Immunological Aspects of Liver Disease

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Introduction

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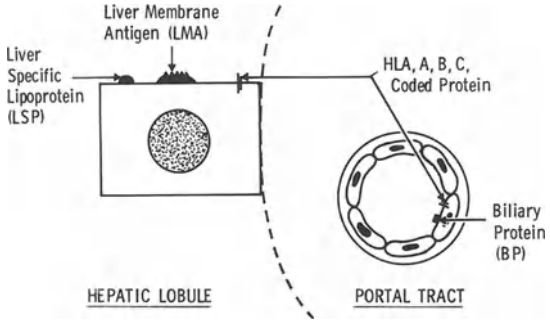
In normal subjects the regulatory apparatus of the immune system permits responses to foreign antigens but suppresses those directed to "self" components. Autoimmune disease occurs as a failure of this system either as a result of a primary defect in the regulatory apparatus (primary autoimmunization) or because of a change in the antigenicity of the tissues (secondary autoimmunization).

Autoaggressive reactions are characterised by the presence of autoantibodies. When these are directed to membrane displayed antigens (Fig. 1) they are probably of importance in the lysis of hepatocytes. Those directed to cytoplasmic antigens may be useful diagnostically but are of unknown pathogenic significance. When no extrinsic aetiological factor can be identified, the process is assumed to be the result of a failure of the regulatory system, allowing the spontaneous expansion of a clone of autoreactive lymphocytes. The defect may be generalised or specific to certain groups of self-antigens and thus the autoimmune disease may be either multi- or unisystemic. The recent development of techniques to enumerate and measure the functional activity of the suppressor lymphocytes which control the effector limbs of the immune system has enabled investigators to test whether the various purported autoimmune diseases do have as their basis a generalised defect in immunoregulation. Assessment of antigen-specific immunoregulatory function is, however, not yet readily available.



Sensitisation to
LMA : Lupoid CAH (Primary autoimmune)
LSP : Lupoid CAH, HBV CAH, Alcohol & drug hepatitis (Primary and secondary autoimmune)
BP : PBC and sclerosing cholangitis (Primary and ? secondary autoimmune)

Fig. 1. Hepatic membrane antigens

Of the various types of chronic liver disease, lupoid chronic active hepatitis and primary biliary cirrhosis are possibly of the primary autoimmune type. The target antigens and effector and general regulatory systems have been investigated in chronic active hepatitis and are discussed in this treatise. The disease exhibits strong linkage disequilibrium with an HLA-B locus antigen (B8), suggesting a genetic basis for the immunoregulatory defect demonstrated in these patients. In addition, the fact that the disease predominantly occurs in women raises the possibility that hormonal or other X-chromosome linked factors might also contribute to the pathogenic process. In the case of primary biliary cirrhosis, we are at the earlier stage of defining the specificity of the autoimmune response and discussing possible pathogenic mechanisms.

The chapter on orthotopic liver transplantation is a general review which includes an account of hepatic rejection and allows a comparison between this pathological process and the autoimmune diseases chronic active hepatitis and primary biliary cirrhosis. It is of interest that the rejection process primarily involves the biliary duct epithelium, sparing the hepatocytes. This selective attack is readily understood when we examine the distribution of the antigenic products of the histocompatibility complex in the human liver. We observe that biliary epithelium is rich in HLA-A, B and C loci coded proteins whereas the hepatocyte membrane is virtually devoid of these proteins (Fig. 2). Since the histocompatibility antigens are the major target in rejection, the biliary focus of the process is readily understood.

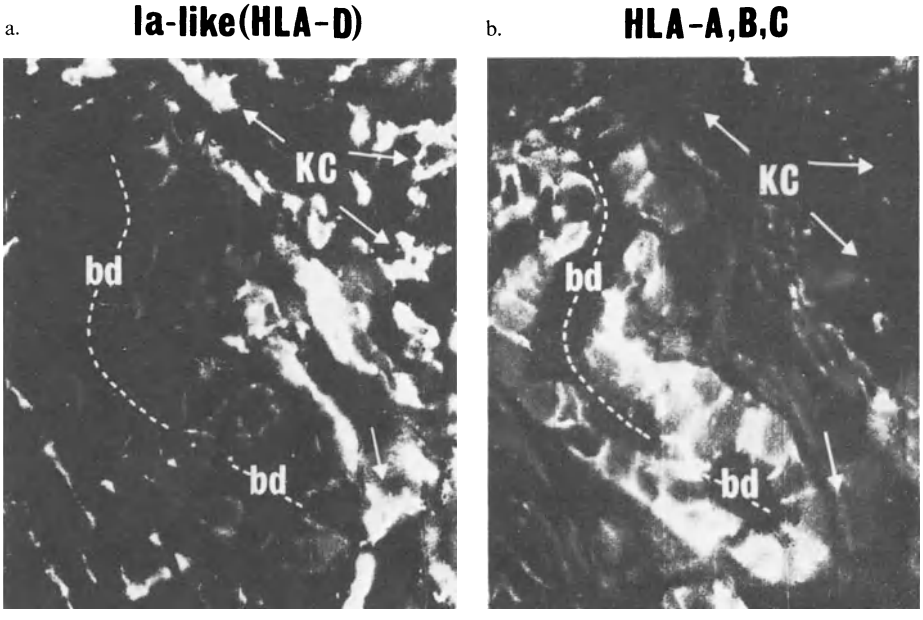


Fig. 2. Normal liver stained by immunofluorescence for 1a-like (HLA-D) and HLA-A, B and C antigens. Note that the 1a antigens are displayed on the Kupffer cells (KC) but not on the hepatocytes or bile duct (bd) epithelial cells. In contrast, HL-A, B and C antigens are present in highest density on the bile duct epithelial cells; they are present to a lesser degree on the Kupffer cells and were not visualised on the hepatocytes. (Thomas, Shipton, and Montano 1982)

The relevance of these observations to the pathogenesis of primary biliary cirrhosis is discussed.

A second group of diseases are initiated by easily identifiable extrinsic factors but have a significant "autoimmune" component. In these patients a normal immunoregulatory system is presumed and the autoimmune response is taken to be the result of an alteration in tissue antigens. The duration of the autoaggressive response is dependent on the continued presence of the trigger factor. Once this factor is removed the response ceases. Chronic viral hepatitis and drug or alcohol induced hepatitis are examples of this process. In these patients the virus, drug or toxin (or their metabolites) probably result in a change in the antigenicity of the hepatocyte membrane antigens and an immune response ensues. The magnitude of this response will be determined by genetic and additional environmental factors, and this will modify the clinical manifestations of the infective or toxic process. This is particularly well illustrated by the variety of syndromes following hepatitis B virus infection. These include acute hepatitis with recovery, fulminant hepatitis or subacute hepatic necrosis with possible death within 3 months, chronic active hepatitis often resulting in cirrhosis, chronic persistent hepatitis and a carrier state with minimal hepatitis. In addition, various extrahepatic lesions, including polyarteritis nodosum, membranoproliferative glomerulonephritis, essential mixed cryoglobulinaemia, papular acrodermatitis (Gianotti disease) and polyneuritis, have been associated with chronic HBV infection but an aetiopathogenic relationship is less clear.

The virus itself is not directly cytopathic and the diversity of lesions described in infected patients has been attributed to variation in the capacity of the host's immune response to eliminate or suppress the infective agent. The occurrence of rapidly progressive hepatitis in agammaglobulinaemic subjects has suggested a major role for non-antibody dependent effector systems in the lysis of infected hepatocytes, and direct studies of the composition of the inflammatory infiltrate suggest a significant involvement of T-lymphocytes. The recognition of virus infected cells by T-lymphocytes has been shown to be dependent on the association of membrane displayed viral antigens with products of the major histocompatibility complex. It is of interest that HLA-A, B and C loci coded proteins are present in low concentrations on hepatocytes, and this may influence the effectiveness of T-lymphocyte mediated cytolysis of infected hepatocytes.

The elimination of the hepatitis B virus in acute hepatitis is presumably dependent on the lysis of infected hepatocytes and neutralisation and phagocytosis of extracellular virus particles. The immunopathology of this process is now well described but our knowledge of (a) the relative importance of the different viral antigens in directing the immune system to the infected hepatocyte, (b) the role of the various cytolytic effector systems and (c) the effect of the modulatory serum and hepatocyte derived lipoproteins on these systems, is still incomplete.

The major enigma in this field is the mechanism of viral persistence and why chronically infected subjects develop either chronic active hepatitis, chronic persistent hepatitis or one of the extrahepatic syndromes. The relative roles of virological and immunological factors in these two areas are still unclear. The majority of Caucasian patients with chronic active or persistent hepatitis are HBe antigen and HBV-DNA polymerase positive, evidence of active viral

replication, whereas carriers with normal histology or minimal hepatitis usually have HBs antigenaemia in the absence of detectable viral replication. The association of viral replication with membrane display of HBs antigen (Fig. 3), and possibly of other viral antigens, may explain the inflammatory activity in these patients. In contrast, carriers with HBs antigenaemia in the absence of HBV particles in the blood exhibit predominantly cytoplasmic HBs antigen with no membrane display: these patients do not usually develop inflammatory liver disease and may continue to produce HBs antigen because of the presence of integrated HBV-DNA. The type of inflammatory liver disease or extrahepatic disease occurring in patients with chronic infection appears also to be related to the type of immune response mounted by the host. A dominant cell-mediated response ("tuberculoid") results in chronic active hepatitis while a dominant humoral response ("lepromatoid") results in extrahepatic disease (PAN or proliferative GN). Although these factors appear to modify the degree of inflammatory response to this chronic infection, the mechanism underlying the persistence of the virus is still not understood.

Finally, the association of HBV infection with development of primary liver cell cancer is well established at the epidemiological, clinical and histopathological levels. However, whether the virus is oncogenic because of its capacity to integrate its DNA into the host genome and the role of co-carcinogens are still unknown.

The study of HBV infection is of major theoretical importance as an example of persistent viral infection but is also of paramount clinical significance, there being approximately 200 million carriers of this virus in the world today. These patients run the risk of developing chronic liver disease or primary liver cancer. For these reasons further knowledge of the mechanisms of elimination of the virus in acute hepatitis and the reasons for persistent infection are urgently required to form a

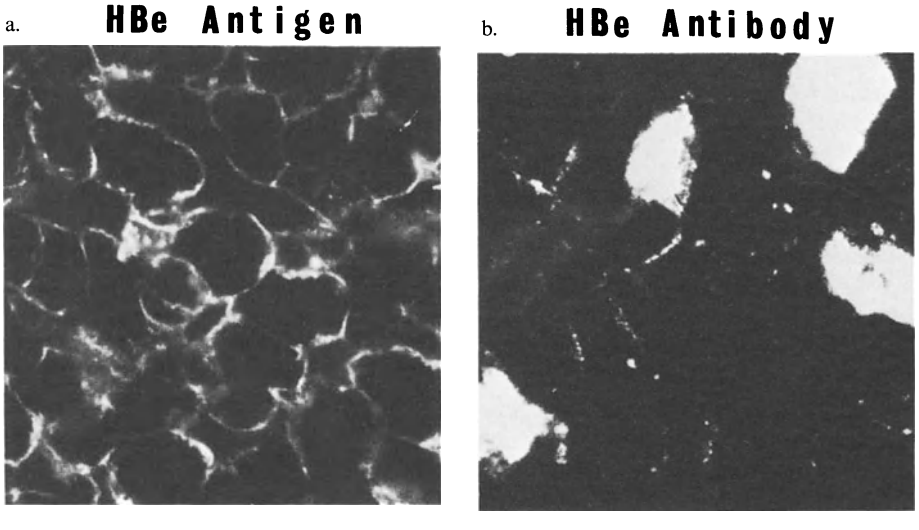


Fig. 3. HBV infected liver from **a** HBe antigen positive and **b** HBe antibody positive patients, stained by immunofluorescence for HBs antigen. Note the membranous or submembranous staining in **a** and the cytoplasmic staining in **b**. (Montano, Goodall and Thomas 1982)

basis for logical therapy. The development of vaccines capable of preventing infection carries with it the hope of preventing not only one common type of chronic liver disease but also a major proportion of the world's primary liver cancers.

Drug and alcohol induced liver disease are examples of the interaction of environmental hepatotoxins with a host exhibiting variability in both its metabolic and immunological response to that toxin. In both cases there is now evidence to support this hypothesis, there being a host immune response to altered membrane antigens.

The diseases used to illustrate primary and secondary autoimmune reactions in chronic liver disease are the best examples available at the present time. Nevertheless, it should be noted that only when all trigger factors have been identified and removed and the inflammatory disease process still continues, can one suspect the presence of a primary defect in the regulation of the immune response. Since one can never be certain that all trigger factors have been excluded, the identification of primary autoimmune disease states awaits the identification of the target antigens involved in the various disease states and the development of techniques to measure the regulation of the immune response to these specific antigens. The genetic and environmental factors controlling antigen recognition and the level of activity of the effector limbs of the immune system must then be determined. Until we reach this stage of understanding, we must content ourselves with a classification dependent on the known aetiological factors (Table 1), presuming that a group with a primary defect in immunoregulation will ultimately be established. The identification of such antigen-specific defects and the purification of soluble factors produced by suppressor T-cells and capable of conferring antigen-specific non-responsiveness on the immune system will herald a new era in the therapeutics of these diseases.

Table 1. Aetiological classification of chronic active hepatitis

Primary autoimmune (lupoid)[a]*:*	
Antinuclear factor (ds DNA)	
Smooth muscle antibody (actin)	
Liver/kidney microsomal antibody	
Mitochondrial antibody	
Secondary autoimmune:	
Viruses:	Hepatitis B
	Hepatitis NANB
Drugs:	Oxyphenisatin
	Methyldopa
	Nitrofurantoin
	Isoniazid
Alcohol:	

N. B. The role of auto-immunity in the pathogenesis of the chronic active hepatitis of Wilson's disease is unknown.

[a] This group has been subdivided on the basis of the specificity of the autoantibody. Whether these represent separate sub-groups resulting from different aetiological processes, is unknown.

Genetic Determinants of Autoimmune Chronic Active Hepatitis

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> "Of all that I have said, the one thing that I should like to be remembered is my conviction that genetics and immunology are two sciences that in the immediate future are likely to be linked in a productive attack on some of the most fundamental problems of biology".
>
> F. M. Burnet, 1948 [4]

Introduction

There has been a remarkable vindication over the past 15 years of Burnet's forecast, with two particular themes of dominant interest to research on immunogenetics. The first is the association in inbred animals between genetic control of immune responses to antigens of limited heterogeneity and genes (Ir genes) of the major histocompatibility complex (MHC) [2, 39]. The second is the increasing refinement of typing methods for human histocompatibility antigens, of relevance to tissue transplantation, and associations between certain immunopathic diseases and HLA specificities [26, 31]. However, despite much experimental work and theoretical speculation, no unifying concept relating MHC-linked Ir genes effects and MHC-linked disease susceptibility has yet emerged.

