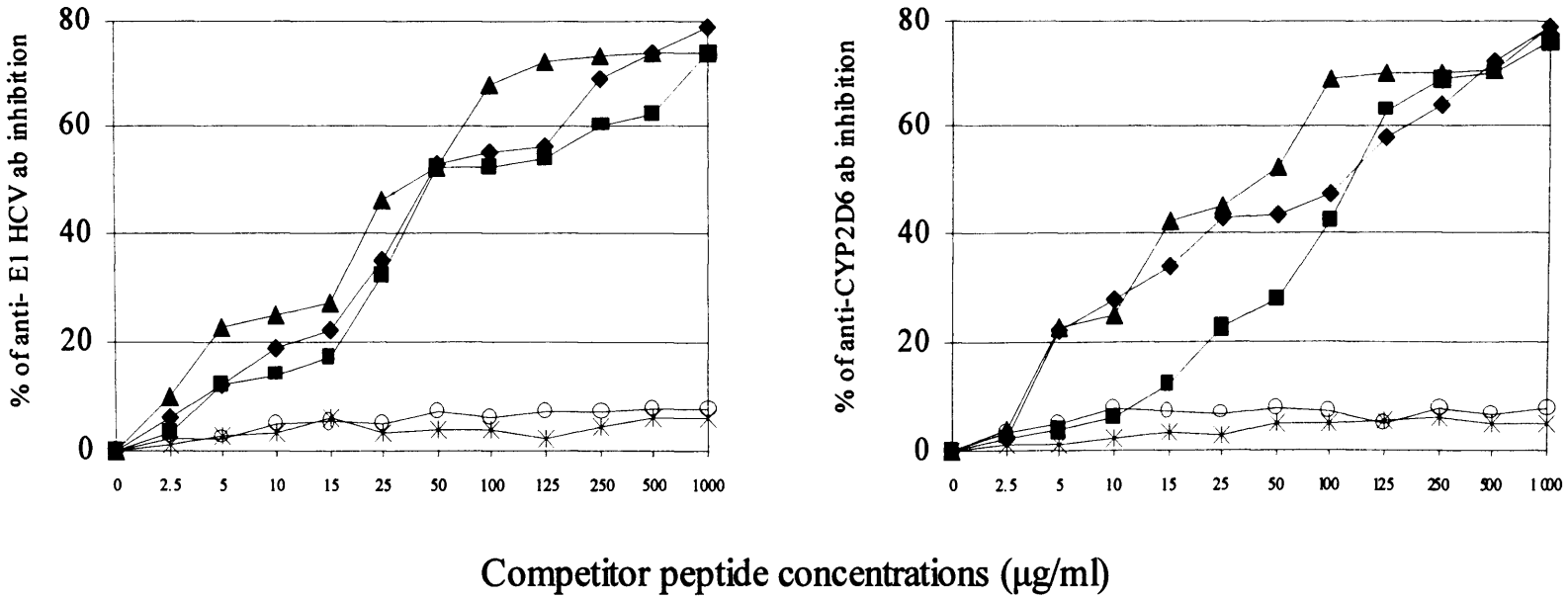


Figure 9.4 *Inhibition of antibody (ab) binding to E1 $HCV_{310-324}$ (C-I) and $CYP2D6_{252-271}$ (C-II) by pre-incubation of the sera with E1 $HCV_{310-324}$ (◆), human $CYP2D6_{252-266}$ (■), human CYP2D6 protein (▲), control peptide (✳) and control protein (M2 antigen) (○), in the presence of competitor peptides at concentrations of 0; 2·5; 5; 10; 15; 25; 50; 100; 125; 250; 500; 1000 µg/ml. The experiment demonstrates the cross-reactive nature of the viral/self reactivity*

9.5 DISCUSSION

This section of the Thesis investigates the evolution from a primary autoantibody response, triggered by HCV infection, to florid autoimmune hepatitis 9 years later. Fine specificity of anti-CYP2D6 responses revealed two major findings: epitope spreading from a single to several other autoepitopes, and a cross-reactive response involving $CYP2D6_{252-271}$, a key autoepitope which shares homology with HCV, HSV, CMV and EBV. The patient was exposed to HSV before surgery as demonstrated by the presence of antibodies to HSV and acquired HCV and CMV infections at the time of the first transplant. It is conceivable that a potentially $CYP2D6_{252-271}$ cross-reactive lymphocyte population, primed by HSV, underwent expansion following exposure to mimicking sequences of HCV, CMV and EBV with consequent production of anti-CYP2D6.

A chronological and possibly causal link between HCV infection and LKM1 AIH has been highlighted by the case of a nurse who developed LKM1 antibodies 4 months after an accidental needlestick injury while caring for an HCV-infected patient, and histologically proven AIH 14 months later (Vento *et al.*, 1997). In support of the involvement of molecular mimicry, HCV and CMV sequences cross-recognised by a mimicking $CYP2D6_{193-212}$, a major B cell epitope of LKM1 antibodies, have been recently identified as targets of viral/self cross-reactive responses (Kerkar *et al.*, 2003). Moreover, Salcedo et al. (2002), found that all patients developing *de novo* AIH had evidence of infection with common viruses such as CMV, EBV or parvovirus.

The fact that viral/self antibodies belong to the IgG isotype, implicate T-cell 'help' in their generation, a notion relevant to the present finding that 4 of the 8 T-cell epitopes on CYP2D6 are identical to those of B-cells and that viral/self cross-reactivity operates at the CD4-T cell level, at least in the case of HCV and HSV (Stevens *et al.*, 1988, Mosmann and Coffman, 1989, Mahon *et al.*, 1995). This raises the possibility that CYP2D6 tolerance breakdown occurs not only at B cell level, but also at the CD4 (Vergani and Mieli-Vergani, 2003). If this is the case, HLA, the most important gene in determining susceptibility to autoimmune diseases, may also be critical for selecting the appropriate viral/self mimics and presenting them to the immune system (Delves and Roitt, 2000b, Delves and Roitt, 2000a). In the present study, initial typing revealed that the girl was HLA B5 positive. As

described in Chapter 8 $CYP2D6_{263-271}$/viral mimics were cross-recognised only when LKM1/HCV infected patients possessed the allotype B51, one of the split antigens of HLA B5. Repeating by molecular techniques the HLA typing, the patient was found to be HLA B51. An algorithm predicts B51 to bind $CYP2D6_{263-271}$. How possession of this HLA class I allele predisposes to a virus/self cross-reactive break of immunological tolerance remains to be established. In primary biliary cirrhosis, another autoimmune liver disease where B and CD8 T-cell epitopes co-localise, a functional association has been shown between these two types of cells, the suggested link being autoantigen uptake by the B cell with cross-presentation to the CD8 T cell, indicative of a unique role for autoantibodies and T-cells in the pathogenesis of the disease (Kita *et al.*, 2002).

CHAPTER 10

Hepatitis B vaccination associated autoimmunity

10.1 BACKGROUND

Molecular mimicry has long been proposed as a pathogenic mechanism for autoimmune disease but documentation of this mechanism has been elusive in humans (Dyrberg and Oldstone, 1986, Oldstone, 1989, Oldstone, 1998, Van de Water *et al.*, 2001). Presently, proof for molecular mimicry relies on the availability of viral mimics able to induce cross-reactive immune responses leading to tissue damage in experimental animals such as Experimental Autoimmune Encephalomyelitis (EAE), the animal model of multiple sclerosis and herpes stromal keratitis (*see* 1.9.1)(Oldstone, 1998, Wucherpfennig, 2001, von Herrath *et al.*, 2003).

The information derived from animal studies is of undoubted value but experimental models of human disease are not always able to reproduce faithfully the human condition. Case studies of immunological cross-reactivity, such as the one presented in the previous Chapter are of interest but there are few (Mackie *et al.*, 1994, Vento *et al.*, 1997, Vento *et al.*, 1996, Vento *et al.*, 1991, Vento *et al.*, 1995). Studies of immunological cross-reactivity in serum samples before and after vaccination against viruses may provide useful information as to how a viral stimulus can induce cross-reactive autoimmune responses. Indeed, it has been proposed that vaccination against infectious agents may activate pathways of molecular mimicry in genetically susceptible hosts, and this may be the basis of adverse reactions to vaccines (Cohen and Shoenfeld, 1996, Poirriez, 2004, Ravel *et al.*, 2004). The present study used vaccination against HBV (HBVacc) as a model for the study of viral/self cross-reactivity.

The current HBVacc is a non-infectious viral subunit consisting of the small hepatitis B virus surface antigen (SHBsAg), a 226 aa long recombinant antigen identical to the 'wild' SHBsAg (Shouval, 2003). Extrahepatic autoimmune manifestations, such as demyelinating disorders and MS have increasingly been linked to HBVacc administration suggesting that the exposure to SHBsAg may be capable of inducing autoimmune adverse reactions (Herroelen *et al.*, 1991, Cohen and Shoenfeld, 1996, Poirriez, 2004, Ravel *et al.*, 2004, Vital *et al.*, 2002, Aron-Maor and Shoenfeld, 2001, Shoenfeld and Aron-Maor, 2000, Maillefert *et al.*, 1999). The fact that the HBVacc is administered in three doses over a period of seven months gives the opportunity to study the emergence of the humoral antiviral immune response and, at the same time, to

witness appearance and evolution of the possible cross-reactive autoimmunity as a consequence of exposure to SHBsAg.

Among various myelin antigens, myelin basic protein (MBP) is the major target of EAE (Raine and Bornstein, 1970, Martin *et al.*, 1992, Steinman, 1996). More recent studies indicate that humoral immunity against myelin oligodendrocyte glycoprotein (MOG) is associated with myelin damage in MS (Genain *et al.*, 1999, Wekerle, 1999, Devaux *et al.*, 1997). Having found through screening of protein databases that known epitopic regions on SHBsAg share extensive homologies with MBP and MOG, the present study investigated whether the viral/self mimics serve as target of cross-reactive immune responses following HBVacc administration in healthy individuals.

10.2 METHODS

10.2.1 Subjects

A total of 234 serum samples stored in the Liver Unit, Institute of Liver Studies, King's College Hospital, London, were tested including 147 samples pre- and post-HB vaccination from 58 adults (median age 42, range 31-62, 38 male), negative for hepatitis B viral markers. Samples were collected before HBvacc (58/58), and post-HBvacc, at three months (48/58) before the second boost, and six months post-HBvacc (41/58) before the third boost. As pathological controls, 87 anti-HBsAg antibody negative patients (median age 45, range 22-74, 59 female) were tested including 23 with PBC, 27 with ALD, 19 with autoimmune thyroiditis, and 18 with SLE.

10.2.2 Anti-viral antibody detection

Commercially available ELISA were used to detect IgG antibodies against SHBsAg (*see* 2.1.2).

10.2.3 Protein database search and analysis

The '*BLASTp 2 sequences*' programme was used (*see* 2.2.1.2.2) to search for aa sequence similarity between SHBsAg and the MBP and MOG myelin antigens.

10.2.4 Peptide synthesis

Twenty-mer biotinylated peptides spanning the SHBsAg/myelin mimics (*see* Figure 10.1) and an irrelevant control peptide were constructed (*see* 2.2.2.3).

10.2.5 Anti-peptide antibody detection

Antibody binding to the peptides was determined by ELISA (*see* 2.2.2.3). The final peptide concentration was 5μg/ml and the dilution of serum samples was 1/100 (*see* 2.2.2.3).

10.2.6 Inhibition studies

To investigate whether the simultaneous reactivity to $SHBsAg_{130\text{-}149}$ and $MOG_{5\text{-}24}$ was due to cross-reactivity, competition ELISA (*see* 2.2.2.4) were performed, measuring

residual anti-SHBsAg$_{130\text{-}14}$ antibody reactivity after incubation respectively with SHBsAg$_{130\text{-}14}$, MOG$_{5\text{-}24}$, recombinant SHBsAg antigen, the irrelevant control peptide and control protein as liquid phase competitors. Antibody detection was carried out under identical conditions to those described in *ELISA*.