The more striking HLA associations with immunopathic diseases in Caucasians have involved a limited number of antigens of the HLA-B and D series, notably HLA-B27 with ankylosing spondylitis and some clinically related spinal arthropathies Cl: HLA-B8 and DR3 which are associated particularly but not exclusively with organ-specific autoimmune diseases and include insulin-dependent diabetes mellitus, myasthenia gravis, Sjögren's disease, chronic active hepatitis (CAH), systemic lupes erythematosus (SLE), and gluten-sensitive coeliac disease, HLA-B7

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and DR2 with multiple sclerosis; and HLA-DR4 with rheumatoid arthritis. The various immunopathic diseases associated with HLA specificities differ so greatly in aetiology and clinical expression that no single explanation can be readily invoked to explain the associations. It is to be noted that the HLA gene influence generally accounts for only a small part of the "risk" for the particular disease associated, so that consideration must be given to other genetic determinants, acting independently of, or interactively with MHC alleles, together with environmental risks including infection which may interact with genetic determinants. Finally, the "weight" which can be attributed to any genetic or environmental determinant of a disease will depend on the diagnostic homogeneity of the disease entity under consideration, a point particularly applicable to CAH.

Autoimmune CAH as a Disease Entity

CAH first became recognized as a disease entity after 1950 [19]. The associated hyperglobulinaemia was an early lead to an immunological contribution to pathogenesis, and this was strengthened by recognition of various disease-associated non-organ specific autoantibodies. Most of the descriptions of the disease involved females derived from populations of Northern Caucasian origin, hinting at racial-genetic factors in pathogenesis. Whilst CAH seemed to cover a spectrum of diseases, referred to collectively as "chronic active liver disease" [11], the classical or prototype form with immunoserological abnormalities was used as the basis for definition by the Fogarty-IASL nomenclature meeting in 1976 [17].

Histopathologists have had a strong influence on definition and classification of CAH [6, 16]. In 1968 an International Group [6] divided chronic hepatitis into two histological types specified as chronic aggressive hepatitis on the basis of progressive parenchymal destruction, and chronic persistent hepatitis on the basis of continuing portal and/or intralobular inflammation without destruction. It has since become evident that these two morphological entities may both have different pathogenetic components.

One clear and generally accepted differentiating characteristic is positivity for the hepatitis B surface antigen (HBsAg), allowing recognition of HBsAg positive CAH attributable to chronic viral infection of the liver, and HBsAg negative CAH which includes the archetypal form of CAH: this in fact is as far as many writers care to go. However, there are other examples of histologically-definable chronic active/aggressive hepatitis in which causal factors are identifiable , namely hypersensitivity reactions to certain drugs, alcohol abuse occasionally, and copper overload as in Wilson's disease. Apart from these, there exist the prototype, lupoid hepatitis [22], characterized by a predominant occurrence among young females, hyperglobulinaemia, and non-organ specific autoantibodies, to nuclei (ANA) and smooth muscle (ASMA), here referred to as autoimmune CAH, and a group neither positive for HBsAg nor autoantibodies, referred to as cryptogenic CAH. Still lacking, however, is an unequivocal set of descriptors for autoimmune CAH, particularly because there are no accepted specifications for the titre and profile of non-specific autoantibodies which indeed may vary during the course of the disease. Moreover a definitive disease-associated liver-specific autoantibody is yet re-

cognized, although there is "candidate status" for antibody to various liver-membrane antigens [27, 29]. Finally whilst there is an overall polarity between HBsAg positive CAH and autoantibody positive CAH, this is not absolute, as minor overlap occurs. The relative percentage frequency from a European centre [28] of four possible groupings among 58 cases of CAH are (a) autoimmune CAH, autoantibody positive, HBsAg negative, 43%; (b) hepatitis B virus (HBV)-associated CAH, autoantibody negative, HBsAg positive, 36%; (c) mixed, autoantibody positive and HBsAg positive, 7%; and (d) cryptogenic, negative for both markers, 23%. Subsequent discussion of genetic determinants will be confined to CAH of the autoimmune type.

Genetic Determinants of Autoimmune CAH

Identifiable genetic influences and markers in autoimmune CAH include (a) those associated with female sex, (b) those associated with genes of the MHC and expressed as HLA antigens, (c) those associated with immunoglobulin V genes, and (d) those associated with genetic control over immunoregulation.

Female Sex

All case studies on autoimmune CAH emphasize the bias towards females in this disease, with female to male ratios of up to 6:1 [18]. The disease occurs most frequently, and most typically, in females aged 10–30 years, and there is a second peak of incidence among females aged 50–70 years [18]. Thus autoimmune CAH has a female sex predilection equivalent to that of other autoimmune diseases, and this can be taken with evidence in man and other species for augmented humoral immune responsiveness in female [24] including higher immunoglobulin levels, greater immunological responses to antigenic challenge, and less stable tolerogenesis as judged by higher background levels of various autoantibodies [15]. It is uncertain whether these various effects are determined predominantly by an immunoregulatory gene on the X chromosome [3], or by oestrogenic or other sex hormones acting upon one or another component of the immune response [42].

HLA Antigens

B locus Antigens. In 1972 we reported [21] a strong association between HLA-A1 (60 %) and HLA-B8 (68%) among 37 patients with chronic active hepatitis (type unspecified, but mostly autoimmune). The significantly increased frequencies of these antigens compared with frequencies in "other liver diseases" and/or healthy controls was corroborated in Europe and the United States of America [20]; the primary association was with HLA-B8 which is in linkage disequilibrium with HLA-A1. The Melbourne study was later extended to include 48 cases, all specified as autoimmune type, and 69% carried HLA-B8 [32]. In West Germany, Freudenberg et al. [9] reported on 108 adult cases fulfilling criteria for autoimmune hepatitis and found a frequency of HLA-B8 of 82% and, relating this to the frequency of B8 among 5046 controls (19%), derived a relative risk for HLA-B8 of 15.4.

Eddleston and Williams [7] have drawn attention to the frequency of other HLA specificities in autoimmune CAH, particularly HLA-B12. The decrease in frequency observed in this antigen might in part be explained by the increase in HLA-B8 but it is of interest that there is a progressive decrease in frequency of HLA-B12 according to time from diagnosis, implying that HLA-B12 is associated with prejudiced survival, and an increase of HLA-B7 in longer-surviving cases was also demonstrated. This implies that there are MHC-linked genes which appear to be associated with disease susceptibility and other MHC-linked genes which appear to affect the outcome of disease. Thus at least two MHC-linked genes play a role in the pathogenesis of CAH.

D Locus Antigens. In a report on D locus antigens, Opelz et al. [34] in the United States described 38 patients with "chronic active liver disease" of whom 32 appeared to have autoimmune-type disease. Whilst there was only a minor increase in HLA-B8 among these cases, the frequency of DW3 (68%) was greatly increased over that among 91 controls, (24%), and it was assumed that the primary HLA association in CAH was with DW3 rather than B8.

DR Locus Antigens. DR antigens in CAH were first examined in the Seventh (1977) International Histocompatibility Workshop, with cases (mostly of autoimmune type) contributed from Melbourne and London. The frequency of WiA3, later specified as DRW3, (now DR3), was significantly increased [13]. Moreover there were weakly significant associations between HLA-B8, DRW3, and autoantibodies, including ANA and ASMA. Subsequently DR locus typing on 48 cases of autoimmune CAH was reported by Mackay and Tait [23], and Table 1 shows the

Table 1. Frequency and percentage ( ) of HLA-DR antigens, HLA-A1 and HLA-B8 in CAH-Type A, liver disease controls and normal controls (from Mackey and Tait [23])

	CAH-A n=43	Liver disease controls n=35	Normal controls 119
DR1	9 (20.9)	7 (20.0)	25 (21.0)
DR2	6 (14.0)	15 (42.9)	31 (26.1)
DR3	32 (74.4)	11 (31.4)	38 (31.9)
DR4	13 (30.2)	9 (25.7)	34 (28.6)
DR5	7 (7.0)	10 (28.6)	26 (21.9)
DRW6	5 (11.6)	6 (17.1)	23 (19.3)
DR7	7 (16.3)	8 (22.9)	25 (21.0)
HLA-A1	ab $\frac{41}{56}$(73.2)	a $\frac{13}{35}$(37.1)	b $\frac{188}{586}$(32.0)
HLA-B8	ab $\frac{41}{56}$(73.2)	a $\frac{11}{35}$(31.4)	b $\frac{155}{586}$(26.5)

P values (χ^2 with Yates' correction)

a CAH-A versus liver disease controls: B8 and DR3 ($P<0.0005$), A1 ($P<0.005$), DR2 ($P<0.01$), DR5 ($P<0.05$)

b CAH-A versus normal controls: A1, B8 and DR3 ($P<0.0005$)

frequencies of seven DR antigens, together with frequencies for HLA-A1 and B8, in autoimmune CAH, other liver diseases, and normal controls reported in this study. There were highly significant differences for DR3 (increased) and DR2 (decreased). This study revealed a high degree of co-occurrence between HLA-B8 and DR3, with 31 of 33 cases positive for either carrying both B8 and DR3. Family studies showed that, in 14 patients positive for B8 and DR3, a haplotype containing B8 and DR3 was present in all 14 index cases, with the A1, B8, DR3 haplotype being present in 10. Thus is could be inferred that when B8 and DR3 coexist in CAH, they are present on the one chromosome, and that a "disease susceptibility gene(s)" exists in linkage disequilibrium with these alleles.

Studies on HLA and disease have bearing on problems related to heterogeneity within diseases. For example, HLA markers have facilitated the differentiation of types of diabetes mellitus, myasthenia gravis, juvenile rheumatoid arthritis, psoriasis, and other diseases. Equally, HLA markers have validated the differentiation of chronic active hepatitis into the type associated with autoimmune serological markers and the type associated with seropositvity for HBsAg in which HLA associations are either weak or lacking (vide infra); moreover the B8-DR3 increase is not seen among cases of cryptogenic CAH.

In two studies [32, 34] in which enquiry was directed as to whether the presence of HLA-B8 or DW3 per se identified a particular subtype of CAH, the B8 or DW3 positive groups included more females and younger cases, with greater evidence for immunological disturbance and dependence on prolonged corticosteroid drug therapy, but the differences were not definitive. However, from data reported at the Eighth Histocompatibility Workshop [41], HLA, B8-DR3 was most strongly associated (82%) with CAH in females aged less than 30 years with positive tests for "hepatitis related" autoantibodies, ANA and ASMA. Among older females with CAH and hepatitis-related autoantibodies, the B8, DR3 association was less striking, and among the small group with positive tests for antimitochondrial antibody (AMA) a disease association with HLA-B8 or DR3 was not evident [23].

CAH other than Autoimmune CAH. There are many studies on HLA antigens in HBsAg-associated CAH, with no striking associations [20, 35]. In some studies the frequency of HLA-B8 was decreased [20] and among Italian cases there were increases in HLA-A3 and BW35, with the phenotypic association of A3-BW35 being increased from 6% to 28.5% [25]. One study [46] on cases of CAH, of uncertain ethnic background and selected on histological criteria, reported an increase in DR4 but not DR3; criteria for case selection could account for this finding.

Geoepidemiology of CAH. This is of interest in that the autoimmune type of CAH has been reported mostly from among populations of Northern European origin, among which HLA-B8 exists in the highest frequency [37], and rarely among Southern European, African, and Asian populations, whereas HBsAg-associated CAH and high carrier rates of HBV predominate in the latter populations among whom HLA-B8 exists in lower frequency, or is lacking. Possibly there are types of HLA-B8 and DRW3-associated immune responses which, whilst carrying predisposition to autoimmunity, are associated with some degree of host defence against acute or chronic HBV infection. This would account for different types of

chronic active hepatitis among different populations, and also in part for contrasting incidences of primary hepatocellular cancer (PHC) in different types of cirrhosis and among populations with differing carrier rates of HBsAg, assuming that predisposition to the HBV, HBsAg carrier status and PHC are interrelated.

Explanation of HLA-B8, DR3 Effect. Any explanation of the association of HLA with disease must take into account the wide range of immunopathic disorders involved, and the antigens, extrinsic or intrinsic, of widely differing specificity with which such diseases may be related. First, for ankylosing spondylitis and related diseases, predominating in males and seemingly associated with "lacunar" immune deficiency, the molecular mimicry concept is attractive. Second, for other HLA-associated diseases, the existence of an "immune response" (immune-susceptibility) gene(s) in linkage disequilibrium with an HLA gene provides the basis for an explanation, if such gene(s) were to specify a cell-surface receptor (Ia) on antigen-presenting cells. Third, the varied character of diseases associated with HLA-B8, DR3, and the existence of defective immunoregulation in at least certain of these diseases, had led to the suggestion that the B8, DR3 specificities may be associated with a gene promoting a defect in the activity of suppressor T cells, resulting in a loss of control over immune responses [7]. Also of interest is the reported existence in autoimmune CAH of cytotoxic cells which effect lysis of avian erythrocytes coated with liver specific antigen, with maximal activity in subjects positive for HLA-B8 [43].

Immunoglobulin Gm Allotypes

In a study on autoimmune CAH, we enquired into possible associations with Gm allotypes [45]. Gm phenotypes were established by testing 50 patients and 180 controls for the allotypic markers Glm (f, z, a, x) and G3m (b0, b1, b3, b5, c3, s, t, g) by passive haemagglutination inhibition, and associations were sought between disease, sex, HLA type, and Gm type. In this study the statistical analysis of the association of HLA and Gm antigens with autoimmune CAH involved log-linear models fitted to multidimensional contingency tables, and procedures were developed to analyse the "three factor interaction" (disease risk affected by non-additive effects of HLA and Gm) and of each of the "two factor effects" (disease risk affected by HLA or by Gm).

It was found that females predominated 5 to 1, 40 of 50 patients (80%) were positive for HLA-B8 compared with 46 of 180 (26%) of healthy controls ($P < 0.001$, RR = 11.6), and 33 of the 39 patients typed (85%) were positive for DR3 compared with 38 of 119 (32%) healthy controls ($P < 0.001$, RR = 11.7). Moreover, there was an excess of patients with the Gm phenotypes a+x+b+, and analysis of the relationship of Gm phenotype to disease according to the presence or absence of HLA-B8 showed that the association of disease with Gma+x+b+ was confined to those patients who were positive for HLA-B8 (observed = 16, expected = 5.22); there was also a slight excess of patients with Gma+x+b− and HLA-B8 (observed = 2, expected = 1.09). Overall, the relative risk for the Gma+x+ haplotype was 2.3 with 95% confidence limits of 1.82–2.97. Gm phenotypes were dichotomised according to the presence or absence of the Gma+x+ haplotype, to examine possible non-additive effects of Gm and HLA-B8; 18 of the 50 patients (36%) had

Gma+x+, and this haplotype was detected in 18 of 40 (45%) of HLA-B8 positive patients compared with none of 10 without HLA-B8. In controls the frequency of Gma+x+ was 24% in those with HLA-B8 and 18% in those without HLA-B8. Statistical analysis showed that the effect of Gma+x+ on disease was significantly influenced by HLA-B8, and vice versa, i.e. Gma+x+ was only associated with disease when HLA-B8 was also present.

There is some precedent for the concept that the MHC and immunoglobulin allotype-linked genes can interact to cause CAH and other autoimmune diseases [45]. Thus it has been shown in animals that the immune response to certain antigens is determined by genes, referred to as Ir genes, segregating within the MHC, and by genes associated with immunoglobulin allotypes; correspondingly, at the cellular level, the immune response depends upon the association of antigen with cell membrane structures coded by MHC genes, and the recognition of antigen by lymphocyte receptors coded by immunoglobulin V genes. In man, we have shown that the immune response to a bacterial antigen flagellin is determined by interactive effects of Gm and HLA linked genes [44]. There is also evidence in other diseases that susceptibility is influenced by Gm as well as HLA-linked genes, notably autoimmune thyrotoxicosis [8] and myasthenia gravis [33] but an interactive effect between HLA and Gm has not been described for these diseases.

If predisposition to autoimmunity were to depend upon the interaction of immune response genes linked to the HLA-B and DR loci with immunoglobulin V region genes linked to the Gm allotype locus, the specificity of the interaction of HLA-B8 with Gma+x+ in CAH may reflect the specificity of recognition of separate determinants on the antigen molecules which are presumed to initiate the autoimmune process. In other words, products of genes linked to the HLA-B8, DR3 haplotype could associate on the surface of antigen-presenting cells with certain determinants of initiating antigen(s) in such a way as to facilitate an appropriate recognition of other antigenic determinants by products of Gma+x+ linked immunoglobulin V genes. The T helper factors may be the functional level at which Gm-linked gene products (V_H) associate with MHC-related products (Ia), since idiotypic cross-reactions between T cell factors and antibodies directed towards the same antigen have been described [1]. Thus the coexistence of Ia-associated and V_H-associated gene products in the one T cell factor in the mouse provide a model for explaining HLA and Gm interaction in humans. Moreover, with antibody diversity currently being explained in terms of a genetically-determined V gene repertoire modulated by gene rearrangement and somatic mutations, inheritance both of "disease-susceptibility" V genes as well as MHC genes would predispose to autoimmune diseases, although how the gene products might interact with an environmental agent is uncertain. We cannot identify any "single event" which sets the disease process of autoimmune CAH on course; the genetic determinants we have described could act by predisposing to a specific infectious agent, to a particular type of initial injury by an infectious or other environmental agent, or to on-going inflammation after various types of initial injury.

Genetic Defect of Immunoregulation

Although multiple cases of CAH within families are seldom reported, clinicians often draw attention to pedigrees wherein patients and family members suffer from

various non-hepatitic immune-mediated diseases including thyroiditis, thyrotoxicosis, glomerulonephritis, Sjögren's disease, fibrosing alveolitis, myasthenia gravis, and ulcerative colitis, or have autoantibody profiles associated with such diseases. There are two published studies, from England [10] and Finland [38] in which first-degree relatives of patients with CAH and primary biliary cirrhosis were tested for various autoantibodies, AMA, ASMA, ANA and microsomal antibodies of thyroid and gastric parietal cells. The increase of autoantibodies among relatives (Table 2) could be interpreted in terms of inherited overall instability of tolergenesis, or weakness of suppressor control, which would be unrelated to HLA inasmuch as the autoantibodies described are not directly dependent upon any HLA specificity, including HLA-B8. In two studies [5, 14], autoimmune CAH has been associated with defective induction by concanavalin A of suppressor T cells, but it has not yet been shown that this defect exists in relatives, as pertains in SLE [30]. Currently we are attempting to establish whether the reported familial aggregation of autoantibodies in immunopathic liver disease is of genetic rather than environmental origin, to investigate whether the trait is homogeneous, and to ascertain whether the trait is linked to HLA or immunoglobulin loci, or to other marker loci.

Predisposition to Liver Injury

The possibility can be canvassed of inherited predisposition of the liver to injury, taking the analogy of inherited target organ (thyroid) predisposition in the autoimmune thyroiditis of the obese chicken [36]. Moreover there is evidence in mice for differences among strains in susceptibility to toxic liver damage [12]. Current studies in our laboratory (unpublished) have shown clear differences in susceptibility to carbon tetrachloride between Balb/c and SJL mice, as judged by biochemical and histological indices of liver injury and repair. The degree to which genetic predisposition to liver injury in man might influence susceptibility to chronic hepatitis is unknown. It could be noted, incidentally, that whilst the risk of toxin-induced liver injury in man, as exemplified by alcoholic cirrhosis, may be influenced by the HLA antigen status, there is no consensus as to the particular HLA alleles involved.

Table 2. Antibodies in relatives of cases of CAH and PBC

Subjects	Positive results %					
	No.	AMA	SMA	ANA	THY	GPC
CAH relatives	a 165	3.6*	8.5*	7.9	27	6.1
	b 58	0	1.7	19*	23*	10*
PBC relatives	a 95	7.4*	11*	16*	27	13
	b 39	5	0	13	15	2.5
Controls	a 260	0.4	1.9	4.6	18	4.6
	b 504	0.6	1.4	3.0	14	2.2

a Galbraith et al. [10]
b Salaspuro et al. [38]

* $P<0.05$

Conclusions

As introduction, we emphasized the heuristic relationship between genetic control over immune response in animals and HLA associations with human diseases, and problems with diagnostic heterogeneity in evaluating genetic determinants of disease. The autoimmune type of chronic active hepatitis (CAH) was singled out for consideration. The female sex predisposition speaks for an effect of an immunoregulatory X-linked gene, or oestrogenic hormones, on immune responsiveness. There is a strong association with HLA-B8, the highest reported relative risk being 15.4, and for DW3 and DR3. In autoimmune CAH, these D locus antigens coexist with HLA-B8, and family studies have shown that B8, DR3 exists as a haplotype. The B8, DR3 association supports the segregation of autoimmune CAH as a disease entity and correlates with the geographical localization of this entity to populations of Northern European origin. The immunoglobulin allotype Gma+x+ is also associated with autoimmune CAH and, of great interest, there is a strong interactive effect between B8 and Gma+x+ in predisposition. In view of the presumed linkages in man between HLA and "immune response" genes, and between genes coding for constant and variable region sequences of Ig molecules, it is presumed that both MHC and V_H genes may interact at a functional level. Another possible immunogenetic determinant is an inherited defect in immunoregulation unrelated to the MHC, as reflected in studies on the immune status of relatives of patients with CAH.

Some observations and inferences on HLA associations are shown in Table 3. In regard to the HLA association, it is possible that there is an immunoregulatory

Table 3. Summary of arguments on HLA-B8, DW3 haplotype

Observation	Inference
A number of different immunopathic disorders are associated with HLA-B8, DW3, DR3	One (or more) immunoregulatory alleles are in linkage disequilibrium with HLA-B8, DW3 haplotype
Coeliac disease and other disorders tend to breed true in families and segregate with HLA-B8, DW3	At least one of modifying alleles may be linked to B8, DW3, DR3 haplotype
Diabetes mellitus of juvenile onset tends to be most concordant in HLA-identical siblings with HLA-DW3, 4	At least one modifying allel can be linked to DW4 haplotype
Autoimmune chronic active hepatitis shows:	
HLA-B8, DW3 association	See above
Effects related to B12	Suggests modifying genes on HLA-B12 haplotypes
Effects related to Gm	Suggests modifying genes on Gm haplotypes
Little familial aggregation	Suggests that there are several modifying loci

locus, mapping near HLA-B and D loci, with an allele in strong linkage disequilibrium with HLA-B8, which facilitates all of the aberrant responses to different antigens. On this view the antigenic specificity of the aberrant response in a particular individual would need to be explained in terms of ancillary modifying genes. These ancillary genes might also be carried on the HLA-B8, DW3 haplotype; if so, the same immunopathic disorder might be observed in any relative who happened to inherit the same HLA-B8, DW3 haplotype. On the other hand, if ancillary genes map within the MHC of the homologous chromosome, as is the case in juvenile onset diabetes mellitus [40], the same immunopathic disorder would only tend to be observed in siblings who happen to be HLA-identical with the index case. Alternatively, the ancillary modifying genes could be linked to immunoglobulin allotype loci or be otherwise unlinked to the MHC; this could give rise to the same disorder in relatives receiving the same set of modifying genes, and to different disorders (or no disorder at all) in relatives receiving different sets of modifying genes. Finally, the antigenic specificity of the various immunopathic disorders related to HLA-B8, DW3 may prove to be partly dependent on environmental differences, consistent with the view that in the course of the evolutionary separation of European populations, natural selection by environmental differences would have had important effects on the frequencies of the HLA-B8, DW3 haplotype in different parts of Europe and beyond.

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The Role of Liver Membrane Antigens as Targets in Autoimmune Type Liver Disease

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1. Introduction

Autoimmune type chronic active hepatitis is clinically characterized by the presence of non-organ-specific autoantibodies, high gammaglobulin levels, circulating liver membrane autoantibodies, and the absence of hepatitis B virus markers. Host immune reactions against liver membrane antigens are regarded to be of significant importance for hepatic injury. Organ-specific determinants are of special interest whereas non-organ-specific determinants are believed to account for extrahepatic symptoms in chronic inflammatory liver diseases. In the present paper a review is given of the published data concerning the identification of liver membrane antigens. Furthermore the present knowledge of immune reactions against these target antigens will be reported and their clinical significance will be discussed.

2. Characterization of Liver Membrane Antigens

2.1. The Liver Specific Protein (LSP)

The liver specific protein (LSP) is part of a macrolipoprotein complex and can be detected in the first peak of Sephadex G 200 chromatography of 100,000 *g* supernatants of fresh human liver homogenates [37]. A further purification and an improved stability of the labile components of this lipoprotein fraction has been achieved by chromatography on Sepharose 6B in a Tris buffer system containing 1 mM EDTA [32]. This high molecular weight liver specific protein complex contains a membrane antigen localized on the cell surface of liver cell membranes as shown by immunofluorescence studies using isolated hepatocytes and heterologous anti-LSP sera [15].

Small amounts of human LSP were purified by affinity chromatography on insolubilized anti-LSP serum prepared in a sheep [6]. After elution from the affinity chromatography columns using 3 M sodium iodide, human LSP showed only one precipitin band with the sheep antiserum whereas native LSP complex reveals both

species-specific and non-species-specific determinants. The non-species-specific determinant is altered by the sodium iodide treatment and seems to be less stable [31].

Molecular weight determinations on calibrated Sepharose 4B and Sepharose 6B columns suggest that the molecular weight of the LSP fraction is between 4×10^6 and 20×10^6 daltons. The lipid and apolipoprotein moieties of the liver specific protein were partially characterized after separation on LH-20 column chromatography. Thin layer chromatography of the different fractions showed that LSP contains large amounts of phosphatides and triglycerides [17]. A further analysis of the phosphatides revealed cephalin, sphingomyelin, lecithin, and lysolecithin. The antigenicity of LSP depends on its lipid content. Delipidated LSP does not react with antisera against native LSP when tested by double immunodiffusion [17]. Polyacrylamide gel electrophoresis of LSP isolated from different species demonstrated a similar mobility of the macrolipoprotein. When the same experiment is performed in the presence of sodium dodecylsulphate (SDS) five major and several minor components can be distinguished demonstrating the existance of different polypeptides [17, 32]. In recent studies immunoelectrophoretic methods were used for the identification of species-specific and non-species-specific determinants of the LSP complex. Human LSP shows in crossed immunoelectrophoresis two different precipitin lines with anti-human LSP serum prepared in a sheep whereas rabbit, rat, mouse, and swine LSP exhibited one precipitin line with similar mobility. No immunoprecipitate was found with sheep and bovine LSP [31]. In addition to the non-species-specific determinant present in several mammals human LSP complex contains a species-specific determinant. Analogous results were obtained by fused rocket immunoelectrophoresis (Fig. 1).

When LSP of these seven different species was tested against the sheep anti-human LSP with fused rocket immunoelectrophoresis a pattern of identity is seen of one human precipitin line with rabbit, rat, swine, and mouse LSP. These species share a common non-species-specific determinant. A second precipitate is only seen with human LSP (Fig. 1).

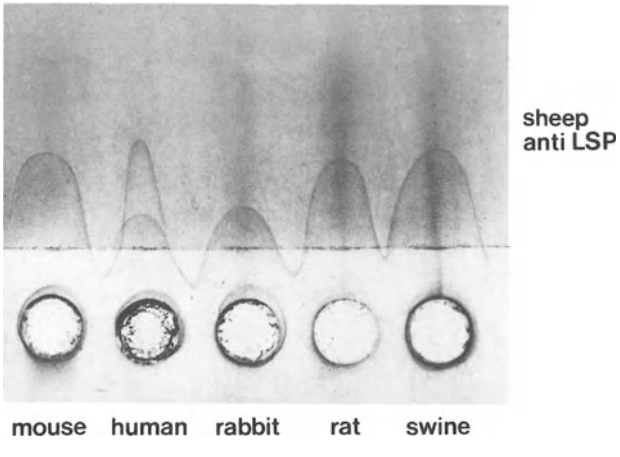

Fig. 1. Fused rocket immunoelectrophoresis: LSP of different species tested against sheep anti-human LSP serum

Autoantibodies of rabbits with experimentally induced chronic active hepatitis (CAH) react against human LSP with additional precipitin peaks in crossed immunoelectrophoresis. Using rabbit LSP only one precipitate is found. This autoantibody specificity is directed against the non-species-specific determinant of LSP as confirmed by absorption experiments with LSP from different species [31].

The hypothesis of different determinants requires that the LSP complex displays microheterogeneity. Microheterogeneity can readily be assessed by isoelectric focusing (IEF). Human and raddit LSP have been isoelectrically focused in a 4.3% macroporous polyacrylamide slab gel through a pH 3 to 10 gradient (Möller, B., Meyer zum Büschenfelde, K.-H., unpublished data). A series of six to seven bands with identical isoelectric points in human and rabbit LSP can be identified (Fig. 2). The protein stained bands indicate a similar microheterogeneity of the LSP in both tested species. The protein bearing pH-regions of the slab gel obtained after IEF were separated and used for short time immunization of rats.

The antisera raised against different protein bands of isoelectrically focused human and rabbit LSP were compared in fused rocket immunoelectrophoresis. Before absorption of rat anti-LSP sera with plasma, kidney, and blood cells, the immunoelectrophoretic pattern of different antigenic proteins in the elution profile of Sepharose 6B by fractionated soluble liver proteins is shown in Fig. 3. The unabsorbed antiserum contains antibodies reacting with different proteins of the first and second peak of Sepharose 6B chromatography. The antisera of all four isoelectrically focused protein zones show a similar immunoelectrophoretic pattern

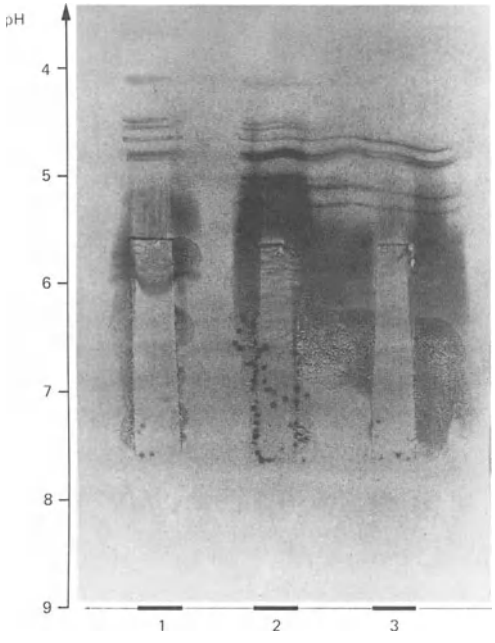

Fig. 2. Isoelectric focusing (pH 3–10) of purified human-LSP [1] and rabbit-LSP [2, 3] prepared by gel chromatography on Sepharose 6B

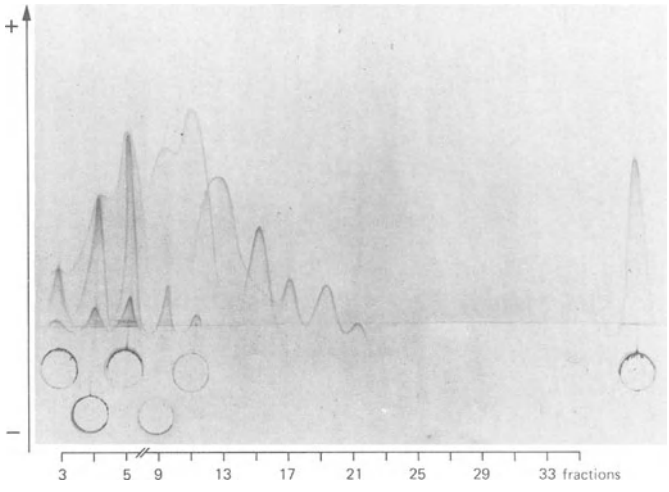

Fig. 3. Fused rocket immunoelectrophoresis: Fractions of rabbit liver proteins obtained after Sepharose 6B chromatography tested against non-absorbed rat anti-rabbit-LSP (immunizing antigen: isoelectric focused LSP, pH gradient 3–10)

in fused rocket immunoelectrophoresis suggesting a similar distribution of several antigenic determinants on each isoelcetrically focused constituent of the LSP complex.