10.3 RESULTS

10.3.1 Protein database search and analysis

Amino acid similarities between SHBsAg (226 aa) and the MPB (304 aa) and MOG (247 aa) antigens are illustrated in Figure 10.1. Overlapping sequences within MOG_{10-22} share a 66-86% local homology with $SHBsAg_{135-140}$ and $SHBsAg_{83-98}$. Another pair of viral/self mimics involves $SHBsAg_{9-15}$ and $MOG_{218-224}$ (100% homology). $SHBsAg_{135-140}$ is part of the immunodominant 'a' B-cell epitope on SHBsAg; $SHBsAg_{83-98}$ is part of the leucine zipper pattern of SHBsAg and MOG_{10-22} extensively overlaps with the encephalitogenic epitope MOG_{1-20} (Landschulz *et al.*, 1988, Genain *et al.*, 1999).
The best homology between SHBsAg and MBP is that of the $SHBsAg_{67-80}/MBP_{82-95}$ pair (8/14 homology) (*see* Figure 10.1).

Antigens	aa position														Identities	Similarities
SHBsAg	(135-140)	P	S	C	C	C	T									
		P	S	C		C	.								4	5/6(82%)
MOG	(10-15)	P	S	C	L	C	S									
MOG	(12-20)		C	L	C	S	F	L	L	L	L					
			C	L				L	.	L	L				5	6/9 (66%)
SHBsAg	(89-98)	L	C	L	I	F	L	L	V	L	L					
		L	C				L	L	.	L	L				6	7/10 (70%)
MOG	(13-22)	L	C	S	F	L	L	L	L	L	L					
SHBsAg	(83-89)	F	L	F	I	L	L	L								
		F	L		L	L	L	L							6	6/7 (86%)
MOG	(16-22)	F	L	L	L	L	L	L								
SHBsAg	(9-15)	L	G	P	L	L	V	L								
		L	G	P	L	.	.	L							5	7/7 (100%)
MOG	(218-224)	L	G	P	L	V	A	L								
SHBsAg	(67-80)	P	I	C	P	G	Y	R	W	M	C	L	R	R	F	
		P	.		P	G		R				.	R		F	6	8/14 (57%)
MBP	(82-95)	P	A	D	P	G	S	R	P	H	L	I	R	L	F	

Figure 10.1 *Amino acid sequence homology between small Hepatitis B surface antigen (SHBsAg) and myelin antigens. Amino acids in standard single letter; Full stop (.), conservative substitution. MOG, myelin oligodendrocyte glycoprotein; MBP, myelin basic protein*

10.3.2 ELISA

10.3.2.1 Before vaccination

Reactivity to at least one of the SHBsAg peptides was found in 8 (14%) pre-vaccinated subjects (including 3 cases with antibodies to the full-length SHBsAg) who were excluded from the study.

Amongst the remaining 50 cases, reactivity to at least one of the MOG peptides was present in 4 (8%) (*see* Table 10.1).

10.3.2.2 At 3 months post-vaccination

Reactivity to at least one of the SHBsAg peptides was present in 41/45 (91%) of the cases (Table 10.1), all of whom reacted with $SHBsAg_{130-149}$ (*see* Table 10.2). Antibodies against the full-length SHBsAg were present in 40/45 (89%) of the cases.

Reactivity to at least one of the MOG mimics was present in 24/45 (53%) of the cases, all but one reacting with MOG_{5-24} (*see* Table 10.1 and Table 10.2).

Double reactivity to at least one SHBsAg/MOG mimicking pairs was present in 24/45 (53%) of the cases (*see* Table 10.1 and Table 10.2).

10.3.2.3 At 6 months post-vaccination

Reactivity to at least one of the SHBsAg peptides was present in 36/39 (92%) of the cases (Table 10.1), all of whom reacted with $SHBsAg_{130-149}$. All 36 cases also had antibodies against the full-length SHBsAg.

Overall, 47/50 (94%) of the post vaccinated cases had anti-SHBsAg antibody protective immunity (median anti-SHBsAg antibody titre:386 mIU/ml, range 16-1000 mIU/ml, cut off = 10 mIU/ml).

Reactivity to at least one of the MOG mimics was present in 17/39 (44%) of the cases.

At 6 months post-vaccination, 3 of the 4 anti-MOG reactive cases before vaccination and 7 of the 24 (29%) of the anti-MOG reactive cases at 3 months post-vaccination had lost their reactivity to MOG_{5-24}.

Double reactivity to at least one SHBsAg/MOG mimicking pairs was present in 16/39 (41%) of the cases.

Overall 30/50 (60%) vaccinees had SHBsAg/MOG double reactivity on at least one occasion (either at 3 or 6 months post-vaccination) compared to none before-vaccination ($p<0.001$) and in 2/87 (2%, $p<0.001$) of the pathological controls (a 52-year

old woman with PBC and a 28-year old woman with SLE). None of the vaccinees or the pathological controls had double reactivity to the SHBsAg/MBP mimicking pair.
None of the vaccinees experienced any signs or symptoms of demyelinating disorders at the 6-month post-vaccination follow-up.

Table 10.1 Single and double reactivity to SHBsAg and myelin mimics in absolute numbers and percentages (%), in 50 vaccinees before and after vaccination.

	Pre-HBVacc baseline n=50	Post-HBVacc 3 months n=45	Post-HBVacc 6 months n=39
$SHBsAg_{130-149}$	0/50 (0%)	41/45(91%)	37/39 (95%)
$SHBsAg_{80-99}$	0/50 (0%)	1/45 (2%)	0/39 (0%)
$SHBsAg_{1-20}$	0/50 (0%)	10/45 (22%)	13/39 (33%)
$SHBsAg_{65-84}$	0/50 (0%)	5/45 (11%)	5/39 13(%)
MOG_{5-24}	4/50 (8%)	24/45 (53%)	14/39 (36%)
$MOG_{210-229}$	1/50 (2%)	8/45 (17%)	5/39 (13%)
MBP_{80-99}	0/50 (0%)	0/45 (0%)	0/39 (0%)
$SHBsAg_{130-149}/MOG_{5-24}$	0/50 (0%)	23/45 (51%)	16/39 (41%)
$SHBsAg_{80-99}/MOG_{5-24}$	0/50 (0%)	1/45 (2%)	0/39 (0%)
$SHBsAg_{1-20}/MOG_{210-229}$	0/50 (0%)	7/45 (16%)	5/39 (13%)
SHBsAg/ (at least 1peptide)	0/50 (0%)	21/45 (47%)	16/39 (41%)
MOG (at least 1peptide)	4/50 (8 %)	24/45 (53%)	16/39 (41%)
SHBsAg (full protein)	0/50 (0%)	40/45 (89%)	36/39 (92%)
SHBsAg/MOG (at least 1pair)	0/50 (0%)	21/45 (47%)	16/39 (41%)

HBVacc, hepatitis B vaccination; SHBsAg, small hepatitis B surface antigen; MOG, myelin oligodendrocyte glycoprotein; MBP, myelin basic protein

Table 10.2 Antibody reactivity to $SHBsAg_{130-149}$ and MOG_{5-24} before and after vaccination

Case #	$SHBsAg_{130-149}$ pre-HBVacc baseline	MOG_{5-24} pre-Hbvacc baseline	$SHBsAg_{130-149}$ post-HBVacc 3 months	MOG_{5-24} post-HBVacc 3 months	$SHBsAg_{130-149}$ post-HBVacc 6 months	MOG_{5-24} post-HBVacc 6 months
1	0	3.7	na	na	7.1	0
2	0	0	4.7	3.3	5.5	0
3	0	0	3.4	0	na	na
4	0	0	6.7	0	6.9	0
5	0	0	3.8	3.3	4.2	0
6	0	0	4.2	4	6	4.8
7	0	0	0	0	0	0
8	0	4.1	7.1	5.2	7.8	0
9	0	0	6.8	0	5.6	0
10	0	0	6.6	2.3	na	na
11	0	0	na	na	6.5	5
12	0	0	4.3	0	5.7	3.7
13	0	0	2.8	2.8	3.9	3.9
14	0	0	2.3	0	2.7	0
15	0	0	na	na	8.1	0
16	0	0	0	0	0	0
17	0	0	3.3	0	6.8	0
18	0	0	3.3	4	4.9	0
19	0	0	2.8	0	2.9	0
20	0	3.2	4.1	2.9	4.1	0
21	0	0	4.7	0	na	na
22	0	0	4.8	0	na	na
23	0	0	3.2	2.3	3.1	3.8
24	0	0	0	0	0	0
25	0	0	3.2	0	3.1	4.4
26	0	0	na	na	7.8	3
27	0	0	2.9	0	2.9	0
28	0	0	3	0	na	na
29	0	0	3	3.2	na	na
30	0	0	3.1	0	3	0
31	0	0	5.1	3	5.1	2.2
32	0	0	3.8	3.7	6.5	3.5
33	0	0	3.3	3	4.8	4.8
34	0	0	2.9	2.7	na	na
35	0	0	5	5.9	5	0
36	0	0	6.1	5	na	na
37	0	0	3.4	3	3.4	3
38	0	0	na	na	4.8	2.9
39	0	4.2	5.2	0	5.9	0
40	0	0	3.5	3	3.5	2.6
41	0	0	2.9	2.4	2.9	2.9
42	0	0	7	0	7	0
43	0	0	3.3	0	3.3	3.1
44	0	0	2.9	2.1	7.8	2.9
45	0	0	0	0	3.8	3.1
46	0	0	5.1	4	na	na
47	0	0	3.3	2.2	3.2	0
48	0	0	3.8	0	na	na
49	0	0	4.6	3.5	na	na
50	0	0	4.9	0	4.7	0

HBVacc, hepatitis B vaccination; SHBsAg, small hepatitis B surface antigen; MOG, myelin oligodendrocyte glycoprotein; na, not available

10.3.3 Inhibition studies

Antibody binding to $SHBsAg_{130-149}$ was inhibited by 73-92% after pre-incubation with the $SHBsAg_{130-149}$; by 69-85% after pre-incubation with MOG_{5-24} and by 74-88% after pre-incubation with full-length SHBsAg, in all 3 cases tested (Figure 10.2). Insignificant inhibition was observed by pre-incubation with the irrelevant control peptide and antigen (Figure 10.2).

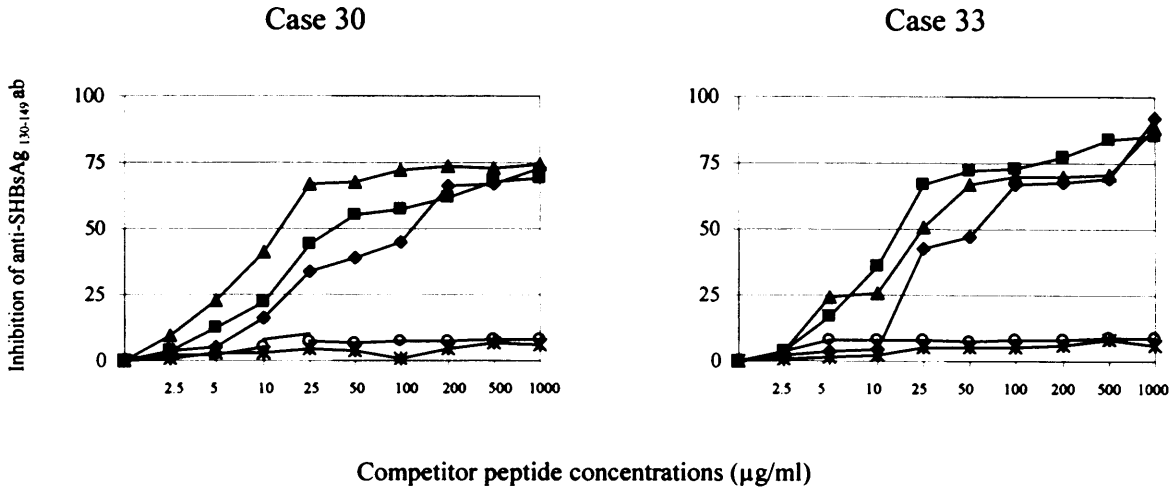


Figure 10.2 *Inhibition of antibody binding against $SHBsAg_{130-149}$ by pre-incubation with $SHBsAg_{130-149}$ (♦), MOG_{5-24} (■), SHBsAg (▲), control antigen (○) and control peptide (*) of case 30 and 33. On the vertical axis is the percentage of inhibition of antibody binding to the peptide in the presence of competitor peptide or antigen at different concentrations. $SHBsAg_{130-149}$, Small Hepatitis B surface antigen; MOG, myelin oligodendrocyte glycoprotein; ab, antibody*

10.4 DISCUSSION

This section of the Thesis has looked for evidence of cross-reactivity between SHBsAg and myelin mimics in normal subjects undergoing hepatitis B vaccination. Not only does SHBsAg share strong homologies with major myelin antigens such as MBP and MOG but specific viral/self pairs were found to be targets of antibody responses induced by the administration of the viral vaccine. As for other mimics described in this Thesis, sequence similarity *per se* did not necessarily lead to anti-peptide reactivity and immunological cross-reactivity (Oldstone, 1987). In the present study the SHBsAg/MBP mimicking sequences and two of four sets of SHBsAg/MOG mimics were not antibody targets (*see* Table 10.1). Inhibition studies have demonstrated the cross-reactive nature of the observed SHBsAg/MOG antibody responses. The selective appearance of viral/myelin cross-reactive responses in some but not all of the mimicking pairs supports the biological significance of the present findings (Oldstone, 1987, Oldstone, 1989, Oldstone, 1998).