The absorbed antiserum forms only one precipitate in the region of the first peak of Sepharose 6B chromatography (Fig. 4). It is identical with the species-specific moiety of LSP and is also detectable in all isoelectric-focused LSP-fractions.

Recently new radioimmunoassay systems for the detection of autoantibodies against LSP were developed [20, 22, 30]. Such highly sensitive test systems may allow further characterization of the liver specific lipoprotein complex LSP and its antigenic determinants.

Behrens and Paronetto [3] recently reported additional immunochemical studies on LSP. Comperative investigations on LSP, using Sepharose 6B first peak, and the kidney equivalent, designated KSP, revealed no major immunochemical differences of both antigen preparations. These authors were not able to produce organ-specific antibodies in rabbits, which may be due to different immunization procedures when compared to previous reports. Nevertheless these authors derived from cytotoxicity studies [4] the conclusion that the LSP preparation contains organ-specific determinants.

2.2 The Liver Membrane Antigen (LM-Ag)

Absorption studies revealed that liver membrane autoantibodies (LMA) which were detected by indirect immunofluorescence [13] could not be absorbed by purified LSP in many sera. This observation was the first evidence that LSP may not be the only target antigen in liver diseases. By affinity chromatography on insolubilized serum from patients with HBsAG-negative CAH a protein was isolated which reacted in crossed immunoelectrophoresis with HBsAg-negative LMA-positive

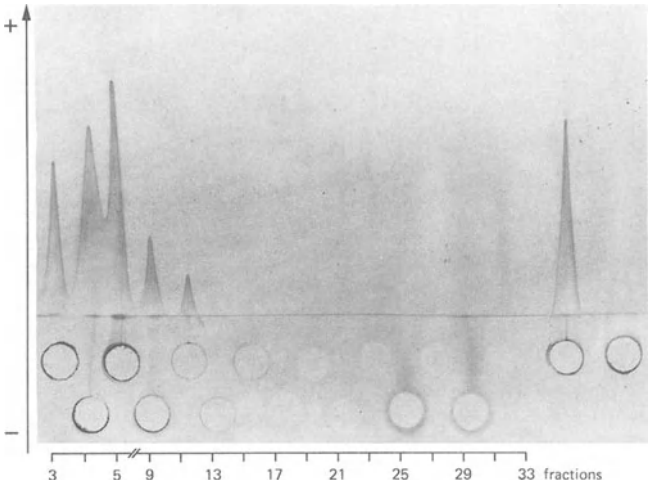

Fig. 4. Fused rocket immunoelectrophoresis: Fractions of rabbit liver proteins obtained after Sepharose 6B chromatography tested against rat anti-rabbit-LSP (immunizing antigen: isoelectric focused LSP, pH gradient 3–10) after absorption with rabbit plasma and rabbit kidney homogenate

CAH serum [36]. This protein was purified from human and rabbit soluble liver proteins. Immunological identity was confirmed by tandem crossed immunoelectrophoresis. The antibody activity to this new antigen, called liver membrane antigen (LM-Ag), could be absorbed by isolated rabbit hepatocytes. LM-Ag could not be purified by affinity chromatography on LMA-negative HBsAg-positive CAH-serum, normal human serum, normal sheep serum, and anti-LSP-serum prepared in a sheep.

Schuurman, H. J., Vogten, A. J. M., Schalm, S. W. (personal communication) were able to prepare liver membrane antigen (LM-Ag) from normal human liver by homogenization, sucrose density gradient centrifugation, treatment of the 1.18–1.20 g/cm^3 inter-face with Triton X 100, and subsequent Sepharose 6B chromatography. Using a reference serum from a patient with HBsAg-negative CAH which contained high titre liver membrane autoantibodies as detected by the LMA-test [13], LM-Ag was located in the second peak of Sepharose 6B. This reference serum was anti-LSP negative. At present it is unknown whether both LM-Ag preparations represent identical antigens.

3. Humoral Immunity Against Liver Membrane Antigens

3.1. Autoantibodies Against Liver Specific Lipoprotein (Anti-LSP)

Jensen et al. [20] first reported a sensitive radioimmuno-precipitation test for the detection of circulating anti-LSP. LSP in this test system was radio-labelled with I^{125} by the Bolton-Hunter technique [5] and LSP-anti-LSP complexes were precipitated by Cowan I staphylococcal cells, which contain protein A in their cell walls that avidly binds the Fc region of IgG. Ninety seven percent (29/30) of patients

with untreated CAH had anti-LSP detectable in their serum. The mean titre was higher in HBsAg negative cases, although the difference was not significant. Ninety five percent (20/21) of patients with acute viral hepatitis had anti-LSP within two weeks of the onset of jaundice. In uncomplicated acute viral hepatitis sera became anti-LSP negative within 12 weeks. Anti-LSP was found in 60% (10/17) of patients with chronic persistent hepatitin (CPH.) A highly significant correlation was observed between antibody titre and histological and biochemical parameters of activity of disease in CAH. No correlation existed with the presence of non-organ-specific autoantibodies. Smooth muscle antibodies (SMA), antinuclear antibodies (ANA) and antimitochondrial antibodies (AMA).

A similar technique was used by Gerber et al. [9] who detected anti-LSP in 63% (38/60) of patients with chronic active hepatitis (CAH) irrespective of the presence of non-organ-specific autoantibodies (ANA, SMA, AMA) or HBsAg. The incidence of anti-LSP was significantly higher in untreated patients. Anti-LSP were further found in patients with primary biliary cirrhosis (PBC), chronic persistent hepatitis. (CPH) and acute viral hepatitis (AVH) not in patients with alcohol induced liver diseases, but anti-LSP were detected in 18% (3/17) of patients with glomerulonephritis (Table 1).

Kakumu et al. [22] used a different radioimmunoprecipitation technique to detect circulating anti-LSP in patient sera. In their studies LSP was labelled using the Chloramine T method [19] and rabbit anti-human IgG serum was used as second antibody. These authors found anti-LSP in 57% of patients with chronic

Table 1. Frequency (% positive) of anti-LSP in patient sera

	Jensen et al. [20]		Kakumu et al. [22]	
	%	No.	%	No
Chronic active hepatitis	97	29/30	57	25/44
Chronic persistent hepatitis	60	10/17	22	5/23
Acute viral hepatitis	95	20/21	40	12/32
Alcohol induced liver disease	–	–	0	0/8
Miscellaneous liver diseases	0	0/14	0	0/22
Cirrhosis of the liver	–	–	38	8/21
Primary biliary cirrhosis	–	–	33	2/6
Drug induced hepatitis	–	–	0	0/11
Primary non-hepatic auto-immune diseases	–	–	0	0/60
Healthy blood donors	–	–	0	0/50

active hepatitis irrespective of HBsAg status. Twenty two percent of patients with CPH, 38% of patients with liver cirrhosis, and 40% of patients with acute viral hepatitis had anti-LSP. In uncomplicated cases anti-LSP disappeared within two months after the onset of disease. In cases which progressed to CAH anti-LSP remained positive and indicated development of CAH before the diagnosis was made histologically. The frequency of anti-LSP was significantly correlated with the presence of non-organ-specific autoantibodies. No correlation was observed with other biochemical data.

Manns et al. [30] developed a radioimmunoprecipitation test for anti-LSP similar to the one described by Kakumu et al. [19]. Anti-LSP was detected in 44% of patients with (Chronic active liver disease), 56% with CPH, 42% with AVH, and 20% with inactive cirrhosis irrespective of HBsAg status. Anti-LSP was also found in 29% of patients with alcohol-induced liver disease, 14% with miscellaneous liver diseases, and 10% of patients with primary non-hepatic autoimmune diseases (Table 1); anti-LSP was not detectable in 31 healthy blood donors. In this study within the group of CALD no correlation was observed transminase between anti-LSP and sex, age, HBsAg, gamma-globulin level, serum glutamic-oxaloacetic (SGOT), or non-organ-specific autoantibodies (ANA, AMA, SMA).

Human LSP was used as test antigen in the assays described by Jensen et al., Gerber et al., and Kakumu et al. Kakumu et al. could absorb the antihuman LSP activity using human and rat LSP, indicating that non-species-specific determinants

Gerber et al. [9]		Manns et al. [30]		Uibo et al. [47]	
%	No	%	No	%	No
63	38/60	44	27/62	39	9/23
56	5/9	56	9/16	–	–
40	4/10	42	14/33	8	2/25
0	0/13	29	4/14	0	0/10
0	0/14	14	1/7	0	0/24
–	–	20	2/10	–	–
43	9/21	–	–	100	45/45
–	–	–	–	–	–
18	3/17	10	6/58	–	–
–	–	0	0/31	–	–

of LSP were targets for anti-LSP autoantibodies in the sera tested by these authors. No information is given in this paper as to whether all sera tested were absorbed by rat LSP. As species-specific and non-species-specific determinants of LSP could be demonstrated by heterologous antisera [31] we tested all groups of patients for anti-human and anti-rabbit LSP. The incidence for anti-rabbit LSP was similar although below the percentage obtained for anti-human LSP. A striking difference was observed in AVH. Only 9% of patients had anti-rabbit LSP whereas 42% had antibodies to human LSP in this group of patients. As antibodies against LSP in AVH were found to be transient in uncomplicated AVH [20, 22] and as in rabbits, antibodies against non-species-specific determinants of LSP were only found in animals with histological signs of CAH [31], it may be speculated whether only antibodies against non-species-specific determinants reflect a real self-perpetuating state of autoimmunity.

Originally LSP was defined by heterologous antisera obtained after absorption with kidney proteins, human plasma, and blood cells [37]. Furthermore the present LSP preparation contains non-organ-specific contaminants [3]. Therefore one may question whether these non-organ-specific contaminants are targets for circulating autoantibodies. A kidney protein fraction prepared in the same way as LSP served as labelled antigen in the anti-LSP radioimmuno precipitation test [30]. Antibodies against the kidney equivalent of LSP were found in 10% (6/62) of patients with CALD, 14% (1/7) of patients with alcohol-induced liver disease, and in 3% (2/58) of patients with primary non-hepatic autoimmune diseases, indicating that naturally occurring anti-LSP are predominantly directed against organ-specific determinants of the LSP preparation.

Very recently Uibo et al. (47) reported the detection of circulating anti-LSP by enzyme linked solid phase immunosorbent assay (ELISA) (Table 1). These authors found circulating anti-LSP with human LSP, rabbit LSP, and bovine LSP as antigen.

The described radioimmunoprecipitation tests for anti-LSP indicate that autoantibodies against the antigen fraction LSP are of diagnostic and prognostic value in inflammatory liver diseases. As all test systems use an antigen of partial purification and the monospecificity of the autoantibodies detected by these test systems has still to be proved, a further purification of these molecule LSP and its antigenic determinants seems necessary. From the reported data on anti-LSP it cannot be determined whether detected autoantibodies are reacting with identical determinations of the LSP antigen complex. Furthermore one has to question whether the organ-specific and non-organ-specific determinants are located on identical or different molecules.

3.2. Liver Membrane Autoantibodies Detected by Indirect Immunofluorescence on Isolated Hepatocytes (LMA)

Liver membrane autoantibodies can further be detected by indirect immunofluorescence on isolated rabbit hepatocytes [13]. When isolated rabbit hepatocytes are incubated with patient serum the existence of circulating liver membrane autoantibodies is indicated by a characteristic linear membrane staining (LMA-test). In the original study [13] LMA were found in 7/10 patients with HBsAg negative CAH

but not in patients with AVH, CPH, HBsAg positive CAH, healthy HBsAG carriers, alcoholic liver disease, and healthy controls. These original observations were followed by a larger clinical study [43]. In this study LMA were predominantly found in HBsAg negative chronic inflammatory liver diseases: 38% of HBsAg negative CAH, 61% of HBsAg negative cirrhosis, and rarely in other liver diseases. Schuurman et al. [42] tested 100 sera for the presence of LMA. While the above reported data resemble LMA of IgG type, Schuurman et al. tested for IgM antibodies as well. They found LMA positive sera (including IgG and IgM antibodies) in 71% of HBsAg negative CAH, 14% of HBsAg positive CAH, 35% of PBC, 57% in drug induced liver diseases, and in 67% of AVH. No LMA were found in alcohol induced liver disease, a group of miscellaneous liver diseases, and in inflammatory bowel diseases. Thus the sensitivity of the LMA test was confirmed for HBsAg negative CAH; LMA in PBC, AVH, and drug induced liver disease were mainly referable to IgM antibodies. The LMA titre in this study was decreased during immunosuppressive therapy.

Similar results were obtained by Junge et al. [21]. These authors could absorb the immunofluorescence by liver homogenate but not by equivalent kidney tissue fractions.

Kawanishi and McDermott [25] detected liver membrane autoantibodies by indirect immunofluorescence. Human and rabbit hepatocytes were isolated after liver perfusion in the presence of 0.1% collagenase. Sera from 10 patients with CALD, preabsorbed with human kidney tissues possessed antibodies against the surface membrane of human and rabbit hepatocytes. The detected autoantibodies were only of IgG class. The five HBsAg negative CALD sera exhibited a linear membrane staining whereas the remaining five sera from patients with HBsAg positive CALD showed a mixed granular and diffuse fluorescence of lower intensity.

3.3 Heterogeneity of Liver Membrane Autoantibodies

The LMA-test seems to be a valuable marker for autoimmune type liver disease. Nevertheless no information is available concerning the target antigen(s) to which these LMA are directed. First it was suggested that LMA resemble antibodies against LSP [12, 20]. Absorption studies revealed that many of the LMA positive sera could not be absorbed by LSP [36]. Recently, testing 231 patient sera for LMA and anti-LSP [30], it could be demonstrated that the LMA-test detects liver membrane autoantibodies against further membrane antigens besides LSP. As the linear membrane immunofluorescence staining of the LMA-test could be absorbed by 100,000 *g* supernatants of liver homogenates the corresponding target antigens had to be looked for in this protein fraction. A solid phase radioimmunoassay (RIA) [29] was developed to further characterize liver membrane autoantibodies. The IgG fraction of 38 patient sera was prepared by ammonium sulphate precipitation and labelled with I^{125} using the Chloramine T method [19]. The IgG fraction was used to coat the assay tubes and as labelled antibody, 100,000 *g* supernatants served as test antigen. Positive results were only obtained with 10/14 sera from patients with HBsAg negative CAH, not with sera from three patients with HBsAg positive CAH, two with CPH, six patients with AVH, six with miscellaneous liver diseases, seven with primary nonhepatic autoimmune diseases, and two healthy blood

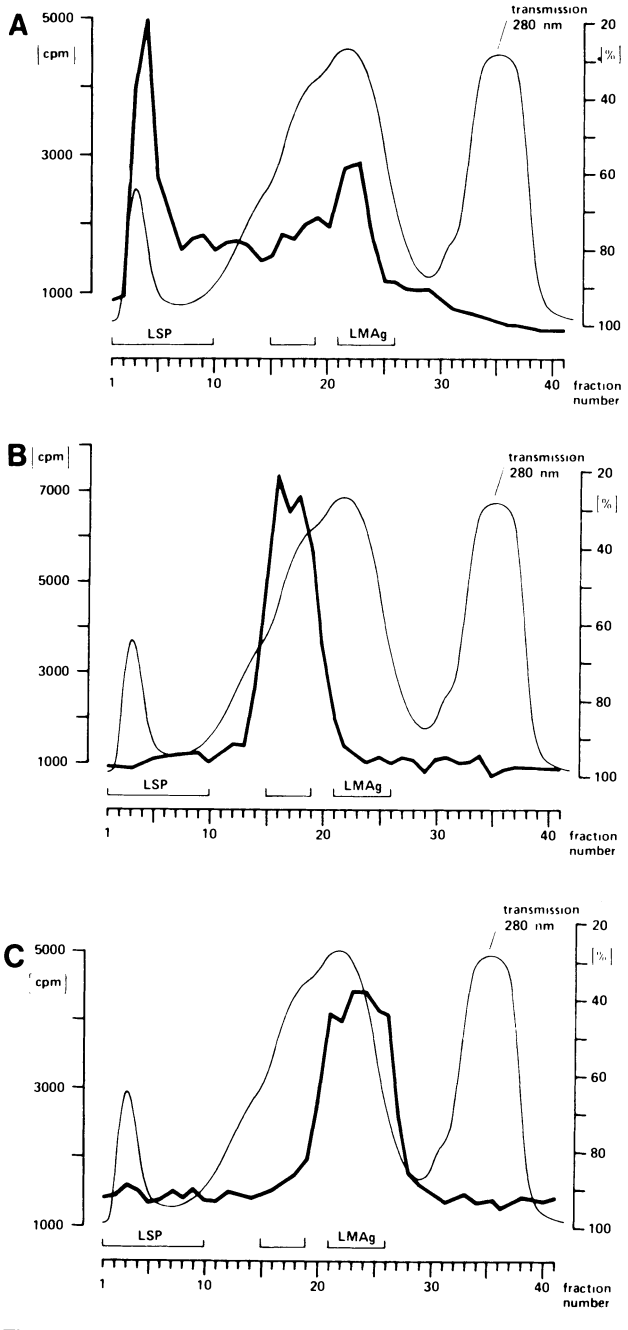

Fig. 5 A-C. Sepharose 6B chromatography of human soluble liver proteins. Fraction 1 represents the first fraction after a void volume of 124 ml. —: RIA for detecting liver membrane antigens, results are presented as counts per minute (cpm). —: protein content indicated as optical transmission at 280 nm (%). **A** Serum from a patient reacting with the LSP- and LM-AG-fraction. **B** Patient reacting with the third antigen fraction. **C** Patient with antibody to LM-Ag. With rabbit liver proteins similar results were obtained for all patients tested

donors. All these sera were tested by the LMA-test as well; LMA-test was positive in 9/14 patients with HBsAg negative CAH, all other sera were negative. The antibody activity detected by RIA could be absorbed by isolated liver cell membranes. No correlation between the RIA results and SMA, ANA, or AMA was observed. Supernatants (100,000 *g*) were chromatographed on Sepharose 6B and all fractions obtained after Sepharose 6B column were tested by RIA. The corresponding antigens were found in three different antigen fractions of Sepharose 6B (Fig. 5). Besides the first peak, resembling LSP, autoantibodies reacted with a fraction that exhibited the characteristics of LM-Ag. A third antigen fraction was localized at fractions with a molecular weight of approximately 2×10^5–3×10^5 daltons. Tests for organ-specificity showed that the RIA positive sera were reacting with equivalent kidney protein fractions, only antibodies reacting with the first peak of Sepharose 6B so far were found to be organ-specific. These data demonstrate a heterogeneity of liver membrane autoantibodies and it may be concluded that in inflammatory liver diseases we are not dealing with one target antigen-antibody system. Coating – cross – and blocking tests revealed an individual-specificity and species-cross-reactivity of many of the detected liver membrane autoantibodies. An interaction with HLA-antigens could be ruled out in these studies. A further characterization and purification of these liver membrane antigens will be the subject for further studies. Well defined autoantibodies from patients' sera should be used in these studies. In addition further investigations will have to deal with the evaluation of the organ-specificity of liver membrane target antigens.

4. Cellular Immune Reactions Against Liver Membrane Antigens

Studies on cellular immunity against liver membrane antigens have relied predominantly on in vitro studies with either crude liver homogenates or LSP preparations as antigens. Three types of assay systems have been employed, namely leucocyte migration inhibition, lymphocyte stimulation, and various cytotoxicity assays.

Each of these assays measures different functions of the immune response. The production of certain leucocyte kinins, such as leucocyte migration inhibition factor (LIF) and monocyte migration inhibition factor (MIF) in response to specific antigens, is the basis for the leucocyte migration inhibition test in its various modifications. The lymphocyte stimulation assay measures the early steps of antigen recognition with proliferation of antigen-specific immunocytes and blast transformation. The cytotoxicity assay systems measure the cytotoxic potential of lymphocytes or lymphocyte subpopulations sensitized against target antigens present on the plasma membrane of target cells. Human hepatocytes, rabbit hepatocytes, cultured liver cells, and antigen coated red cells have been used in the past. The results are influenced by the complex processes of antigen recognition by sensitized immunocytes and the differentiation into cytotoxic effector cells.

4.1. Leucocyte Migration Inhibition

We have used a two stage leucocyte migration inhibition assay to study cellular immune reactions in patients with CAH [35]. Twenty nine of 34 (85%) of untreated

patients with hypergammaglobulinaemic, autoimmune CAH and 8/19 patients with kryptogenic cirrhosis showed significant leucocyte migration inhibition. A subsequent study expanded these findings on defined subgroups of CAH (38). Seventeen of 20 (85%) of untreated patients with autoimmune CAH, 46% of patients with type B CAH, and 29% of patients with HBsAg negative, autoimmune marker negative CAH showed significant inhibition of leucocyte migration.

Bacon et al. investigated cellular immunity in 32 patients with chronic active hepatitis and obtained evidence for cellular sensitization in 75% of patients against a crude liver extract [2]. Miller et al. studied 16 patients with CAH and found leucocyte migration inhibition in 11 patients (40). Four patients treated with immunosuppressive agents failed to show migration inhibition. Lee et al. reported leucocyte migration inhibition in 67% of HBsAg negative, anti-HBs negative patients with CAH, and a reduction of migration inhibition in response to immunosuppressive therapy [28].

4.2. Lymphocyte Stimulation

The first report of lymphocyte stimulation in response to soluble liver homogenate came from Tobias et al. who observed significant lymphocyte stimulation in two of four patients with PBC and in one of two patients with CAH [46]. Thestrup-Petersen et al. studied lymphocyte stimulation in response to LSP in 34 patients with various liver diseases. Eight of ten patients with CAH or CPH and two of five patients with non-alcoholic cirrhosis showed a positive in vitro reactivity to LSP. There was no significant correlation between the stimulation with LSP and the presence or absence of HBsAg or other biochemical abnormalities [44].

4.3. Cytotoxicity Assay Systems

Cytotoxicity of peripheral blood lymphocytes against a number of target cells has been reported in the past. There is a lack of agreement of the results, which is due to several factors, such as methodological differences and differences in the target cells studied. The results obtained with different target cells and the likely target antigens in these assays will be discussed below. The topic of effector cell mechanisms is dealt with in the paper by Wands published in this issue.

4.3.1. Rabbit Hepatocytes. Rabbit hepatocytes as target cells have been extensively studied. The hepatocytes were isolated by perfusion and the use of collagenase. Cytotoxicity was assessed by determining the percentage of viable plastic adherent hepatocytes after incubation with patient lymphocytes. A high effector-target cell ratio of 400:1 was necessary for optimal cytotoxicity. Increased cytotoxicity was observed in 20/22 patients with CAH, HBsAg positive or HBsAg negative. The cytotoxicity could be blocked by addition of small amounts of human LSP, thus suggesting that LSP had been the target antigen of the cytotoxic attack [45]. In a subsequent study all 15 untreated cases of CAH showed increased cytotoxicity, which became negative in four of nine patients studied over a prolonged period. Patients who had a negative cytotoxicity under immunosuppressive therapy had a better prognosis than those patients under therapy with positive cytotoxicity. There

was a positive correlation between cytotoxicity and the histological grading of disease activity, in particular with the extent of piece-meal necrosis [7]. There was no correlation between cytotoxicity and the presence or absence of HBsAg.

Facchini et al., using the same method, found increased cytotoxicity against rabbit hepatocytes in patients with CAH [8]. These authors observed no correlation with the presence or absence of HBsAg, autoimmune markers, gammaglobulins, or enzyme elevation. Patients under steroid therapy had a decrease of cytotoxic activity.

Kawanishi et al. studied antibody dependent cellular cytotoxicity of peripheral blood lymphocytes from normal subjects against rabbit hepatocytes in the presence of HBsAg positive and HBsAg negative CAH sera [25]. Two patterns of IgG binding could be distinguished: sera from HBsAg negative CAH patients showed a diffuse linear membrane pattern and sera from HBsAg positive patients showed both a granular and a weaker diffuse immunofluorescence. The immunofluorescence could be abolished by prior absorption of these sera with lyophilized human liver homogenate. Human embryonal intestinal cells did not show IgG binding. A significant cytotoxicity of peripheral blood lymphocytes against rabbit hepatocytes in the presence of CAH serum but not in the presence of normal serum was observed in HBsAg negative and HBsAg positive CAH sera.

4.3.2. Human Hepatocytes. When autologous human hepatocytes are used as target cells in cytotoxic assays the problems of HLA restriction of T cell cytolysis can be avoided. These advantages are, however, offset by the fact that human hepatocytes have to be isolated by enzymatic methods, that the cell yield is usually low, and that these cells have only a limited viability. The studies using human hepatocytes have yielded discrepant results. Wands et al. found an increased cytotoxicity of peripheral blood lymphocytes toward autologous liver cells, which returned to normal values in patients under prednisolone therapy [51]. In a similar study, increased cytotoxicity was found in 53% of patients with CALD, but in 32% there was a significantly decreased cytotoxicity when compared to normal controls. The remaining patients had a normal cytotoxicity. Patients exhibiting cytotoxicity against hepatocytes had a serum factor which was able to inhibit the phytohaemagglutinin-induced lymphocyte transformation of normal lymphocytes [10]. Paronetto et al. studied cytotoxicity of lymphocytes against cultured autologous hepatocytes and observed increased cytotoxicity in eight of ten patients with CAH. Cytotoxicity was seen in both HBsAg positive and HBsAg negative patients [41].

Vergani et al. studied cellular cytotoxicity against autologous hepatocytes after 48–96 h in culture [48]. They found significantly increased cytotoxicity in 10/16 patients. All six untreated patients showed cytotoxicity, but only 4/10 patients under immunosuppressive treatment were cytotoxic to autologous hepatocytes. There was a statistical association between cytotoxicity and the extent of histologically assessed liver damage. The cytotoxicity could be blocked in all cases by the addition of human LSP preparation, thus suggesting that LSP was the target antigen.

One of the problems with this approach is, for obvious ethical reasons, the control group was not studied in an autologous system but consisted of normal

lymphocytes studied with CAH hepatocytes. Hepatocytes which were killed by patients' lymphocytes were also killed to some degree by normal lymphocytes, which may have been due to decreased viability or to membrane fixed immunoglobulin by these hepatocytes.

4.3.3. Cultured Cells. Chang liver cells have been used by several investigators with different results [1, 16, 24, 49, 51, 52]. This may be due to the fact that some Chang cells are contaminated with, or are in fact, HeLa cells. We have studied the membrane expression of LSP on several Chang cell strains and have observed differences of membrane expression [18]. This could explain some of the discrepancies reported in the literature and underlines the importance to control the expression of the putative target antigens.

Increased cytotoxicity in eight patients with CAH against Chang cells was observed by Wands et al. [52]. Different results were obtained by Vierling et al. who studied spontaneous (SCMC) and antibody dependent (ADCC) cytotoxicity against Chang cells and a mouse sarcoma cell line [49]. There was no difference in SCMC and ADCC in patients with CAH when compared to controls; patients with PBC showed a decreased cellular cytotoxicity. When comparing the cytotoxic effect on Chang cells or a mouse sarcoma line, no differences were encountered, suggesting that the cytotoxicity was not organ-specific. We have studied SCMC and ADCC in HBsAg positive CAH and non-A, non-B CAH. SCMC and ADCC were increased in patients when compared to normal controls. Sera of patients were inhibitory to SCMC and ADCC when compared to homologous AB serum [16]. The cytotoxicity was partially inhibited by the addition of human LSP. An inhibitory effect of autologous CAH serum was also observed by Kakumu et al. [23]. A study by ourselves of patients with non-A, non-B CPH disclosed increased SCMC but normal ADCC when compared to normal controls [16]. Kawanishi studied the cytotoxicity of CAH serum on Chang sells by lymphocytes from normal subjects. He found a significantly increased cytotoxicity by CAH serum when compared to normal serum. The cytotoxicity could be blocked by addition of heat-aggregated IgG and prior absorption of sera with liver homogenate [24].

4.3.4. *LSP-Coated Target Cells.* Vogten et al. developed a test system, in which human LSP was non-covalently bound to avian erythrocytes [50]. The observed increased cytotoxicity in approximately 50% of 62 CAH patients, whereas only 5/100 normal persons and 2/8 patients with PBC showed cytotoxicity. The cytotoxic effect could be blocked by addition of human LSP and heat-aggregated IgG.

Behrens et al. coated a mastocytoma line either with human LSP or the kidney equivalent KSP and studied cytotoxicity of human granulocytes from normal persons in the presence of CAH serum [4]. Several sera from patients contained antibodies against liver and kidney antigens. Inhibition experiments revealed that the cytotoxicity could be blocked by addition of the appropriate coating antigen but in many cases also by other antigens. Cross-inhibition of LSP coated target cells with KSP was not always observed, suggesting that the LSP preparation contained an organ-specific component. The authors concluded, that the cytotoxicity against LSP coated target cells is not always an organ-specific phenomenon.