The question of a connection between vaccination and autoimmune disorders is surrounded by controversy (Shoenfeld and Aron-Maor, 2000). A heated debate is going on regarding the causality between anti-HBV vaccination and demyelinating disorders such as multiple sclerosis (Shoenfeld and Aron-Maor, 2000). The focus of the present study was the application of HBV vaccination as a model for the study of immunological cross-reactivity rather than the study of the safety and efficacy of the anti-HBV vaccination. Serum samples from HBV vaccination recipients with established autoimmune disorders have not been tested in the present study. All of the vaccinees were free of autoimmune phenomena before vaccination and remain free of any adverse reactions during the follow up. Clues as to the pathogenic link between the vaccine and specific autoimmune disorders, therefore, could not be established. Cross-reactive immunity was found only after vaccination, though decreasing in magnitude over time with a significant proportion of vaccinated subjects maintaining the anti-viral response but losing that against self. These findings suggest that upon vaccination, induction of an anti-viral response is initially capable of inducing cross-reactive anti-self immune responses which decrease over time, possibly as a result of peripheral tolerance mechanisms (*see* 1.2.2). This scenario may explain why very rarely adverse post-vaccination autoimmune reactions occur (Herroelen *et al.*, 1991, Cohen and

Shoenfeld, 1996, Shoenfeld and Aron-Maor, 2000). Equally important may be the finding that the minority of vaccinees with an anti-myelin reactivity pre-HBV vaccination almost universally lost this reactivity post-vaccination. It is possible to speculate that the viral components of the vaccine and in particular the self mimicking SHBsAg sequences may play a role as altered peptide ligand, i.e. may represent sequences unable to induce cross-reactive responses but able to promote tolerance to a given autoepitope (Sloan-Lancaster and Allen, 1996). Such mechanism could probably be of benefit in patients with multiple sclerosis in whom HBV vaccination or immunomodulatory treatment with SHBsAg mimics as altered peptide ligand might contribute to restoration of tolerance towards myeling antigens (Sloan-Lancaster and Allen, 1996).

The hepatitis B vaccine is the first vaccine which has been shown to prevent cancer, and it has saved - and will save - millions of lives (Shouval, 2003). But is also a vaccine against a virus which has been pathogenetically linked to autoimmune adverse reactions (Herroelen *et al.*, 1991, Cohen and Shoenfeld, 1996, Poirriez, 2004, Ravel *et al.*, 2004, Vital *et al.*, 2002, Aron-Maor and Shoenfeld, 2001, Shoenfeld and Aron-Maor, 2000, Maillefert *et al.*, 1999). In view of the observed SHBsAg/MOG cross-reactivity, the vaccine's possible role as a trigger for the induction of demyelinating adverse reactions through molecular mimicry must be further investigated.

CHAPTER 11

General Discussion

This Thesis has focused on the investigation of molecular mimicry and immunological cross-reactivity as a possible mechanism involved in the induction and/or maintenance of liver autoimmunity.

In the first part (Chapters 3-7), the principle of molecular mimicry was exploited to reveal microbial agents able to cross-react with PDC-$E2_{212-226}$, the major mitochondrial autoepitope in PBC (Oldstone, 1989). Evidence for mimicry is readily obtained by scanning databases for sequence homologies between proteins of microbial and human origin (Oldstone, 1998, Bogdanos *et al.*, 2000). Through protein database searches, the present study has found that human PDC-$E2_{212-226}$ shares remarkable amino acid similarities with microbial mimicking sequences of proteins unrelated to PDC. Previous studies demonstrating microbial PDC-E2/self PDC-E2 cross-reactivity have failed to convince several investigators who have argued that such homologies (and the observed cross-reactive responses) are not pathogenetically meaningful since they can readily be predicted, taking into account the phylogenetically highly conserved nature of PDC-E2 among species (Van de Water *et al.*, 2001). The potential significance of the present findings is emphasized by the fact that the identified microbial homologues were better mimics of human PDC-$E2_{212-226}$ than other microbial PDC-E2 mimics such as *E. coli* PDC-$E2_{231-245}$ which has been repeatedly suggested to be involved in the induction of human anti-PDC-E2 autoimmunity.

It has to be stressed, however, that sequence similarity does not necessarily lead to structural/conformational similarity and, hence, need not equate with actual cross-reactivity (antigenic mimicry) (Oldstone, 1987, Quaratino *et al.*, 1995). On the other hand, there is a growing number of examples where evidence of microbial infection in autoimmune disease is also associated with immunological cross-reactivity between autoepitopes and mimicking microbial peptides (Oldstone, 1998). These cases have been taken as evidence of a pathogenetic role for such molecular mimicry. However, they may also be interpreted as trivial epiphenomena: strong structural similarity between epitopes of different antigens simply leading to cross-recognition by independently stimulated lymphocyte populations. In such a debate therefore it is important to bear in mind cases where such trivial cross-reactivity might be expected, but is not observed.

In the case of PBC three variants of such non-reactivity might be cited.

1) Mycobacterial heat shock proteins contain a unique peptide sequence that is a close structural mimic of the dominant autoepitope of PDC-E2, and cross-reactivity

between those peptides is seen in sera from a significant proportion of Spanish PBC patients, but not in a large number of pathological controls from the same region (*see* Chapter 3). Moreover, in a large group of British PBC patients and controls no such disease-specific recognition of mycobacterial antigen was observed indicating a geographical/genetic restriction of the observed phenomenon.

2) On various clinical grounds *Helicobacter pylori* would have seemed to be an excellent candidate in a pathogenetic scenario for PBC. Yet sera (as well as T-cells), from PBC patients, whilst reacting against the immunodominant PDC-$E2_{212-226}$ and the PDC antigen do not recognise the urease peptide that very closely mimics it (*see* Chapter 6).
3) The X sub-unit of the caseinolytic Clp complex (ClpX) of *E. coli* contains a peptide sequence that is the closest mimic of PDC-$E2_{212-226}$. Nevertheless, sera from PBC patients do not recognise the ClpX peptide. However, there is disease-specific recognition of the ClpP component of that complex in a significant percentage of PBC patients. In this case, it seems likely that, in a proportion of patients, mimicry is operative at the CD4 T-cell level, helping in the production of antibodies against the other constituent of the same complex (*see* Chapter 7).

These findings indicate that cross-reactivities between autopitopes and mimicking peptides of infecting organisms, when they are observed, are probably not trivial, and may well have a bearing on the mechanism of disease. This seems to be the case of *E. coli* mimics cross-reactively recognising human PDC-$E2_{212-226}$, particularly in those patients with evidence of recurrent urinary tract infection (Chapter 5). These observations expand further our view of a microbial involvement and in particular of ECOLI-urinary tract infection in the appearance and/or maintenance of anti-mitochondrial antibody responses by a cross-reactive mechanism.

More complex and less well understood is the finding that one third of patients with PBC had an IgG3 subclass antibody response against a lactobacillus sequence that mimics the major anti-mitochondrial autoepitope, which was not found in control subjects (Chapter 4). That the disease-specific anti-BGAL LACDE responses are of IgG3 isotype, implicates T-cell 'help' in their production, and further supports the view that cellular immune responses are 'key' players in the perpetuation of the autoimmune attack (Snapper and Mond, 1993, Stavnezer, 1996, Snapper *et al.*, 1997,Stevens *et al.*, 1988, Mosmann and Coffman, 1989, Mahon *et al.*, 1995).

These results also raise additional questions, which need to be addressed, particularly about the precise mechanisms by which B and T cell cross-reactive immune responses are implicated in the induction of PBC-specific antimitochondrial immune responses (Gershwin *et al.*, 2000, Van de Water *et al.*, 2001). If a B-cell receptor happens to recognise an epitope on a microbial protein that is a mimic of an existing auto-reactive T-cell epitope, and this mimicking sequence is preferentially presented by the cell (by virtue of its protection from degradation during antigen processing), then there may be an enhancement of production of antibodies recognising that microbial sequence. Moreover, new T-cell clones would be recruited into this expansion of the immune response whenever an APC, such as a dendritic cell, happened to present a further mimicking epitope derived from the processing of another microbial protein, and hence prime another potentially auto-reactive T-cell receptor (Liang and Mamula, 2000, James and Harley, 1998). Furthermore, if the repertoire of B-cell antigen receptors, generated in such a scenario, catalysed the uptake of other microbial proteins bearing mimicking sequences that were then themselves presented to the T-cell repertoire, there would be a further enhancement of the autoimmune cascade.

The findings of the present work and those of recent studies demonstrating the existence of PDC-E2 cross-reactive microbial mimics of common pathogens support the concept of a 'multiple hit' mechanism of molecular mimicry whereby a short sequence on human PDC-E2 becomes a cross-reactive target of several microbial mimics, a process that, in susceptible individuals, may culminate in tolerance breakdown (Vilagut *et al.*, 1997, Selmi *et al.*, 2003, Van de Water *et al.*, 2001, Shimoda *et al.*, 2000).

Three further points need to be made: Firstly, microbial involvement in autoimmune disease is likely to be a 'hit and run' phenomenon, so the lack of bacterial products in the liver of PBC patients may be anticipated and is not incompatible with immunological evidence of previous infection (*see* Chapter 9) (Oldstone, 1987, von Herrath, 2000). Secondly, while the results presented here are relevant to the evolution of the antibody repertoire in PBC, they do not shed direct light on the identity of the autoantigen abnormally expressed in biliary epithelium in the disease, which is recognised by these antibodies (Van de Water *et al.*, 1993, Gershwin *et al.*, 2000). Finally, although the observed microbial/self cross-reactive responses are specifically found in patients with PBC, their pathogenic significance is still unclear (Neuberger and Thomson, 1999, Gershwin *et al.*, 2000, Van de Water *et al.*, 2001). It has to be stressed that these responses have been studied at the time of overt disease and therefore may

just represent 'footprints' of an ongoing process that took place several years before the onset of the full-blown clinical picture or may well be secondary to the immunological breakdown in the disease (von Herrath, 2000).

In the second part of the Thesis (Chapters 8-10), the role of immunological cross-reactivity has been similarly investigated in viral hepatitis-associated autoimmunity. Of interest, in the case of HCV associated LKM1 autoimmunity, it was found that a 'multiple hit' mechanism of molecular similarity between CYP2D6, HCV and HSV did probably underpin the production of a cross-reactive response (Chapter 8). The finding of the present study that the possession of HLA B51 allele differentiates the cross-reactive from the non-cross-reactive LKM1 positive HCV infected patients needs further investigation.

These cross-reactive responses have been demonstrated in patients' serum samples obtained long after the disease has been established, and are therefore unable to provide clues as to the underlying mechanisms preceding the full blown clinical picture. To circumvent this limitation the present study has employed two different approaches. The first has focused on the investigation of immunological cross-reactivity in a girl with primary and secondary LKM1 response following HCV infection acquired at the time of liver transplantation and followed-up for 14 years (Chapter 9). This girl developed a florid autoimmune hepatitis nine years after the first appearance of disease-specific autoantibodies in her serum (Mackie *et al.*, 1994). The study of the fine specificity of her anti-CYP2D6 responses revealed both an epitope spreading from a single to several other CYP2D6 epitopes, and additionally, and most importantly, a cross-reactive response involving the major CYP2D6 autoepitope and its HCV, HSV, CMV, and EBV mimics indicating that a 'multiple hit' viral/self cross-reactive mechanism may have been instrumental in the appearance of the autoimmune response. The second approach involved the study of cross-reactive immunity between the hepatitis B surface antigen, the viral component of the hepatitis B virus vaccine, and its mimics on myelin antigens (Chapter 10). Cross-reactive immunity was found only after vaccination, though in decreasing magnitude over time, and with the vaccinated subjects maintaining the anti-viral response but losing that against self. The finding that a cross-reactive viral/self immune response frequently occurs after vaccination but decreases with time, possibly as a result of peripheral tolerance mechanism(s), may explain why post-vaccination autoimmune adverse reactions are rare (Shoenfeld and Aron-Maor, 2000). Of interest is

the finding that the minority of vaccinees with an anti-myelin reactivity pre-HBV vaccination lost this reactivity post-vaccination. This suggests that the viral components of the vaccine and in particular the self mimicking SHBsAg sequences, may well play a role in the suppression of pre-existing autoreactive responses giving support to the notion that molecular mimicry can be a mechanism of prevention (instead of acceleration) of autoimmunity (Sloan-Lancaster and Allen, 1996, Christen and von Herrath, 2004).

In summary, this Thesis has highlighted potentially different aspects of molecular mimicry in liver autoimmunity and presented examples demonstrating the complexity and diversity in which immunological cross-reactivity may affect autoimmunity. The present studies have demonstrated that disease-specific microbial/self cross-reactive responses do occur and may be of pathogenic significance. Tracking and quantitating the cross-reactive immunological process following viral infection in large number of individuals at risk of developing autoimmune manifestations may provide an insight into the real impact of molecular mimicry in human autoimmune liver diseases.

However, as for other autoimmune disease, definitive proof for molecular mimicry as an initiator or perpetuator of autoimmunity will remain difficult to obtain in humans.

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Cytochrome P4502D6$_{193-212}$: A New Immunodominant Epitope and Target of Virus/Self Cross-Reactivity in Liver Kidney Microsomal Autoantibody Type 1-Positive Liver Disease[1]

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Cytochrome P4502D6 (CYP2D6), target of liver kidney microsomal autoantibody type 1 (LKM1), characterizes autoimmune hepatitis type 2 (AIH2) but is also found in patients with chronic hepatitis C virus (HCV) infection. To provide a complete linear epitope B cell map of CYP2D6, we tested peptides spanning the entire sequence of CYP2D6. In addition to confirming previously described antigenic sites, we identified four new epitopes (193–212, 238–257, 268–287, and 478–497). CYP2D6$_{193-212}$ is immunodominant and was the target of 12 of 13 (93%) patients with AIH2 and 5 of 10 (50%) HCV/LKM1-positive patients. Because LKM1 is present in both AIH2 and a viral infection, we tested whether Abs to CYP2D6$_{193-212}$ arise through cross-reactive immunity between virus and self. We identified a hexameric sequence "RLLDLA" sharing 5 of 6 aa with "RLLDLS" of HCV$_{2985-2990}$ and all 6 aa with CMV$_{130-135}$. Of 17 CYP2D6$_{193-212}$-reactive sera, 11 (7 AIH and 4 HCV) reacted by ELISA with the HCV homologue, 8 (5 AIH and 3 HCV) with the CMV homologue, and 8 (5 AIH and 3 HCV) showed double reactivity. Autoantibody binding to CYP2D6$_{193-212}$ was inhibited by preincubation with HCV$_{2977-2996}$ or CMV$_{121-140}$. Recombinant HCV-nonstructural protein 5 and CMV-UL98 proteins also inhibited Ab binding to CYP2D6$_{193-212}$. Affinity-purified CYP2D6$_{193-212}$-specific Ab inhibited the metabolic activity of CYP2D6. The demonstrated similarity and cross-reactivity between CYP2D6$_{193-212}$ and two unrelated viruses suggests that multiple exposure to viruses mimicking self may represent an important pathway to the development of autoimmunity. ***The Journal of Immunology,*** **2003, 170: 1481–1489.**

Liver kidney microsomal Ab type 1 (LKM1)[3] is the diagnostic marker of autoimmune hepatitis type 2 (AIH2), AIH1 being characterized by the presence of antinuclear Ab and/or smooth muscle Ab (1). LKM1 is also present in up to 10% of patients with chronic hepatitis C virus (HCV) infection, where it appears to confer severity to the disease process, as shown by Giostra et al. (2), and to predispose to adverse reactions during treatment with IFN (3). LKM1, first described by Rizzetto et al. in 1973 (4), is known to recognize a 50-kDa protein in rat microsomes located primarily in the smooth endoplasmic reticulum (5) and a 48-kDa protein in human liver microsomes (6). This protein in human liver was later identified as cytochrome P4502D6 (CYP2D6) (7–9). The catalytic function of this cytochrome is inhibited in vitro by incubation with LKM1-positive serum (7).

The presence of B cell epitopes on CYP2D6 has been investigated by several groups (Fig. 1*A*), and the region spanning 254–271 has been reported to be highly antigenic in LKM1-positive AIH (10–12). Within this region, Manns et al. (11) reported that CYP2D6$_{263-270}$ is recognized by 62% of AIH2 patients, and Yamamoto et al. (12) reported that CYP2D6$_{257-269}$ is the most frequently recognized antigenic site in LKM1 AIH (85% of sera tested). They also described three other epitopes spanning 321–351, 373–389, and 410–429 regions of CYP2D6, recognized respectively by 53, 7, and 13% of the AIH2 cases (12). In contrast to patients with AIH2, reactivity to the CYP2D6$_{254-271}$ region was not detected in chronic HCV patients seropositive for LKM1 (13). Two epitopes (200–214 and 321–339) recognized by LKM1 in chronic HCV infection were described by Parez et al. (14). More recently, Klein et al. (15) reported that the CYP2D6 sequence 196–218 is recognized by 68% of patients with AIH2, but by only 18% of LKM1/HCV-positive patients. Common to all of the above reports is the selection of putative antigenic sites for testing, an approach that could ignore other important epitopes. The aim of the present study was to provide a complete B cell map of CYP2D6 linear epitopes using a representative number of LKM1-positive sera from patients with AIH2 or chronic HCV infection. Epitopes recognized by LKM1-positive serum would then be analyzed for similarities with HCV and other pathogens, possible triggers of LKM1 production.

Materials and Methods

Subjects

Twenty-three LKM1-positive patients were studied. Thirteen had classical LKM1-positive AIH (AIH2) diagnosed according to the recently revised

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[3] Abbreviations used in this paper: LKM1, liver kidney microsomal Ab type 1; AIH2, autoimmune hepatitis type 2; HCV, hepatitis C virus; CYP2D6, cytochrome P4502D6; NS5B, nonstructural protein 5B; AMMC, 3-[2-(*N*,*N*-diethyl-*N*-methylamino)ethyl]-7-methoxy-4-methylcoumarin.

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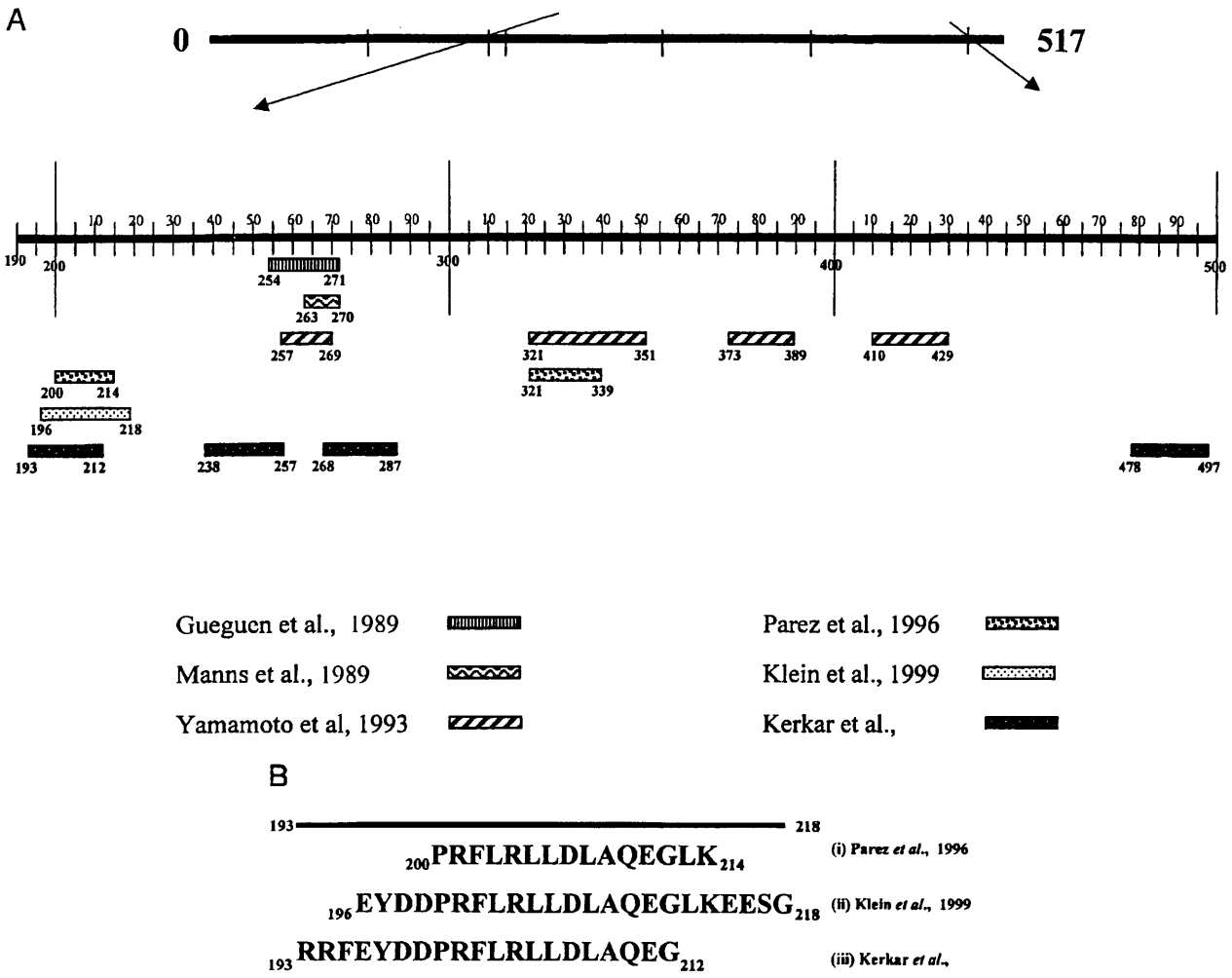

FIGURE 1. *A*, Epitope map of CYP2D6 illustrating documented epitopes: CYP2D6$_{254-271}$ by Gueguen et al. (8); CYP2D6$_{263-270}$ by Manns et al. (9); CYP2D6$_{257-269}$, CYP2D6$_{321-351}$, CYP2D6$_{373-389}$, and CYP2D6$_{410-429}$ by Yamamoto et al. (12); CYP2D6$_{200-214}$ and CYP2D6$_{321-339}$ by Parez et al. (14); CYP2D6$_{196-218}$ by Klein et al. (15); CYP2D6$_{193-212}$, CYP2D6$_{238-257}$, CYP2D6$_{268-287}$, and CYP2D6$_{478-497}$ as described in this report. *B*, Detailed epitope map of CYP2D6$_{193-218}$. *Bi*, CYP2D6$_{200-214}$ described by Parez et al. (14) and recognized by 62.5% of LKM1-positive HCV patients, but not by AIH2 patients. *Bii*, CYP2D6$_{196-218}$ described by Klein et al. (15) and recognized by 68% of AIH2 and 18% of LKM1/HCV patients. *Biii*, CYP2D6$_{193-212}$, the new epitope described in this report recognized by 92% of AIH2 and 50% of LKM1/HCV patients.

criteria of the International Autoimmune Hepatitis Group (16). All had raised transaminases at presentation and were negative for HCV by PCR as well as other serological markers for hepatitis A-E and IgM to EBV and CMV. All had characteristic interface hepatitis on liver biopsy and responded to immunosuppressive treatment (Prednisolone with and without Azathioprine). Eleven patients were female with a median age of 10.6 years and a range from 3.1 to 18 years. LKM1 was detected by standard immunofluorescence, and the LKM1 titers at the time of testing ranged from 1/40 to 1/5120 with a median of 1/640. Four children had other autoimmune conditions: autoimmune thyroiditis in one, insulin-dependent diabetes mellitus in the second, sclerosing cholangitis in the third, and inflammatory bowel disease in the fourth. Three children were tested at diagnosis, four during remission on immunosuppressive therapy, and six during relapse.