5. Animal Models for Autoimmune CAH

Evidence for a pathogenetic role of LSP in chronic active hepatitis originated from experiments in which rabbits were long-term immunized with human liver subfractions containing organ-specific determinants. In these experiments the first peak of a Sephadex G-100 column from 100,000 *g* supernatants of human liver (HLP) or rabbit liver (RLP) were used [12, 14, 26, 27, 33, 34]. Animals immunized with HLP over a period of 143 weeks i. p. with complete Freund's adjuvant developed CAH or cirrhosis after this time. The combined administration of HLP together with RLP caused development of histological lesions in only 13% of rabbits. There was a correlation between the histological liver lesions and cellular immunity against allogeneic liver proteins as assessed by skin testing [14, 33]. As in addition only rabbits that had developed CAH after long-term immunization had circulating autoantibodies against allogeneic rabbit LSP, it was suggested that the loss of tolerance against a species-non-specific determinant of LSP was responsible for the development of CAH [31]. More recently the development of inflammatory liver lesions in rabbits was demonstrated after long-term immunization with purified LSP, using the first peak obtained after Sepharose 6B chromatography as immunizing antigen [8, 47].

6. Conclusion

The reported data on liver membrane antigens demonstrate that these antigens are targets for humoral and cell mediated immunity in human inflammatory liver diseases. Undoubtedly some of these determinants are organ-specific. Future work will have to concentrate on a further purification of these antigens. Special interest should concentrate on the attempt to separate organ-specific from non-organ-specific determinants. If these determinants are located on identical molecules, then organ-specific determinants can only be identified and characterized by monospecific autoantibodies. At present studies for CMI and humoral immunity against liver membrane antigens have to include appropriate control experiments with equivalent protein preparations from organs other than liver.

The described liver membrane antigens are constituents of normal liver cell membranes. Nothing is known about their functional integrity. Are they associated with membrane enzymes, or linked to receptors of the liver cell membrane, i. e. hormone receptors, receptors for drugs or toxins? Are they located at the sinusoidal or biliary poles of the hepatocyte membrane?

Further purification and definition of these antigens will facilitate the development of more sensitive and more reproducible assays enabling a more precise evaluation of the clinical relevance of liver membrane autoantibodies. It has still to be investigated whether special types of liver membrane autoantibodies are characteristic for subgroups of chronic active liver diseases. Furthermore prospective studies will be needed to determine whether liver membrane autoantibody titres decline under immunosuppressive treatment.

There are several problems with the cytotoxicity studies reported in patients with chronic liver diseases. The use of autologous hepatocytes is limited by the low viability and low yield of cells from these preparations. In the published studies the

Table 2. Putative target antigens of immune reactions in various forms of chronic active hepatitis

Hepatitis	Immune reactions	
	Humoral	Cellular
CAH Type B	anti-HBs, anti-HBc, anti-LSP	LSP, HBsAg (?)
CAH Type NANB	anti-LSP(?) anti-NANB-antigen	LSP(?), viral antigens (?)
Autoimmune CAH	anti-LSP, LMA	LSP, LM-Ag
Drug-induced CAH	(?)	Hapten-modified membrane antigens (?)
Alcohol hepatitis	anti-LSP anti-hyalin (?)	LSP alcoholic hyalin

cytotoxicity in patients was studied in an autologous system but was compared with normal lymphocytes against allogeneic patient hepatocytes. This fact limits the usefulness of this system, since control experiments are thus not performed in a syngeneic system. Another problem is the use of collagenase for the isolation of hepatocytes, since membrane alterations cannot be excluded.

The use of cultured cells of hepatic origin presents different problems. It has to be assessed critically, whether cultured cells express liver specific antigens on the cell membrane including LSP. Most of these cell lines are rather dedifferentiated and have lost most of their liver specific biochemical functions. Also the problem of contamination with mycoplasms must be remembered. Our results using Chang cells have stressed this problem. The discrepant results using Chang cells as target cells of cytotoxicity assays by different authors might well be explained by differences in membrane expression of liver specific antigens or contamination by other cell types.

An alternative to the use of autologous hepatocytes and cultured cells is the use of red blood cells coated with viral antigens or with liver specific protein. In this case the specificity of the cytotoxicity assay is controllable by inhibition experiments with either purified LSP, unrelated membrane proteins, or other cell types. However, since LSP probably bears non-organ-specific as well as organ-specific determinants the inhibition by LSP is insufficient to demonstrate organ specificity. In this area more work on the specificity of the target antigens is important.

These various test systems used to study CMI in inflammatory liver diseases indicate that the immune phenomena measured are important factors in the immunopathogenesis. Nevertheless the present assays are rather complicated and therefore restricted to special research laboratories. For many of the described assay systems little is known about the target antigens involved or the exact effector mechanisms. In addition, in many cases the authors did not study the organ-specificity of the detected immune-phenomena. A further unanswered question is whether in a single patient humoral and CMI may coexist or are mutually exclusive.

Concerning the importance of the described immune-phenomena for the immunopathogenesis, one can state that these immune reactions are important factors for the course of inflammatory liver diseases, but we can only speculate as to

whether the origin of the disease is mediated by a primary autoimmune attack possibly mediated by a genetic predisposition. In addition, viruses and even drugs have been suggested to be involved in the primary loss of tolerance against liver membrane antigens.

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Immunologic Effector Mechanisms in Hepatitis B-Negative Chronic Active Hepatitis

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Introduction

Hepatitis B surface antigen (HBsAg) -negative chronic active hepatitis (CAH) is a progressive, destructive, inflammatory liver disease of unknown etiology. It is characterized by hepatic parenchymal necrosis and fibrosis, with subsequent development of macronodular cirrhosis in the majority of patients. The cardinal histologic findings consist of infiltration of the portal tracts by mononuclear cells which extend beyond the limiting plate and into the parenchyma, where their presence is associated with hepatocellular destruction known as piecemeal necrosis. One form of HBsAg-negative CAH primarily affects women and has a high incidence of serologic abnormalities. Antibodies against autologous tissues such as thyroid, gastric parietal, adrenal, and renal tubular cells are frequently present [69], and positive tests for antinuclear antibodies and "lupus erythematosus cells" have prompted the designations "lupoid hepatitis" and "autoimmune" CAH for this entity. Evidence of active hepatitis B infection is absent upon serologic testing, and additional genetic, epidemiologic, and serologic studies suggest that this disease is distinct from HBsAg-positive [7] and drug-induced CAH.

While the presence of occult viral infection has not been excluded, development of autoantibodies and an association with other suspected autoimmune diseases such as thyroiditis, pernicious anemia, and Addison's disease has fostered speculation that this type of hepatitis is due to autoimmunity [69]. This concept is supported by a high degree of association between lupoid hepatitis and HLA B8, a haplotype with an increased prevalence in these reputed autoimmune diseases [36]. This important observation suggests a genetic predisposition for development of the disease. Experimental evidence demonstrating autoimmunity as a primary pathologic mechanism in lupoid hepatitis has been difficult to obtain, however. Nevertheless, much effort has been devoted to the investigation of this possibility, and in this paper we will examine the evidence for an etiologic role of the immune system in the pathogenesis of HBsAg-negative CAH.

Liver Membrane Antigens

Because of the suspicion that immunologic mechanisms may injure the hepatocyte, there has been increasing emphasis on characterization of liver cell surface antigens. In 1972 Meyer zum Büschenfelde and Miescher described two antigens obtained from supernatants of human liver homogenates fractionated via column chromatography [41]. One protein, labeled LP-1, was shown to be a low density macromolecular lipoprotein from the hepatocyte membrane, while LP-2 was derived from the cytoplasm. Studies suggested that these proteins were organ-specific, but not species-specific [28]. That is, human liver-specific protein (LSP) demonstrated partial antigenic cross-reactivity with LSP similarly prepared from other mammalian species. LSP proved to be highly labile, but its antigenicity could be preserved by using EDTA during preparation [16]. Despite the fact that it contained many other organ-nonspecific and uncharacterized proteins, LSP preparations were used by several groups to gather evidence for immunologic hepatic damage in CAH.

In early work, Meyer zum Büschenfelde and colleagues exploited the species-nonspecificity of LSP in an attempt to demonstrate a role for immunity to this lipoprotein in the pathogenesis of CAH. They injected rabbits with LSP in complete Freund's adjuvant repeatedly over many months. Eventually, many of the animals developed a liver lesion characterized by portal tract infiltration and piecemeal necrosis [40]. Development of this histologic picture appeared to correlate with delayed hypersensitivity reactivity to LSP [39]. All the rabbits produced antibodies to LSP and those which developed CAH-like lesions could be demonstrated to have antibodies coating their hepatocytes. It should be noted, however, that all rabbits developed mononuclear infiltrations of their kidneys and lungs, which raised the question of whether LSP was truly specific for liver. Nevertheless, these investigations suggest that humoral and cellular immunity to LSP was associated with hepatocellular damage.

Humoral Immunity

Initial attempts to detect antibodies against liver-specific antigens in sera from patients with CAH proved difficult. Using a passive hemagglutination technique, antibodies to LSP were found in only a few cases of CAH and only in low titers [38]. More recently, Hopf et al. examined suspensions of viable human hepatocytes for the presence of surface-bound IgG by direct immunofluorescence [25]. They found positive hepatocyte membrane staining in five of six untreated patients with CAH and in two of 27 others on immunosuppressive therapy. Similar results were obtained in HBsAg-positive and -negative patients. Patients with acute hepatitis A, chronic persistent hepatitis, and other liver diseases did not have detectable anti-hepatocyte membrane antibodies and thus served as negative controls. In addition, the fluorescent pattern on hepatocyte surface membranes from HBsAg-negative patients was noted to be linear, while in HBsAg-positive cases it appeared granular [26]. Furthermore, in those HBsAg-negative patients with "autoimmune hepatitis," a serum antibody which bound to membranes of isolated rabbit hepatocytes in a

linear fashion could be detected. This putative liver membrane antibody (LMA) was absent in HBsAg-positive patients. LMA could be absorbed from serum by preparations of LSP but not by homogenates of human nonhepatic tissues [26, 58], and appeared to be confined to HBsAg-negative CAH. The two distinct immunofluorescent patterns on autologous hepatocytes and the presence of LMA only in HBsAg-negative CAH suggested an immunologic difference between HBsAg-negative and positive CAH. It was not determined whether the hepatocyte-bound immunoglobulin was important in producing cell damage, however. Moreover, it is conceivable that the autoantibody observed was actually directed against smooth muscle rather than a hepatocyte membrane antigen. Actin/myosin are known to be part of a cell's cytoskeleton, lying just beneath the plasma membrane. Thus it is possible that anti-smooth muscle antibody, which is present in the majority of HBsAg-negative CAH patients, might bind to hepatocytes. Moreover, Hopf's observations were at variance with a study by Alberti et al., who reported granular IgG deposits on the hepatocellular surfaces of both HBsAg-positive and -negative CAH patients [1].

The subsequent development of a sensitive radioimmunoassay for anti-LSP enabled Jensen et al. to further examine sera from patients with chronic liver disease for antibodies to LSP [30]. Unlike Hopf's findings measuring LMA, anti-LSP was detected in almost all patients with acute hepatitis and untreated CAH (both HBsAg-positive and -negative), and also in patients with chronic persistent hepatitis (CPH) at lower levels. This result was confirmed by other investigators [31]. Anti-LSP titers correlated with biochemical and morphologic indices of disease activity in patients with CAH but not in those with acute hepatitis, suggesting the possibility that this antibody might play a causative role in the former but not the latter condition. Jensen et al. postulated that anti-LSP might be the final common pathway of liver cell damage in both HBsAg-negative and positive CAH. Alternatively, these observations might have represented a humoral immune response to antigens liberated by hepatocyte necrosis which thus played no pathogenetic role in CAH [15].

The divergent results noted in these studies may be due to the fact that anti-LSP is not identical to LMA. Nevertheless, they do raise the question of whether LMA is truly specific for HBsAg-negative CAH. The immunofluorescence patterns described by Hopf are interesting, however, because they call to mind the patterns seen in glomerulonephritis, where linear staining is due to an autoantibody against glomerular basement membrane and granular staining is due to immune complex deposition. By analogy to glomerulonephritis, therefore, an autoantibody might be present in lupoid hepatitis, while immune complexes might produce the granular pattern in HBsAg-positive CAH [45]. Deposition of HBsAg-anti-HBs complexes on hepatocytes could occur via Fc and complement receptors on their surfaces [27]. However, although circulating immune complexes have been described in both HBsAg-positive and -negative CAH when associated with rash, arthralgias, and arthritis, they cannot be demonstrated in the majority of patients who lack these features [66]. Furthermore, while most prevalent in HBsAg-positive liver disease, circulating immune complexes have been described in a variety of viral and nonviral liver diseases [60]. It is thus unlikely that immune complexes are of primary importance in producing hepatocellular damage in HBsAg-positive CAH.

Cellular Immunity

The leukocyte migration test [57] was utilized early in the search for evidence of cell-mediated immunity in CAH. Initially, a crude extract of homogenated liver was used as the test antigen, producing both inhibition and stimulation of migration in leukocytes from CAH patients relative to normal controls [2, 56]. While inhibition of leukocyte migration appears to be a valid measure of specific cell mediated immunity [5, 8], it is difficult to interpret data consisting of both inhibition and stimulation as positive results. Stimulation may have resulted from suboptimal antigen concentration [43]. Therefore, Miller et al. prepared LP-1 as described by Meyer zum Büschenfelde for further experiments [43]. Lymphocytes from 11 of 16 patients with CAH inhibited leukocyte migration after exposure to LP-1 while only one showed stimulation. Half the patients were receiving prednisone, which was subsequently shown to diminish migration inhibition [35]. In addition, once testing for HBsAg became available, it was found that antigen-positive cases behaved similarly to antigen-negative ones [35]. These findings provide evidence suggestive of cell-mediated immunity to hepatocytes in patients with CAH. However, these assays rely on an indirect parameter of cell-mediated immunity, namely detecting release of a lymphokine.

Attention therefore turned to demonstrating cellular cytotoxicity by peripheral blood mononuclear cells (PBMC) in vitro. The initial investigations employed rabbit hepatocytes as target cells, since LSP had been shown to be present on such cells and to demonstrate partial antigenic cross-reactivity with human LSP [28]. Thomson et al. studied cytotoxicity of lymphocytes from CAH patients to isolated rabbit hepatocytes as assayed by the visual depletion of plastic-adherent target cells [62]. Peripheral blood mononuclear cells from 20 of 22 patients with CAH were significantly more cytotoxic than PBMCs from healthy controls and patients with other chronic liver diseases. It is important to note that 18 of the patients were receiving immunosuppressive agents, and the two without enhanced killing were from this group. The HBsAg status of the CAH patients was not determined. Blocking studies were performed to determine the specificity of this reaction. Both human and rabbit LSP preparations markedly inhibited cytotoxicity when added to the target cells with the PBMC. In contrast, a human kidney protein fraction prepared in an identical manner to LSP failed to inhibit cytotoxicity, suggesting specificity of the cytotoxic cells for LSP. Moreover, antibodies to LSP raised in guinea pigs also blocked the reaction. These antibodies were shown to bind to the target cells by direct immunofluorescence, although an important control using normal guinea pig serum to rule out nonspecific blocking by cross-reacting antibodies was not reported. These observations are consistent with cell-mediated killing, with LSP producing blockade by binding to lymphocyte surface receptors for antigen, and anti-LSP covering antigenic sites on the target cell surfaces to prevent interaction with lymphocytes. However, the type of PBMC effecting killing was not identified.