Ten sera were from patients with liver disease attributed to chronic HCV infection. Six were female with a median age of 37 years and a range of 19 to 55 years. All were HCV RNA-positive (Amplicor; Hoffmann-LaRoche, Basel, Switzerland). LKM1 titers at the time of testing were 1/160–1/640 with a median of 1/640. One was receiving IFN-α, two had stopped IFN-α 5 mo and 4 years before this study, and one was on steroid treatment after having developed a transaminase flare 4 mo into IFN-α treatment. These patients have been previously described within a larger series of LKM1/HCV-positive patients treated with IFN-α (17). Six patients were not treated. Serum from seven healthy controls, four male and three female with a median age of 13 years (range 10–30 years), were also studied.

Peptides

Thirty-four 20-mer synthetic peptides spanning the entire 517-aa sequence of the protein and overlapping by 5 aa were constructed by 9-fluorenylmethyloxycarbonyl chemistry (Mimotopes, Clayton, Victoria, Australia) (Table 1). Biotin was linked to the peptides through a spacer with alternating serine (S) and glycine (G) residues to avoid steric hindrance and reduce hydrophobicity (18). Peptides conformed to the following format: biotin-SGSG-peptide-amide, where SGSG is the spacer. A peptide encoding a randomly generated sequence of amino acids, biotin-SGSG-HEDYVNQSLRPTPLEISVRA-amide, was used as the negative control peptide. All peptides were >90% pure after reverse-phase HPLC. The identity of each peptide was confirmed by mass spectrometry. The reactivity of LKM1-positive serum against each of the 34 peptides constructed was tested by ELISA.

Recombinant proteins

CMV alkaline exonuclease, the UL98 gene product, was a kind gift from Dr. D. J. Tenney from Bristol-Myers Squibb (Pharmaceutical Research Institute, Wallingford, CT). Recombinant human CMV-UL98 was produced using a baculovirus-insect cell expression system and was purified by high salt cell lysis, Q Sepharose chromatography, and phosphocellulose chromatography. The final material was dialysed against 20 mM Tris-HCl (pH 7.5), 25% glycerol, 0.1 mM EDTA, and 1 mM DTT to an NaCl concentration of 5 mM. The protein resolved as a single ~65-kDa band on

Table I. *Peptide key*[a]

Peptide No.	Protein	Sequence No.	Amino Acid Sequence
1	CYP2D6	1–20	MGLEALVPLAVIVAIFLLLV
2	CYP2D6	16–35	FLLLVDLMHRRQRWAARYPP
3	CYP2D6	31–50	ARYPPGPLPLPGLGNLLHVD
4	CYP2D6	46–65	LLHVDFQNTPYCFDQLRRRF
5	CYP2D6	61–80	LRRRFGDVFSLQLAWTPVVV
6	CYP2D6	76–95	TPVVVLNGLAAVREALVTHG
7	CYP2D6	91–110	LVTHGEDTADRPPVPITQIL
8	CYP2D6	106–125	ITQILGFGPRSQGVFLARYG
9	CYP2D6	121–140	LARYGPAWREQRRFSVSTLR
10	CYP2D6	136–155	VSTLRNLGLGKKSLEQWVTE
11	CYP2D6	151–170	QWVTEEAACLCAAFANHSGR
12	CYP2D6	166–185	NHSGRPFRPNGLLDKAVSNV
13	CYP2D6	181–200	AVSNVIASLTCGRRFEYDDP
14	CYP2D6	193–212	RRFEYDDPRFLRLLDLAQEG
15	CYP2D6	208–227	LAQEGLKEESGFLREVLNAV
16	CYP2D6	223–242	VLNAVPVLLHIPALAGKVLR
17	CYP2D6	238–257	GKVLRFQKAFLTQLDELLTE
18	CYP2D6	253–272	ELLTEHRMTWDPAQPPRDLT
19	CYP2D6	268–287	PRDLTEAFLAEMEKAKGNPE
20	CYP2D6	283–302	KGNPESSFNDENLRIVVADL
21	CYP2D6	298–317	VVADLFSAGMVTTSTTLAWG
22	CYP2D6	313–332	TLAWGLLLMILHPDVQRRVQ
23	CYP2D6	328–347	QRRVQQEIDDVIGQVRRPEM
24	CYP2D6	343–362	RRPEMGDQAHMPYTTAVIHE
25	CYP2D6	358–377	AVIHEVQRFGDIVPLGMTHM
26	CYP2D6	373–392	GMTHMTSRDIEVQGFRIPKG
27	CYP2D6	388–407	RIPKGTTLITNLSSVLKDEA
28	CYP2D6	403–422	LKDEAVWEKPFRFHPEHFLD
29	CYP2D6	418–437	EHFLDAQGHFVKPEAFLPFS
30	CYP2D6	433–452	FLPFSAGRRACLGEPLARME
31	CYP2D6	448–467	LARMELFLFFTSLLQHFSFS
32	CYP2D6	463–482	HFSFSVPTGQPRPSHHGVFA
33	CYP2D6	478–497	HGVFAFLVSPSPYELCAVPR
34	CYP2D6	498–517	NGVPSPQPAPSPEALMYNKA
35	CMV	121–140	GSDDYVWLSRLLDLAPNYRQ
36	HCV	2977–2996	KLTPLPEARLLDLSSWFTVG
37	HCV	ALA[b]	AAAAAPEARLLDLSSWFTVG
38	HCV	ALA	KLTAAAAARLLDLSSWFTVG
39	HCV	ALA	KLTPLPEAAAAAAASSWFTVG
40	HCV	ALA	KLTPLPEARLLDLAAAAAVG
41	HCV	ALA	KLTPLPEARLLDLSSAAAAA
42		Control	HEDYVNQSLRPTPLEISVRA

[a] 1–34, Amino acid sequences of 34 synthetic peptides in standard single letter code, spanning the full length of the CYP2D6 molecule. Each peptide overlaps by 5 aa with the preceding and subsequent peptide. 35, 36, Viral peptide homologues of $CYP2D6_{193-212}$. 37–41, Alanine-substituted peptides derived from $HCV_{2977-2996}$. 42, Control peptide.

[b] ALA, Alanine-substituted peptides.

a Coomassie-stained SDS-PAGE. It had a specific activity of 28 mg of activated calf thymus DNA digest/mg protein/30 min with a protein concentration of 83 μg/ml. HCV-nonstructural protein 5B (NS5B) (genotype 3a) was a kind gift from Dr. Seong of Yonsei Engineering Complex (Yonsei University, Seoul, Korea). Recombinant protein migrated as a single band at ~60 kDa on SDS-PAGE and was enzymatically active as assessed by a gel-based biochemical RdRp assay. The protein was dialyzed into 25 mM sodium phosphate (pH 6.8), 150 mM NaCl, 0.5 mM EDTA, 5 mM DTT, 0.5% Tween 20, and 55% glycerol at a final concentration of 60 μg/ml. Recombinant metabolically active CYP2D6, produced in a baculovirus-insect cell expression system and extracted as a microsomal preparation (10 mg/ml), was purchased from Cambridge BioScience (Cambridge, U.K.).

ELISA

A total of 200 ml/well of 2% BSA/PBS was added for 1 h at 20°C to a 96-well polysterene plate precoated with 5 mg/ml streptavidin (Mimotopes) to prevent nonspecific binding. After addition to each well of 100 ml of biotinylated peptide diluted to 1/1000 in PBS containing 0.1% sodium azide and 0.1% BSA, the plate was mildly agitated in a shaker (Dynex Technologies, Guernsey, U.K.) for 1 h at 20°C. One hundred microliters of patient serum diluted to 1/200 in 2% BSA/PBS containing 0.1% sodium azide was added to each well and incubated under mild agitation at 20°C for another hour. Thereafter, 100 ml of HRP-conjugated goat anti-human IgG (Sigma-Aldrich, Poole, Dorset, U.K.) diluted to 1/1000 in 2% BSA/PBS was added and incubated for 1 h without shaking at 20°C. The optimum concentrations of reagents at various steps of the immunoassay were determined in preliminary experiments by checkerboard titration. Initially, using a wide range of dilutions, the working conditions of the assay were identified and, subsequently, using smaller dilutions around the optimum, the final concentration of the test samples and reagents was determined (19). After each of the above steps, the plate was washed five times with PBS containing 0.1% Tween 20. Freshly prepared *o*-phenylene-diamine in citrate phosphate buffer was used as substrate, and the reaction was stopped with 4N sulfuric acid. Absorbance values were read at 490 nm on a spectrophotometer. In each plate, two wells were used as blanks, in which serum and peptide were omitted, and two additional wells were used for a positive and a negative control. The positive control consisted of an LKM1-positive serum (titer 1/5120), which in preliminary experiments was shown to react with $CYP2D6_{253-272}$, containing a previously described immunodominant epitope (10). A randomly generated scrambled control peptide incubated with serum from a healthy individual was used as negative control. Each serum tested against experimental peptides was also tested against the control peptide. The final absorbance value was calculated by subtracting control peptide from the experimental peptide absorbance. Subtracted values exceeding 0.11 were considered positive, this value representing mean + 3 SD of 126 readings using serum from seven healthy subjects against 14 randomly selected CYP2D6 peptides. A synthetic peptide was considered to encompass an epitope when the subtracted absorbance was >0.5.

CMV IgG was tested by ELISA using CMV IgG kit (Captia; Trinity Biotech, Bray, Ireland).

Serum from the 10 LKM1-positive patients with chronic HCV infection was tested for rheumatoid factor IgM Abs using a commercially available kit (Rheumatoid Factor kit; Sigma-Aldrich).

Database search

Protein databases SWISS-PROT and PIR were searched using PROTEIN-INFO to search for homologies between the newly identified epitopes and human microorganisms. Homologous peptides were constructed by 9-fluorenylmethyloxycarbonyl chemistry as detailed above (Mimotopes) (Table I).

Inhibition studies

To investigate whether the reactivity to $CYP2D6_{193-212}$ and homologous viral peptides was due to cross-reactivity, competition ELISAs were performed using $CYP2D6_{193-212}$ in solid phase and using $CYP2D6_{193-212}$, $HCV_{2977-2996}$, $CMV_{121-140}$, and the irrelevant peptide as competitors in liquid phase. Test serum was diluted 1/400 with peptide solutions to give final peptide concentrations of 0.1 mg/ml, 0.5 mg/ml, and 1 mg/ml, respectively. The solutions were incubated at 37°C for 2 h and then tested for anti-$CYP2D6_{193-212}$ reactivity by ELISA.

Alanine substitution studies

To further investigate the contribution of the $HCV_{2977-2996}$ region $_{2985}$RLLDL (which is identical with $CYP2D6_{204-208}$ and $CMV_{130-134}$) to Ab reactivity to the full 20-aa sequence, five peptides were constructed in the format biotin-SGSG-peptide-amide, each with five contiguous alanine substitutions across the length of $HCV_{2977-2996}$ (Table I). Alanine-substituted peptides were tested for reactivity to patient serum by ELISA. $LKM1^+$ patient serum was tested at a dilution of 1/400, and HRP-conjugated goat anti-human IgG Ab was used at a dilution of 1/2000. All other experimental conditions were as described in *ELISA*.

Reactivity of LKM1-positive serum to recombinant proteins

ELISA microtiter plates (Nunc International, Roskilde, Denmark) were coated overnight at 4°C with recombinant CYP2D6, HCV-NS5, and CMV-UL98 proteins at a concentration of 2 μg/ml in sodium bicarbonate buffer (pH 9.6). After washing, plates were blocked with 100 μl of 5% BSA in PBS for 1 h at 37°C. One hundred microliters of serum from a patient with LKM1-positive AIH at a dilution of 1/200 was added after washing. Further steps were as described in *ELISA*.

Inhibition of reactivity to $CYP2D6_{193-212}$ by recombinant proteins

Inhibition of reactivity to the immunodominant epitope by whole protein was investigated by performing a competition ELISA using $CYP2D6_{193-212}$ in solid phase and using recombinant HCV-NS5 and CMV-UL98 pro-

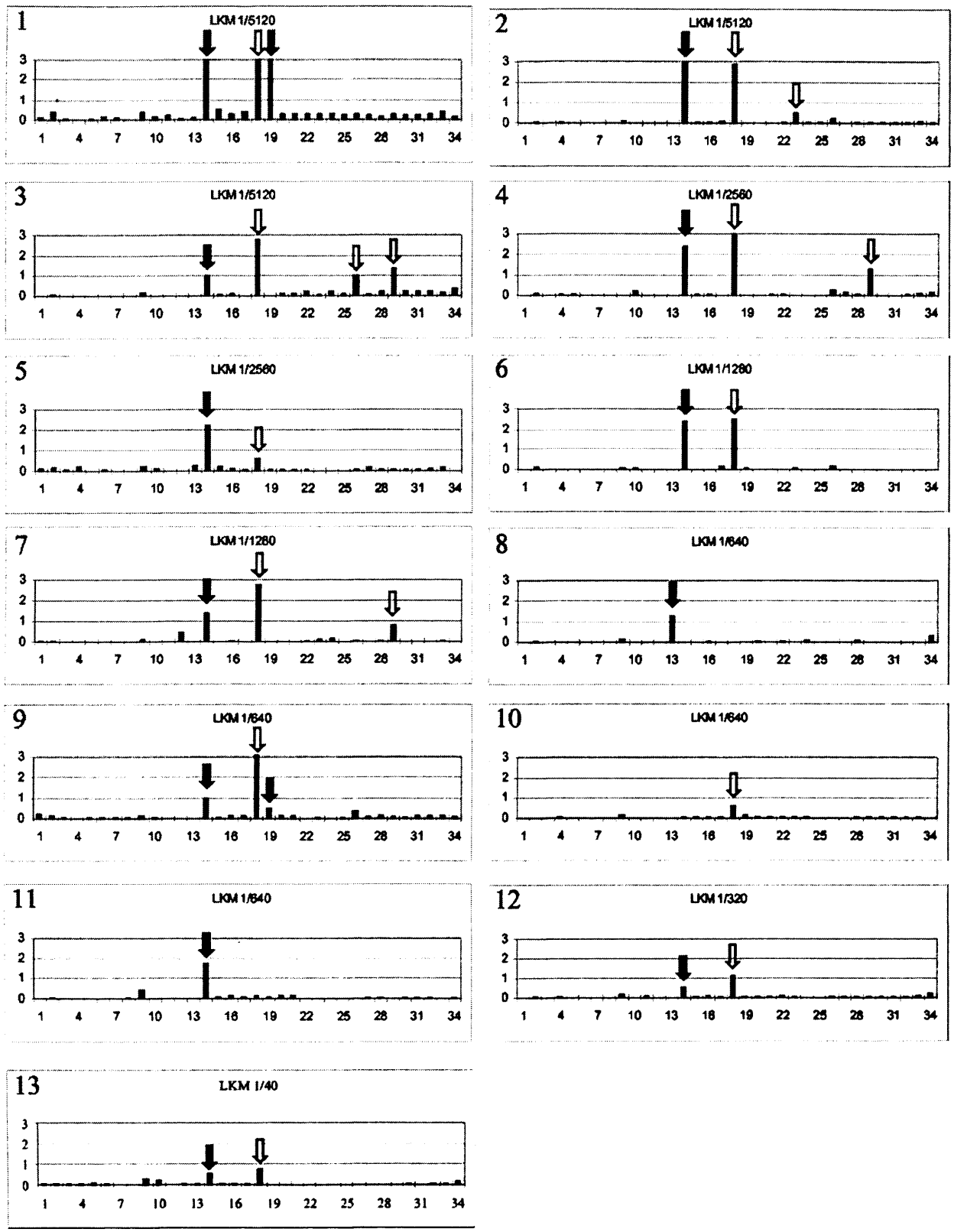

FIGURE 2. Reactivities of sera from 13 patients with AIH2 (*A*) and 10 LKM1-positive patients with chronic HCV infection (*B*) against 34 overlapping 20-mer peptides spanning residues 1–517 on CYP2D6. Absorbance values on the *y*-axis are plotted against the 34 individual peptides on the *x*-axis. The filled arrows mark the four newly identified epitopes: CYP2D6 $_{193-212}$ (peptide 14) is recognized by 12 AIH2 (patients 1–9, 11, 12, and 13) and 5 LKM1/HCV cases (patients 14, 15, 17, 18, and 20); CYP2D6$_{238-257}$ (peptide 17), recognized by LKM1/HCV patient number 22; (*Figure legend continues*)

B

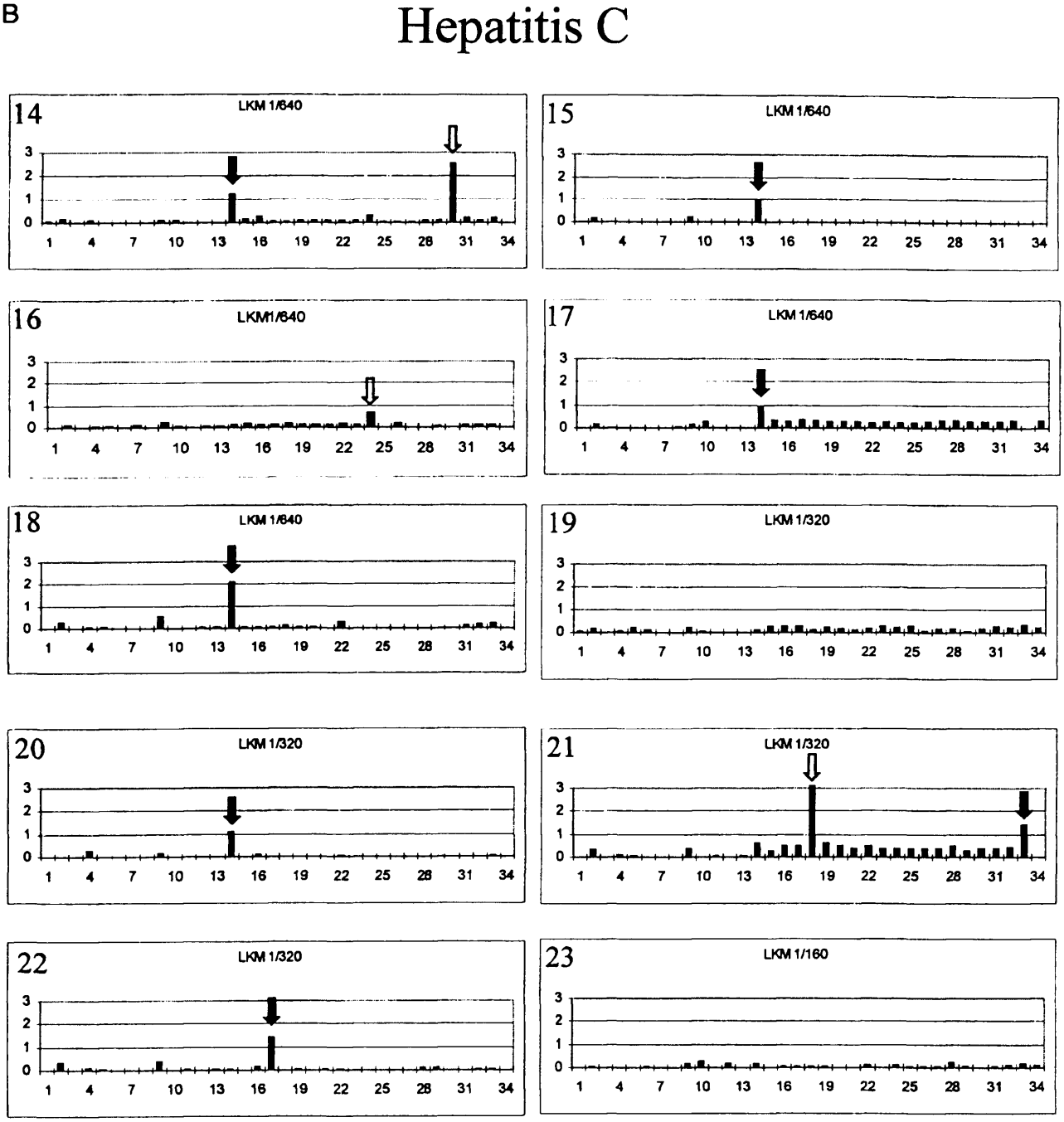

FIGURE 2. *continued.*

CYP2D6$_{268-287}$ (peptide 19), recognized by two AIH2 cases (patients 1 and 9); and CYP2D6$_{478-497}$ (peptide 33), recognized by LKM1/HCV patient number 21. The open arrows mark previously described epitopes: CYP2D6$_{253-272}$ (peptide 18), is recognized by 11 AIH2 cases (patients 1–7, 9, 10, 12, and 13) and LKM1/HCV patient number 21; CYP2D6$_{328-347}$ (peptide 23), recognized by AIH2 patient number 2; CYP2D6$_{343-362}$ (peptide 24), recognized by LKM1/HCV patient number 16; CYP2D6$_{373-392}$ (peptide 26), recognized by AIH2 patient number 3; CYP2D6$_{418-437}$ (peptide 29), recognized by three AIH2 cases (patients 3, 4, and 7) and LKM1/HCV patient number 14.

teins as competitor in liquid phase. Test serum (at a final dilution of 1/200) and recombinant proteins (at a final concentration of 0.3 μg/ml) were incubated for 2 h at 37°C and subsequently were tested for reactivity to CYP2D6$_{193-212}$ as described in *ELISA*.

Affinity purification of LKM1-positive serum

An LKM1-positive serum sample (reactive to CYP2D6$_{193-212}$, HCV$_{2977-2996}$, and CMV$_{130-134}$) was affinity purified over a Tetralink tetrameric avidin column (Promega, Southampton, U.K.) complexed with biotinylated CYP2D6$_{193-212}$ per the manufacturer's instructions. Briefly, 1 ml of biotinylated CYP2D6$_{193-212}$ at a concentration of 0.25 mg/ml was passed over a tetrameric avidin column (equilibrated in PBS). CYP2D6$_{193-212}$-specific Ab was captured by passing 5 ml of a "triple-reactive" LKM1-positive serum (diluted 1/4 in PBS) over the prepared column a total of five times. The column was washed with PBS to remove unbound material and captured Ab was eluted into 2 M Tris-HCl (pH 8.0) using 50 mM glycine (pH 3.0). The eluted Ab was concentrated to give a final volume of 1 ml (to match the original serum volume).