In a subsequent report from the same group using a similar assay system, Cochrane et al. measured cytotoxicity of PBMCs before and after enriching them for lymphocyte subpopulations [11]. Twelve of 17 CAH patients' cells were cytotoxic to rabbit hepatocytes; four of the patients were positive for HBsAg. Preparations

enriched for non-T cells by removal of erythrocyte (E)-rosetted cells were cytotoxic in all 12 cases, whereas preparations enriched for T cells by removal of erythrocyte-antibody-complement (EAC)-rosetted cells were cytotoxic in only one case. HBsAg-positive and -negative cases reacted similarly. In six patients the addition of aggregated IgG significantly reduced cytotoxicity. The authors concluded that the effector cell was a K cell mediating an ADCC reaction directed against a liver-specific membrane lipoprotein. Antibody-dependent cellular cytotoxicity (ADCC) is produced by PBMCs which bear receptors for the Fc piece of IgG. These Fc receptors enable them to bind to and lyse target cells which are coated with antibody. The PBMCs capable of ADCC include null cells (lacking T and B cell markers and known as killer cells or K cells) monocytes, and perhaps some T cells [48].

There were, however, several problems in these studies. T cell-enriched populations could have been more conventionally prepared from the cells sedimented after E rosetting, because the use of EAC rosetting to deplete K cells is controversial. While some K cells might bear a receptor for complement [49], the majority probably do not [64]. Still, some Fc receptor-positive cells might have been removed by this technique, because when one does not prepare EAC with pure IgM, some IgG Fc pieces might remain available after complement treatment, yielding EA rosetting [51]. In this study EAC were prepared with unspecified antibodies. Furthermore, a more rigorous proof that aggregated IgG was inhibiting cytotoxicity by blocking K cell Fc receptors, rather than acting in a nonspecific fashion, would have required the use of aggregated F $(ab')_2$ fragments of IgG as a negative control. Moreover, this assay provided no ready source of anti-LSP to coat the target cells. The authors postulate that antibody might have been secreted by B lymphocytes, or that immune complexes passively transferred on Fc receptor-positive cells might have dissociated and bound to the target cells. Lastly, had the potential for ADCC been present, the added anti-LSP should have enhanced rather than inhibited cytotoxicity.

Similar studies by Kakumu et al. used Chang cells as targets and found the effector cell in an E rosette-negative, EAC rosette-positive lymphocyte population [33]. Cytotoxicity was not depleted by removal of glass adherent cells (monocytes), and was again diminished by addition of LSP or complexed IgG [32]. While these workers also concluded they were observing ADCC, questions raised by the absence of anti-target cell antibodies and the ability of LSP to inhibit cytotoxicity were not answered. The latter point is particularly important, because some Chang cells have been demonstrated to lack LSP on their surface membranes [44]. In addition, another study suggests that PBMCs from patients with CAH have a diminished capacity for ADCC [6]. Similar cytotoxicity studies employing rabbit and rat hepatocytes as target cells did not further clarify this situation [18, 21]. Nevertheless, the demonstration of IgG bound to the surface membrane of hepatocytes from patients with CAH [25, 26] raises the possibility that ADCC might occur in vivo.

Another study suggested that the monocyte/macrophage was the effector cell. Vogten et al. used avian erythrocytes coated with LSP as target cells [65]. Half the CAH patients studied had cytotoxic PBMCs in this system, compared to 5% of healthy controls. Cytotoxicity was abolished by depletion of cells phagocytic for

iron filings (monocytes), contradicting the finding of Kakumu et al. that depletion of glass adherent cells did not diminish cytotoxicity [33]. As in previous studies [11, 62] cytotoxicity was diminished by LSP, anti-LSP, and aggregated IgG.

Finally, Vierling et al. studied cytotoxicity to Chang and E1-4 tumor cells [63]. They found no difference in ADCC between PBMCs from CAH patients and those from normal controls. In addition, the capacity for natural killer (NK) activity and mitogen-induced cytotoxicity was similar in CAH patients and controls.

Taken together, these reports present multiple contradictory findings. To date, the identity of the subpopulation(s) of PBMCs from CAH patients that mediates cytotoxicity in vitro has not been identified. However, despite the apparent contradictions, these studies have demonstrated cellular cytotoxicity to a variety of target cells and thus provided a plausible mechanism for destructive autoimmunity in CAH.

The tumor and animal cells used as target cells in these assays have limitations because they might not behave as reliable models for in vivo cytotoxicity. Therefore, several groups sought direct evidence for cytotoxicity against autologous hepatocytes isolated from liver biopsy specimens. Wands and Isselbacher reported enhanced killing of both autologous and Chang liver cells by PBMCs from CAH patients relative to that seen in CPH patients and healthy controls, although only two CAH patients were negative for HBsAg [68]. Paronetto and Vernace reported similar findings using early subcultures of autologous liver cells, although in their assay cells from CPH patients also demonstrated enhanced killing [46]. Thus, patients with CAH have circulating lymphocytes that are capable of destroying their own liver cells in vitro. Other studies provided support for this concept but the results were not as unequivocal. Geubel et al. found significantly greater hepatocyte killing by autologous lymphocytes than by normal allogeneic lymphocytes in one half of CAH patients studied, while one third of patients actually showed less killing than normals [23]. These studies provided evidence for the occurrence of cellular immune hepatic injury in vivo in CAH, and confirmed the enhanced cytotoxicity described in the other assay systems. In addition, these investigators found that cytotoxicity was markedly decreased after treatment of the patients with immunosuppressive agents such as prednisone and azathioprine, providing experimental support to the rationale for this therapy.

Endogeneous factors suppressing cellular immunity also drew increasing attention. It had been noted in several studies that autologous serum contained a factor(s) which appeared to inhibit cytotoxicity of PBMCs in CAH patients [32, 46, 67, 70]. In some [6] but not all cases, this factor suppressed ADCC and might reasonably be assumed to consist of immune complexes. Other factors seemed to suppress cellular proliferation. In Geubel's study [23], he noted that sera from those patients with cytotoxicity less than control inhibited mitogen-induced transformation of normal lymphocytes. Other workers noted a similar phenomenon in patients with cholestatic liver disease [59] and demonstrated that this was associated with an increased level of circulating bile acids [34]. Others have isolated immunosuppressive proteins from human liver homogenates [54]. These factors have not been well characterized and their importance in vivo is unknown.

Finally, there was growing evidence from animal studies that suppressor T cells played a key role in regulation of the immune response [47]. Such cells could also be

demonstrated in man [53]. It therefore seemed a reasonable hypothesis that lack of normal suppressor function might allow a persistent cellular immune response directed against hepatocyte antigens in CAH [17]. Following the description of suppressor cell generation after incubation of PBMCs with concanavalin A (Con A) [55], Hodgson et al. studied Con A-stimulated PBMCs from 14 CAH patients, 11 of whom were negative for HBsAg [24]. They found defective suppression of mitogen-stimulated blastogenesis by Con A-stimulated PBMCs from the majority of CAH patients. Con A-stimulated PBMCs from patients with acute hepatitis and other inflammatory diseases produced levels of suppression comparable to that seen with cells from healthy donors. This observation raised the possibility that defective immunoregulation might create a permissive environment for harmful unchecked cellular and humoral immune reactivity. It is quite possible, however, that the defect observed in suppressor cell generation did not contribute to the development of CAH but rather arose secondary to the disease itself.

Lymphocyte Subpopulations

Several groups sought insight into immune effector mechanisms by studying lymphocyte subpopulations in patients with CAH. The earliest studies examined lymphocytes in peripheral blood. De Horatius found a decrease in the absolute and relative numbers of T lymphocytes as measured by E-rosetting in HBsAg-negative patients, while there was a less than compensatory increase in B lymphocytes detected by immunofluorescence [14]. In contrast, Galili et al. reported normal T and B lymphocyte proportions as measured by E- and EAC-rosetting [22]. Thomas et al. found decreased T and increased null cell populations in all types of liver disease studied and concluded the changes were secondary to liver dysfunction [61]. Columbo et al. agreed that total T lymphocytes were decreased in HBsAg-negative CAH, but found increased "active" T cells in that subgroup that had not developed cirrhosis [12]. Since "active" T cells, characterized by rapid rosette formation with sheep erythrocytes, may correlate with delayed-type hypersensitivity reactions [20], these results are compatible with active immune liver damage which diminishes as cirrhosis ensues. Other workers, found greater depression of T cells in the presence than in the absence of established cirrhosis [19]. No conclusions could be drawn from these studies.

Subsequent investigations examined liver-derived lymphocytes, since changes in PBMCs might not necessarily reflect the characteristics of the cells found at the site of tissue injury. Miller et al. prepared single cell suspensions of lymphocytes from liver biopsy specimens from patients with chronic hepatitis [42]. T and B lymphocytes were enumerated by E- and EAC-rosetting, respectively. There was a predominance of T cells in the hepatic infiltrates of all patients. This was not unique to CAH, as Husby et al. had reported a similar finding in alcoholic liver disease [29]. However, there was a much smaller ration of T : B cells in HBsAg-negative than in HBsAg-positive CAH (2 : 1 compared to 20 : 1). In addition, the percentage of T cells in peripheral blood was depressed in HBsAg-positive cases but not in negative cases. The authors suggested this was further evidence that HBsAg-positive and negative CAH have different etiologies. In addition, the presence of "null"

lymphocytes in the livers of HBsAg negative CAH patients suggested a role for K or NK cells in hepatocyte death.

These finding were not confirmed by Fargion et al., who found almost as many B as T cells in liver biopsies of CAH patients, and reported similar T : B cell ratios for HBsAg-positive and -negative cases [19]. Their technique differed from that of Miller, however, detecting T and B cells by immunofluorescence and EAC-rosetting, respectively, in intact histological sections.

The data is thus inconclusive, and its interpretation is compromised by several additional factors. Firstly, it is difficult to compare studies because different techniques were used, the details of which are important. For example, EAC-rosettes might detect macrophages and K cells as well as B cells, resulting in a falsely elevated B cell count. Fluoresceinated intact antibodies, rather than F $(ab')_2$ fragments thereof, might bind to Fc receptors on non-B cells, again inflating the B cell count. Microaggregates of fluoresceinated IgG could produce a similar artifact if this reagent was not subjected to ultracentrifugation prior to use. Secondly, serum factor(s) in liver disease may interfere with detection of T lymphocytes by E-rosetting [10, 52]. Additional T cells might have been detected had the authors attempted to rosette with neuraminidase-treated sheep erythrocytes, a procedure which has been shown to enhance E-rosette formation [71]. Thus, firm conclusions about lymphocyte subpopulations in CAH can only be drawn after further work has resolved these uncertainties.

Discussion

Taken together, these investigations provide indirect evidence for the presence of autoimmunity in CAH. However, none of the studies, with the possible exception of the induced rabbit hepatitis of Meyer zum Büschenfelde [40], suggest that this immunity is a cause rather than an effect of the disease. In addition, areas of controversy remain which hinder interpretation of much of the data.

Studies employing LSP depend on the reported liver-specificity of this lipoprotein. Yet this material is prepared under conditions that, despite column chromatography, leave it contaminated with other large and/or hydrophobic hepatocellular molecules [15], and further purification has not been reported. Furthermore, LSP preparations are labile, with antigenic characteristics that deteriorate progressively depending on such variables as buffers and temperature [9]. Optimal preparations require fresh liver from operative specimens, as the yields decrease postmortem [37]. Raising antibodies to LSP can also be difficult. Even if the LSP is not denatured during preparation, an animal injected with it might respond preferentially to other organ-nonspecific antigens present.

The most recent effort to clarify this situation was performed by Behrens and Paronetto who re-evaluated the organ-specificity of LSP [3]. They compared LSP and LP-2 with similarly prepared kidney tissue analogues, KSP and KP-2. They were unable to demonstrate liver specificity for LSP or LP-2 or the antibodies raised to them, finding the caracteristics of the kidney preparations virtually identical. Furthermore, they examined sera from CAH patients for the presence of antibodies to these antigens. The sera were able to induce ADCC to erythrocytes coated with

one of the kidney antigens as well to those coated with LSP or LP-2 [4]. They concluded that LSP and LP-2 are not liver specific, and that antibodies produced in CAH detect liver cell membrane antigens sharing common determinants with kidney cells. In addition, they observed that their LP-2 was a protein from the cell membrane rather than the cytoplasm, as previously described [41]. This conclusion was supported by the ability of human LP-2 preparations to induce anti-hepatocyte antibodies and hepatitis in rabbits [40]. They conceded, however, that their LSP might contain liver-specific antigens of low concentration, undetectable by the methods employed. In a critical review, Chisari concluded that at least one and perhaps several liver-specific antigens appear to exist, but they are labile, elusive, and not detectable in every preparation [9]. It is therefore difficult to compare results of different laboratories when each group is using a different source of liver lipoprotein. Clarification of this problem will await further purification and characterization of LSP.

There are additional unanswered questions raised by studies of humoral effector mechanisms. Several studies suggest that immunoglobulin binds to hepatocytes in vivo in patients with CAH. The specificity of a granular distribution reported for HBsAg-positive CAH [26] has been disputed [1], however, and the possibility exists that the linear pattern seen in HBsAg-negative CAH [26] was due to anti-smooth muscle antibody. In addition, it is not known whether redistribution of hepatocellular membrane molecules after antibody binding ("patching") played a role in the creation of the observed immunofluorescent patterns. Moreover, nonspecific binding of fluoresceinated IgG to hepatocyte Fc receptors was not formally excluded with F $(ab')_2$ reagents. The role played by hepatocyte-bound antibody in CAH is also unknown. Neither complement activation nor ADCC have been convincingly demonstrated in vitro, and the possibility that these immunoglobulins act as blocking antibodies which protect hepatocytes has not been excluded. Furthermore, the antigenic specificity of these antibodies has not been conclusively established.

Investigations of cellular immunity in CAH have also produced conflicting results. Firm evidence implicating a specific mononuclear cell subpopulation(s) as the effector cell has not been produced. This is partly due to the lack of suitable target cells, which makes this area difficult to study. The most meaningful target cells are those syngeneic to the lymphocyte donor, such as autologous hepatocytes. Zinkernagel and Doherty have demonstrated that, at least for mediating cytotoxicity towards virally infected cells in inbred mice, T cells must recognize self-histocompatibility antigens to function optimally [72]. However, when autologous hepatocytes are used as target cells, one cannot be certain that neoantigens were not created during preparation. Furthermore, it might not be valid to compare killing of hepatocytes by autologous PBMCs with that from healthy but allogeneic controls. Finally, when xenogeneic or tumor cells are used as targets, cell lysis might reflect natural killer activity rather than antigen-specific cytotoxicity.

In addition, there are hazards in those experiments where LSP was used to inhibit cellular immunity and thus demonstrate the specificity of the reaction. A species of low-density lipoprotein in normal serum can inhibit immune responses by binding to receptors on the lymphocyte surface [13]. Because LSP is a low density lipoprotein, it is conceivable that it could modulate cellular immune responses.

Lastly, "autoimmune hepatitis" is a difficult disease to study because it might not have a single cause. Indeed, this entity might represent a spectrum of diseases. In addition, it is probable that not all the patients with HBsAg-negative CAH in these investigations had the "autoimmune" variety. Many could have had, CAH secondary to hepatitis B with a serum level of HBsAg too low to be detected, or non-A, non-B hepatitis, or CAH due to occult use of drugs such as oxyphenisatin [50]. Additional testing for antibody to hepatitis B core antigen might have shown some of these patients to actually have CAH due to hepatitis B.