Inhibition of CYP2D6 metabolic activity by LKM1-positive serum

A commercially available kit was used to assess inhibition of CYP2D6 metabolic activity by LKM1-positive serum and affinity-purified Ab (CYP2D6/3-[2-(*N*,*N*-diethyl-*N*-methylamino)ethyl]-7-methoxy-4-methylcoumarin (AMMC) inhibitor screening kit; Gentest). The assay uses recombinant microsomal CYP2D6 (1.5 pM) in conjunction with AMMC as substrate (1.5 mM) and cofactor, producing a fluorescent metabolite (3-[2-(*N*,*N*-diethylamino)ethyl]-7-hydroxy-4-methylcoumarin hydrochloride) that is detected using a fluorescence plate reader (CytoFluor Series 4000; Applied Biosystems, Foster City, CA; excitation at 390 nm, emission at 460 nm). Serial dilutions of 1/25 to 1/18,225 of a known CYP2D6 inhibitor (quinidine, initial concentration 0.5 μm) and LKM1-positive serum from patients with AIH and HCV infection, as well as the affinity-purified Ab, were added to 96-well black microtiter plates (Labsystems Fluoro 96; Thermo Labsystems, Vantaa, Finland) containing CYP2D6 followed by the addition of substrate. Control reactions included CYP2D6 with no substrate, CYP2D6 with substrate (no inhibitor), and blank wells, which were otherwise subjected to the same experimental conditions. The reaction was allowed to progress for 30 min at 37°C, after which it was terminated with 80% acetonitrile, 100 mM Tris. Plates were read using the parameters described above. Percentage inhibition was calculated as follows: [(1 − (mean fluorescence with test compound − mean fluorescence of blank well/mean fluorescence without inhibitor − mean fluorescence of blank well)] × 100.

Structural homology modeling of CYP2D6

Swiss-Model and the Swiss-Pdb Viewer (http://www.expasy.org/swissmod) were used for investigating and analyzing the derived CYP2D6 protein structure (20). CYP2D6$_{193-212}$ was modeled locally on the homologous region in *Bacillus megaterium* cytochrome P450 BM-3 (21, 22).

Results

Seven sera from healthy individuals were tested against 14 randomly selected peptides to establish a cutoff for Ab reactivity. The mean (SD) subtracted (from the random control peptide) OD was 0.0137(0.0366), giving a cutoff value of 0.11 (mean + 3 SD).

Sequences incorporating previously described epitopes (12) were recognized at the following frequencies: CYP2D6$_{253-272}$ by 11 of 13 (85%) AIH2 and 1 of 10 (10%) LKM1/HCV patients, CYP2D6$_{418-437}$ by 3 of 13 (23%) AIH2 and 1 of 10 (10%) LKM1/HCV patients, and CYP2D6$_{343-362}$ by 1 of 10 (10%) LKM1/HCV patients. Individual serum from AIH2 patients recognized the sequences CYP2D6$_{328-347}$ and CYP2D6$_{373-392}$ (Fig. 2, *A* and *B*, open arrows). Four new epitopes were identified (Fig. 2, *A* and *B*, filled arrows). These comprised CYP2D6$_{193-212}$, which was recognized by the serum from 12 of 13 (93%) AIH2 and 5 of 10 (50%) LKM1-positive patients with chronic HCV infection, CYP2D6$_{268-287}$ by two AIH2 sera, and CYP2D6$_{238-257}$ and CYP2D6$_{478-497}$ by individual sera from LKM1/HCV patients. Within the immunodominant epitope CYP2D6$_{193-212}$, the motif "RLLDLA" was found to have 5 of 6 aa identical and one similar to the hexameric sequence "RLLDLS" of HCV$_{2985-2990}$ (located within HCV-NS5, the RNA-dependent DNA polymerase), A and S being conserved residues (23). The same motif also shared a 6-aa identity with CMV$_{130-135}$ (located within the CMV alkaline exonuclease) (Fig. 3). All 17 sera (12 AIH2 and 5 LKM1/HCV) reacting with the newly identified epitope CYP2D6$_{193-212}$ were tested by ELISA against the homologous HCV and CMV biotinylated viral peptides. Seven AIH2 and four LKM1/HCV sera reacted with both CYP2D6$_{193-212}$ and HCV$_{2977-2996}$, whereas five AIH2 and three LKM1/HCV sera reacted with both CYP2D6$_{193-212}$

Table II. *Recognition of homologous viral peptides CMV$_{121-140}$ and HCV$_{2977-2996}$ by LKM1-positive sera all reactive with the immunodominant epitope*[a]

Patient	HCV$_{2977-2996}$	CMV$_{121-140}$	CYP2D6$_{193-212}$
AIH 1	**0.47**	**0.13**	**3.16**
AIH 2	**0.75**	**0.26**	**2.70**
AIH 3	**0.45**	0.01	**1.50**
AIH 4	0.01	0.02	**2.25**
AIH 5	0.01	0.01	**0.48**
AIH 6	0.02	0.01	**1.37**
AIH 7	**0.25**	**0.25**	**1.03**
AIH 8	0.06	0.01	**0.23**
AIH 9	0.01	0.01	**0.10**
AIH 10	**0.16**	0.05	**0.24**
AIH 11	**0.39**	**0.39**	**3.01**
AIH 12	**0.48**	**0.56**	**1.36**
HCV 1	0.02	0.01	**0.97**
HCV 2	**0.11**	**0.20**	**2.10**
HCV 3	**0.26**	0.08	**0.95**
HCV 4	**0.27**	**0.10**	**1.10**
HCV 5	**0.53**	**0.24**	**1.20**

[a] Twelve patients with AIH2 and five with chronic HCV infection. Values denote absorbance values obtained at 490 nm. Sera were considered positive when absorbance was ≥0.11 (in bold).

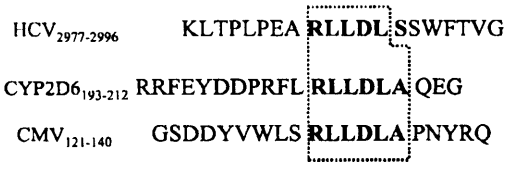

FIGURE 3. Amino acid sequence homology between the HCV$_{2977-2996}$, CMV$_{121-140}$, and CYP2D6$_{193-212}$. Amino acids are in standard single letter code. Identical residues between CYP2D6$_{193-212}$ and the homologous viral peptides are within the box.

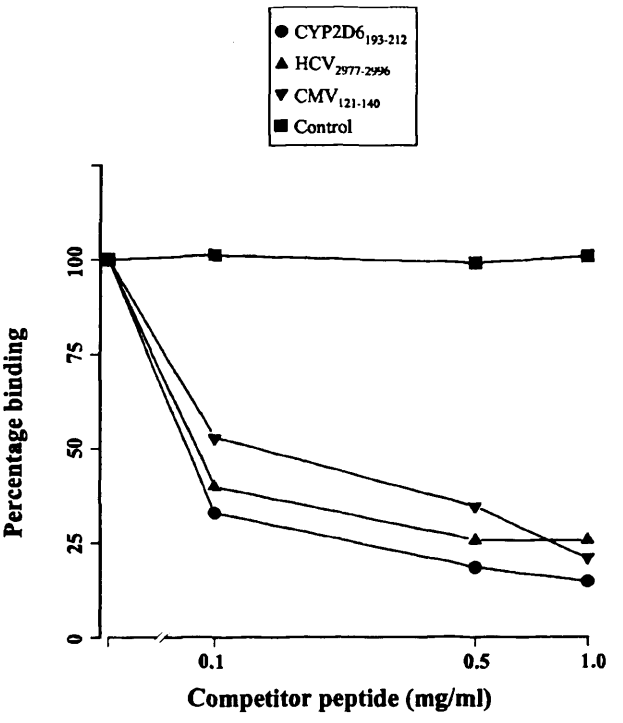

FIGURE 4. Inhibition of Ab binding to CYP2D6$_{193-212}$ after incubation with increasing amounts of CYP2D6$_{193-212}$ (●), HCV$_{2977-2996}$ (▲), CMV$_{121-140}$ (▼), and control peptide (■). Percentage binding is on the *y*-axis. Decrease in binding of >75% is seen after preincubation with 1 mg/ml of self and homologous viral peptides, but not with the control peptide.

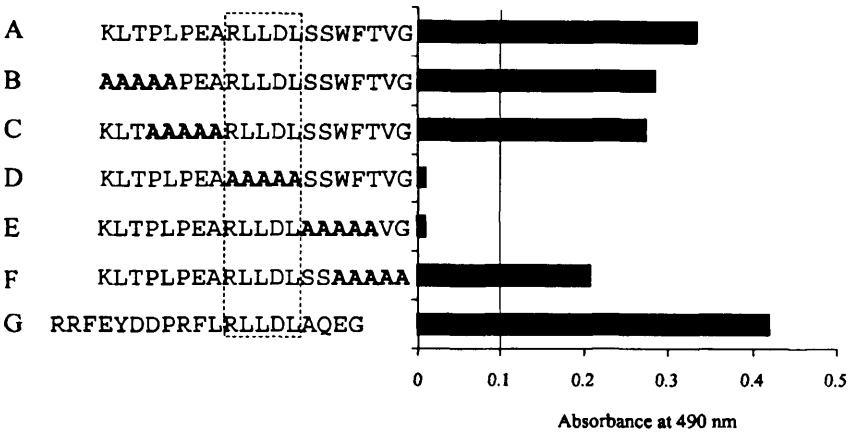

FIGURE 5. Reactivity of LKM1-positive serum to peptide $HCV_{2977-2996}$and its alanine-substituted homologues. *A*, $HCV_{2977-2996}$. *B–F*, Substitution of $HCV_{2977-2996}$ with five contiguous alanine residues across the full length of the native peptide. Amino acids are represented in standard single letter code. Alanine-substituted regions are indicated in bold. The region of identity between $CYP2D6_{193-212}$ and viral peptides is boxed. *G*, $CYP2D6_{193-212}$. The previously established cutoff for a positive reaction is indicated by a line in the *x*-axis. Reactivity to $HCV_{2977-2996}$ is abolished by alanine substitution of the region of homology and the contiguous downstream five residues.

and $CMV_{121-140}$. Triple reactivity to $CYP2D6_{193-212}$/$HCV_{2977-2996}$/$CMV_{121-140}$ was observed in five AIH2 and three LKM1/HCV patients (Table II). Ab binding to $CYP2D6_{193-212}$ was inhibited by addition of increasing amounts of competitor peptide. In the presence of 1 mg/ml of competitor, absorbance was reduced by 70–90%, whereas it remained virtually unaltered by addition of the scrambled control peptide (Fig. 4).

Alanine substitution of the $HCV_{2977-2996}$ region $_{2985}$RLLDL completely abolished reactivity by LKM1 Abs, as did substitution of the contiguous downstream 5-aa segment. All other alanine-substituted peptides produced comparable serum reactivities to wild-type $HCV_{2977-2996}$ and to the homologous CYP2D6 immunodominant peptide $CYP2D6_{193-212}$ (Fig. 5).

Of eight patients reactive with $CMV_{121-140}$ whose serum was available for testing, four (one AIH2 and three LKM1/HCV) were positive for anti-CMV IgG. All 10 sera from LKM1/HCV patients were negative for rheumatoid factor IgM Abs.

Local homology modeling of CYP2D6 using the cytochrome P450 BM-3 crystal coordinates as a template predicts $CYP2D6_{193-212}$ to be located within a helical domain in a solvent-accessible surface region of the protein, compatible with Ab recognition on whole protein. Therefore, we tested the ability of LKM1-positive sera to recognize whole protein to confirm the physiological significance of the observed peptide reactivities. Two LKM1-positive sera, one from a patient with AIH and another from a patient with HCV infection, were tested for reactivity to recombinant CYP2D6, HCV-NS5, and CMV-UL98. A cutoff for recognition was established as the mean absorbance at 490 nm + 3 SD of 43 serum samples from healthy individuals (cutoff OD_{490} = 0.07) (24). Both LKM1-positive sera strongly recognized the recombinant proteins (Fig. 6). We then investigated the ability of these recombinant proteins to inhibit reactivity to the immunodominant CYP2D6 peptide, $CYP2D6_{193-212}$, by a liquid phase ELISA inhibition assay. Preincubation of LKM1-positive serum with HCV-NS5 and CMV-UL98 resulted in 60.5 and 61.2% inhibition of reactivity against $CYP2D6_{193-212}$ (Fig. 7), confirming that LKM1 Abs that recognize $CYP2D6_{193-212}$ are cross-reactive with the HCV and CMV peptide homologues.

To further test recognition of conformationally intact CYP2D6 by LKM1 serum and the LKM1 Ab species reactive to $CYP2D6_{193-212}$, we assessed the ability of LKM1-positive serum and affinity-purified LKM1 Abs specific for the $CYP2D6_{193-212}$ epitope to inhibit CYP2D6 enzymatic activity using a fluorometric assay. Both LKM-positive serum from a patient with AIH and from another with HCV infection inhibited CYP2D6 enzymatic activity, producing ~40% inhibition at a dilution of 1/225. Affinity-purified Ab specific for the cross-reactive immunodominant CYP2D6 epitope, $CYP2D6_{193-212}$, was also able to inhibit metabolic activity of CYP2D6, producing ~20% inhibition at a dilution of 1/225. This suggests that $CYP2D6_{193-212}$-specific Abs contribute ~50% of the inhibitory activity of LKM1 serum, demonstrating the preservation and antigenicity of this epitope within native

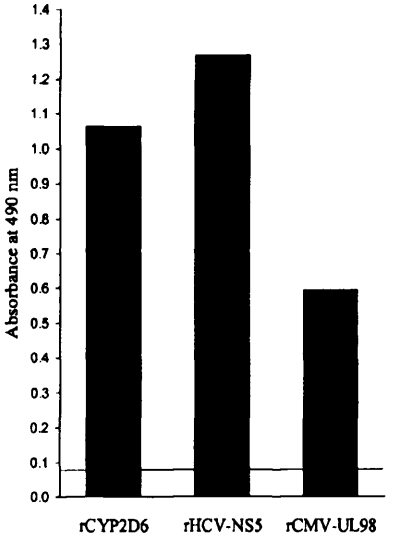

FIGURE 6. Reactivity of LKM1-positive serum to recombinant CYP2D6 (rCYP2D6), HCV-NS5 protein (rHCV-NS5), and CMV-UL98 protein (rCMV-UL98) by ELISA. A cutoff for positive reaction was determined as OD_{490} = 0.07 using serum from healthy individuals and is represented as a horizontal line.

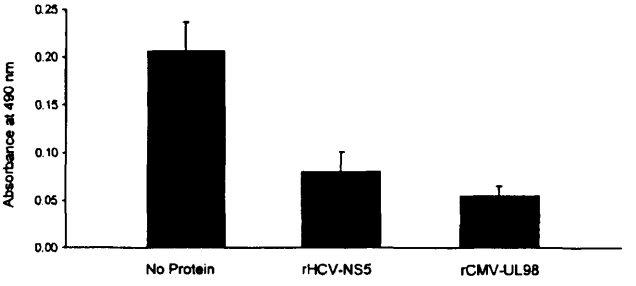

FIGURE 7. Inhibition of Ab reactivity of LKM1-positive serum to $CYP2D6_{193-212}$ by preincubation with 0.3 μg/ml of rHCV-NS5 and rCMV-UL98 or in the absence of competitor protein. Residual Ab reactivity to $CYP2D6_{193-212}$ was detected by ELISA. Error bars represent + SD of six separate experiments.

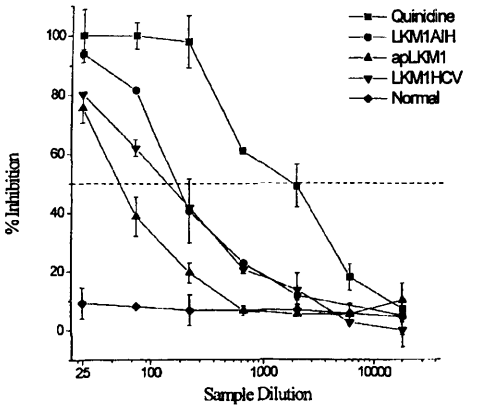

FIGURE 8. Inhibition of the metabolic activity of CYP2D6 by LKM1-positive serum. AMMC was used as substrate and formation of the fluorescent metabolite 3-[2-(*N,N*-diethylamino)ethyl]-7-hydroxy-4-methylcoumarin hydrochloride detected using excitation and emission parameters of 390 nm and 460 nm, respectively. Quinidine was used as a standard positive control (starting concentration = 0.5 μM) and serum from a healthy individual used as a negative control (Normal). In addition to sera from patients with LKM1-positive AIH (LKM1AIH) and LKM1-positive HCV infection (LKM1HCV), affinity-purified Ab (against CYP2D6$_{193-212}$) from an LKM1-positive patient was tested for inhibitory activity (apLKM1). Data are represented as percentage decrease in metabolite formation relative to no test sample/inhibitor. Fifty percent inhibition is indicated as a dashed horizontal line. Error bars represent ± SD of duplicate samples; where no error bars are shown both samples gave identical readings.

CYP2D6 (Fig. 8). In contrast, serum from a healthy individual failed to inhibit enzymatic activity.

Discussion

In this study of complete B cell epitope mapping, we found a new immunodominant epitope on cytochrome P4502D6, CYP2D6$_{193-212}$, recognized by LKM1-positive serum both from patients with AIH2 and chronic HCV infection, which is likely to be the target of virus/self cross-reactive immunity. The fact that reactivity to this epitope has gone unnoticed to date is probably due to the selective epitope construction used in the past to map antigenic sites on the cytochrome. Searching for LKM1 epitopes, Gueguen et al. (10) produced 10 CYP2D6 constructs through the expression of CYP2D6 cDNA or its rat orthologous fragments in a prokaryotic system. Through the analysis of the results of Ab binding to these constructs, they inferred that a region of 33 aa (239–273) was the major antigenic site in AIH, being recognized by all LKM1 AIH sera tested. Reactivity in this region was dissected further by the use of three synthetic peptides (241–260, 254–271, and 264–281), and the sequence 254–271 was found to be the focus of recognition. Although describing CYP2D6$_{254-271}$ as a major epitope on CYP2D6, the authors acknowledged that their strategy might have overlooked other epitopes. Based on Gueguen et al.'s (10) and Yamamoto et al.'s additional data (12) with new CYP2D6 constructs and using information derived from algorithmic prediction of antigenic sites (25), Yamamoto et al. (12) constructed 12 peptides spanning the region 241–429. Epitope 257–269, 5 aa shorter than the epitope described by Gueguen et al. (10), was recognized by 14 of 15 sera from patients with AIH2, confirming that this region is a major antigenic site. They also found that the sequences 321–351, 373–389, and 410–429 were recognized by eight, one, and two of the 15 sera, respectively. Akin to the previous study, however, a selective approach was used, with the possibility of failing to detect other reactive epitopes. In contrast, in the present study all potential epitopes were investigated systematically using peptides spanning the entire length of the CYP2D6 protein. With this approach, we were able to confirm both existence and recognition frequency of the previously described epitopes. In agreement with Yamamoto et al. (12), we found that CYP2D6$_{253-272}$, encompassing the hitherto considered main epitope (10), was recognized by 85% of our patients with LKM1 AIH and by only 1 of 10 LKM1-positive HCV-infected patients.

Of the four new epitopes identified in the present study (CYP2D6$_{193-212}$, CYP2D6$_{238-257}$, CYP2D6$_{268-287}$, and CYP2D6$_{478-497}$), CYP2D6$_{193-212}$ is immunodominant. Hints were already present in the literature, indicating the antigenic importance of this CYP2D6 region. After the previous finding of Yamamoto et al. (13) that LKM1 sera from HCV-infected patients react preferentially with a region comprised within amino acids 208–349 of CYP2D6, Parez et al. (14) constructed a series of peptides selected on the basis of their high probability of being antigenic sites, as predicted by computer analysis. They found that peptide 200–214 contained an epitope recognized by LKM1 in HCV infection, but not in AIH. On the basis of this finding and in the apparent unawareness of our initial reports describing the new epitopes (26, 27), Klein et al. (15) constructed a peptide 4 aa longer than that of Parez et al. (14) at both the N and C ends (CYP2D6$_{196-218}$) and obtained surprising results. In contrast with Parez et al. (14), they found that only 18% of LKM1-positive HCV-infected patients, but 68% of LKM1-positive AIH patients, reacted with the new peptide.

The methodological strategy used in the present paper has enabled us to clarify the antigenic characteristics of the region 193–218 on CYP2D6 and to explain, at least in part, these contrasting results (Fig. 1*B*). We demonstrate that the "AIH epitope" resides within the 193–212 sequence, with arginine, arginine, and phenylalanine at positions 193–195 conferring virtually universal recognition. Sequences after 212 do not appear to have antigenic properties, as suggested by the fact that our own peptide 208–227 is ignored by both AIH and HCV LKM1-positive sera. This also indicates that the "HCV epitope" is contained in the preceding peptide spanning amino acids 193–212, recognized by 50% of our HCV-positive patients. Though it may be argued that the 3-aa difference at the N terminus could explain this higher reactivity frequency among our HCV-positive patients when compared with an 18% frequency described by Klein et al. (15), this contention is not supported by the data of Parez et al. (14), who found a frequency similar to ours using peptide 200–214, not containing amino acids 193–199. Because the three studies have used assays of similar sensitivity, methodological differences are unlikely to explain these discrepancies. It is possible that the autoantibody concentration was lower in the patients studied by Klein et al. (15), who defined their LKM1 sera as exceeding a 1/80 dilution without providing the exact titer. Our data show that the epitope recognized in HCV infection is not present beyond amino acid 208, because CYP2D6$_{208-227}$ was not recognized by any LKM1/HCV-positive sera (28).

Interestingly, the dominant CYP2D6$_{193-212}$ epitope found in the present study shares similarities with HCV and CMV. Modeling studies locate the immunodominant CYP2D6$_{193-212}$ peptide to the surface of CYP2D6, making it accessible to Ab recognition in the context of the physiologically folded protein (21, 22). Similarly, the homologous HCV$_{2977-2996}$ peptide, part of the HCV RNA-dependent DNA polymerase (NS5), lies within a solvent-accessible loop structure of the "thumb" domain of this protein, allowing it to serve as an epitope on the native folded protein (23). No crystal coordinates are available for the CMV alkaline exonuclease, which contains the region CMV$_{121-140}$ homologous to

$CYP2D6_{193-212}$, but preliminary secondary structure analysis suggests it to be solvent accessible.

We have experimentally demonstrated these homologous self and viral sequences to be targets of cross-reactive virus/self Abs, suggesting the involvement of a molecular mimicry mechanism (29), where the physiological immune response to a pathogen targets sequence-sharing self Ags. This mechanism was invoked by Manns et al. (11) to explain the production of LKM1 in HCV infection, after having noted that $CYP2D6_{263-271}$ and the HCV polyprotein $HCV_{2772-2780}$ share sequence similarities. This hypothesis, however, was not tested in studies of cross-inhibition. We demonstrate that serum from LKM1-positive patients with AIH or HCV infection react concurrently with $CYP2D6_{193-212}$, $HCV_{2977-2996}$, and $CMV_{121-140}$. Multiple reactivity is due to an Ab capable of recognizing both virus and self, as demonstrated by inhibition studies. Furthermore, we confirm by alanine substitution of the homologous HCV epitope $HCV_{2977\ 2996}$ that Ab reactivity to this sequence maps to the region with homology to $CYP2D6_{193\ 212}$ and $CMV_{121\ 140}$. Finally, we show by ELISA and a CYP2D6 metabolic assay that $CYP2D6_{193\ 212}$-specific Abs recognize and inhibit the activity of the native enzyme and that the observed peptide cross-reactivities among CYP2D6, HCV-NS5, and CMV-UL98 also operate on the whole Ags when tested in a competition assay.

The cross-reactive Abs detected are of the IgG isotype, implicating T cell "help" in their generation. Because T and B cell epitopes frequently overlap, it is possible that the cross-reactive humoral epitopes identified may also serve as T cell epitopes (29). Thus, the emergence of LKM1 may be the ultimate manifestation of molecular mimicry at the level of the T cell, giving rise to a T-dependent and class-switched cross-reactive humoral response (30).

In conclusion, we found a new immunodominant epitope of LKM on CYP2D6, reactivity to which is likely to arise as a consequence of its similarity with viral Ags. Multiple exposure to viral sequences shared with self may lead to the break of immunological tolerance through a multihit mechanism in predisposed individuals.

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