Conclusion

"Autoimmune" hepatitis is a form of chronic active hepatitis without a known etiology. Epidemiologic, genetic, and serologic evidence suggest that it is distinct from HBsAg-positive CAH. Unlike HBsAg-positive CAH, it is highly associated with HLA B8, and antibodies against autologous tissues are frequently present. In addition, there is an increased association between this type of CAH and suspected autoimmune diseases such as thyroiditis, pernicious anemia, and Addison's disease, which are also highly associated with this HLA haplotype. These observations have fostered speculation that this form of CAH is caused, at least in part, by an attack on the liver by the immune system. Much effort has been expended in pursuit of evidence for this possibility. These investigations suggest that both cellular and humoral immunity to liver cells exists in patients with this disease. Studies have shown that lymphocytes from these patients are cytotoxic to autologous and rabbit hepatocytes and demonstrate cellular immunity to antigens derived from hepatocyte plasma membranes. In addition, autologous hepatocytes have been shown to be coated with IgG. Furthermore, evidence has been obtained that defective immunoregulation might exist in this disease which could foster cellular and humoral immune hepatic damage. Nevertheless, there has been no demonstration that these phenomena are a cause, rather than an effect, of "autoimmune" hepatitis.

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Drug-Induced Chronic Hepatitis

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Introduction

Many drugs can produce hepatic damage, either as the result of a direct toxic effect on the liver which is dose-dependent or as an unpredictable effect which may or may not be a true drug hypersensitivity. When due to a direct hepatotoxin, hepatocellular necrosis varying in severity will be produced. All individuals are susceptible, the lesion is reproducible in animals and there is a direct dose relationship. Even after severe hepatic necrosis, should the patient survive, agents producing a direct hepatotoxic reaction seldom lead to a chronic lesion.

Hepatic injury due to drug allergy occurs in only a small proportion of patients taking the drug. It is not dose-dependent and cannot be produced in experimental animals. The hypersensitivity reaction may be largely confined to the liver, as with chlorpromazine, or may occur as part of a generalized hypersensitivity reaction, including skin rashes and arthritis. The hepatic damage may be indistinguishable histologically from acute viral hepatitis, or there may be little necrosis and it may be predominantly cholestatic. A mixed picture is common. There may be a circulating eosinophilia or an eosinophilic infiltration with or without granuloma formation on liver biopsy. Positive skin test reactions to the drug, specific circulating antibodies or lymphocyte transformation can only rarely be demonstrated [46]. In many instances the drug probably acts as a hapten, but surprisingly little is known about the basic underlying immunological mechanisms. It is not known why certain individuals show this susceptibility, although in some patients there is a personal or family history of atopic disorders, but no specific HLA type has been identified. In some instances there is the possibility that the adverse effect of the drug is associated with its altered metabolism; for example, slow acetylators of isoniazid are more likely to have a hepatitis-like reaction than those who metabolise the drug rapidly [15].

The following forms of chronic hepatitis can occur after drug administration, though their frequency varies considerably.

1) Chronic persistent hepatitis with or without continued administration of the drug.

2) Chronic cholestasis, which may or may not resolve on cessation of the drug.
3) Chronic granulomatous hepatitis.
4) Chronic active (aggressive) hepatitis.
5) Inactive post-necrotic cirrhosis following submassive necrosis.

All forms of chronic liver disease following drug administration are rare, although the role of drugs in the induction of chronic active hepatitis is being recognised with increasing frequency, and it is this area which will receive particular attention.

Drug-Induced Chronic Active Hepatitis

Chronic hepatitis has been defined as prolonged inflammation of the liver lasting for six months or more [16]. The characteristic histological lesion in chronic active hepatitis is piecemeal necrosis with or without cirrhosis. Chronic infection with hepatitis B or non-A, non-B viruses is the commonest cause worldwide, but in the developed countries a large proportion are of unknown aetiology. Some of these may be due to an autoimmune hepatitis, others are associated with alpha-l-antitrypsin deficiency [45]. Over the past ten years, an association with drug ingestion has been recognised with increasing frequency. This subgroup is particularly important to identify because stopping the drug often results in resolution of the hepatic lesion, particularly if cirrhosis has not been established.

In some instances, subacute or bridging hepatic necrosis may be present histologically early in the development of the hepatic abnormality, but subsequently resolves completely. Such cases do not strictly fulfil the criteria for chronic active hepatitis. For example, the extensive bridging necrosis resulting from halothane-induced hepatitis usually resolves, both histologically and biochemically, without residual disease [22]. In a study from Johns Hopkins [32], 18 of 42 patients with bridging hepatic necrosis on liver biopsy had a presumed drug aetiology, and similarly a significant proportion of the cases described by Ware et al. [41] could be attributed to drugs. The bridging necrosis which is associated with drugs does not have the same adverse prognosis as when associated with viral hepatitis [4]. The increasing awareness of drugs as a cause is illustrated in Table 1, where the high proportion of drug-induced chronic active hepatitis in some recent series is much greater than the low frequency in large earlier series.

In the absence of specific immunological markers for drug-induced chronic active hepatitis, the diagnosis is based on clinical and histological grounds. Since the disease may be insidious and the drug intake unclear, diagnosis may be very difficult. A typical example of this difficulty occurred with the recognition that the component of some laxatives, oxyphenisatin, can produce chronic active hepatitis.

Oxyphenisatin, methyl-dopa, and nitrofurantoin are the drugs which have most frequently been incriminated in association with chronic active hepatitis. Less well established are associations with isoniazid, propylthiouracil, aspirin, paracetamol, sulphonamides, dantrolene, and halothane. Although many of these drugs produce an acute hepatitis-like lesion, sometimes fatal, or a chronic persistent hepatitis while the drug is being taken, it is much less common for them to produce a cirrhosis or chronic active hepatitis.

Table 1. Proportion of patients with drug-induced chronic active hepatitis

Drug-induced	Total number	Reference
14	21	Goldstein et al. 1973 [10]
5	12 (women over 30 years)	Cooksley et al. 1973 [5]
41[a]	85	Dietrichson 1975 [6]
12	41	Lindberg et al. 1977 [17]

[a] Nineteen challenged with oxyphenisatin. Eight showed deterioration in liver function.

Oxyphenisatin

The first cases of chronic active hepatitis which could clearly be attributed to a drug occurred with this substance, a constituent of many laxatives. In an elegant clinical study, Reynolds et al.[24] reported two patients with classic features of chronic active hepatitis with autoantibodies, who responded to corticosteroid drugs and withdrawal of the laxatives, and relapsed clinically, biochemically, and histologically when the oxyphenisatin component of the drug was introduced (Table 2). Both patients were women in their thirties. They reported five further cases in which there was strong evidence to support a similar aetiology. Subsequently, additional cases have been reported from Australia [5, 10, 42], Scandinavia [6, 9], and the United States [20]. The clinical and histological features closely resemble those found in autoimmune chronic active hepatitis. The onset is usually insidious, but may be acute. A positive LE cell phenomenon and autoantibodies, such as antinuclear antibody and smooth muscle antibody, are often present. Most patients are female, although it is not clear whether this is due to the greater frequency with which woman take laxatives. It is usual for the lesion to resolve clinically, biochemically, and histologically after the laxative containing oxyphenisatin is stopped. Some patients have been shown to have the histocompatibility antigen HLA B1 and 8. It seems likely that this disorder occurs on the basis of a true hypersensitivity reaction, since many individuals take the drug without any abnormality of liver function tests. Nevertheless, because of the difficulty in diagnosis, it has been withdrawn in most countries.

Methyldopa

Soon after its introduction into clinical usage, this commonly used antihypertensive drug was noted to produce an immunological abnormality in serum, namely a positive Coomb's test. Subsequently, occasional cases of jaundice were reported, though they were usually mild and liver function often returned to normal despite continued use of the drug [7]. However, it soon became clear that more severe and even fatal hepatitis could occur [19, 25, 35]. Both acute and chronic hepatitis have been described, and both appear to be more common in women. Cases resembling chronic active hepatitis with or without positive immunological phenomena or cirrhosis have been reported [34, 35]. In one case with a positive LE cell phenomenon, progression from subacute hepatic necrosis to cirrhosis occurred with disappearance of the immunological markers [27].

Table 2. Results of liver function tests in two patients with chronic active hepatitis after challenge with oxyphenisatin.
After Reynolds et al. 1971 [24]

Case No.	Bilirubin mg/100 ml	Serum glutamic-oxaloacetic transminase Karmen U/ml
1: Day 1	0.4	39
Day 2	0.3	14
Day 3	1.1	158
Day 4	1.9	120
Day 5	1.0	109
Day 6	1.2	34
2: Day 1	0.6	22
Day 2	0.5	28
Day 3	0.4	29
Day 4	1.7	645
Day 5	3.9	240

Because the spectrum of severity of liver disease is so wide, and because cases of fatal hepatic necrosis have been reported after reintroduction of the drug during challenge experiments, any abnormality of liver function should be taken seriously. The drug should then be discontinued and not reintroduced, particularly since good alternative hypotensive agents are available.

Nitrofurantoin

Nitrofurantoin is a commonly used urinary tract antiseptic, and is often given to women for long periods of time. It is estimated that approximately five million courses of nitrofurantoin or one of its analogues are given yearly in the United States and Canada [36]. There is a wide spectrum of hepatic abnormalities associated with nitrofurantoin toxicity, ranging from an acute hepatitis with or without cholestasis to a granulomatous hepatitis and chronic hepatitis with or without cirrhosis [29]. Apart from the hepatic side effects, other abnormalities may occur, including haemolytic anaemia, leucopenia, pulmonary fibrosis, and peripheral neuropathy.

Over the past few years, a number of cases of chronic active hepatitis have been reported [2, 11, 29]. Sharp and associates [29] reported five cases of chronic active hepatitis associated with nitrofurantoin, including two deaths, and reviewed the literature on a total of 20 patients. Of the 20 patients they reviewed, 18 improved clinically and biochemically when the drug was withdrawn, but four developed cirrhosis. Two patients who had been on the drug for more than one year developed progressive hepatic failure with severe hepatic necrosis and died.

A number of interesting immunological abnormalities have been observed in patients showing adverse reactions to nitrofurantoin. Antinuclear antibody was present in 12 of the 20 patients in the above series, and another characteristic feature was elevation of the gammaglobulin and a low serum albumin. Additional

autoantibodies include smooth muscle antibody, but all were negative for HBsAg. Eosinophilia is usually not pronounced. Of interest are the other associated abnormalities, such as pulmonary fibrosis, and in two patients with chronic active hepatitis reported by Klemola et al. [14], pulmonary infiltrates were present as well as the chronic active hepatitis.

In one of two patients with nitrofurantoin-induced chronic active hepatitis reported by Black et al. [2], specific lymphocyte transformation to nitrofurantoin was observed within three months of presentation, but had become negative seven months later. Another immunological abnormality which has been noted in patients showing an adverse effect to nitrofurantoin is the presence of immune complexes of polyclonal IgG antibody and autologous albumin. It is not known why some patients receiving nitrofurantoin develop an abnormal immune response, but Hatoff et al. [11] have reported a patient with nitrofurantoin-induced chronic active hepatitis who had the HLA-B8 antigen. The significance of this is unclear, although Lindberg et al. [17] have detected HLA-B8 in 42% of patients with drug-induced chronic active hepatitis, compared with 23% in a control population.

As is the case with drug-induced systemic lupus erythematosus, it is not clear whether these immunological markers indicate that the drug is unmasking a genetic predisposition to the development of liver disease, or whether they are a consequence of the tissue damage. Tolman [36] has reviewed this issue critically and points out that patients receiving nitrofurantoin have been shown to have high titres of circulating antibodies and hypergammaglobulinaemia in the absence of liver disease, and that although clinical improvement may occur after withdrawal of the drug, the autoantibody may persist. As with other forms of drug-induced liver disease, virtually all the cases reported have been women, but this may merely reflect the higher frequency of long-term nitrofurantoin administration to women [36].

Miscellaneous Drugs

Many other drugs have been reported to produce chronic active hepatitis, but these are small series or individual case reports. They are usually incriminated on the basis of improvement or disappearance of the histological lesion and abnormal liver function tests on removal of the drugs but, even so, the association is often not clearcut [20, 46]. Although severe hepatic necrosis and chronic active hepatitis is rare with isoniazid, the extensive use of this drug not only to treat patients with active tuberculosis but also those with a positive tuberculin test makes these adverse reactions of special importance. Mild disturbances of liver function producing a chronic persistent hepatitis (see below) are more common, but cases of chronic active hepatitis with or without cirrhosis have been reported [1, 18, 23]. Disturbances of liver function with sulphonamides are rare, but occasional cases of acute hepatic necrosis have been reported. Tonder et al. [37] have reported a case of chronic aggressive hepatitis which improved on removal of the sulphonamide and where there was deterioration of liver function within hours of its reintroduction. Similarly, rare cases of chronic active hepatitis have been attributed to propylthiouracil [8] and the long-acting skeletal muscle relaxant dantrolene [39]. Much less certain is the alleged association with chronic paracetamol [3] and aspirin [28, 43] ingestion.

Although there now seems little doubt that halothane can occasionally produce fulminant hepatic failure [30] and that minor histological abnormalities may be quite common [38, 44] the development of chronic active hepatitis is poorly documented. Thomas [33] reported a case of chronic aggressive hepatitis which followed an acute lesion and Klatskin and Kimberg [13] have reported an anaesthetist who had recurrent episodes of halothane hepatitis which progressed to cirrhosis. We ourselves have seen a patient who had repeated halothane anaesthetics, developed an acute hepatitis which progressed to a chronic aggressive hepatitis with cirrhosis and splenomegaly over a period of six months. Most cases of halothane hepatitis, however, either die or, despite extensive bridging necrosis, recover without residual liver damage [22].

Chronic Persistent Hepatitis

Many of the drugs mentioned above can go on to produce a chronic persistent hepatitis with spotty hepatic necrosis and enlargement of the portal tracts, but without a true chronic aggressive hepatitis on biopsy. This can occur particularly with isoniazid and nitrofurantoin as well as with aspirin, and it is important to monitor liver function when patients are on these drugs. The abnormality in liver function and the histological lesion usually regresses on withdrawing the drugs.

Granulomatous Hepatitis

Granulomas may occur in the liver as part of a generalized reaction to a number of drugs, but are often transient. In some a chronic granulomatous hepatitis may occur and these drugs include phenylbutazone [12], allopurinol [31], and methyldopa [21].

Chronic Cholestasis

This is extremely rare, though cases of prolonged cholestasis with features of primary biliary cirrhosis have been described [26, 40].

Cirrhosis

Progression to cirrhosis may occur with any of the drugs listed above associated with chronic active hepatitis. The evidence that a post-necrotic cirrhosis can follow acute hepatic necrosis is much less clearcut and, as indicated earlier, severe hepatic injury due to paracetamol or halothane usually resolves completely if the patient survives.

Conclusion

Drug-induced chronic hepatitis is being recognised with increasing frequency, and in some series of chronic active hepatitis costitutes a large proportion of the cases. The identification of the drug as a causative agent is based on clinical, biochemical, and histological grounds with regression on its removal and relapse after challenge. The drugs most commonly associated with chronic active hepatitis are oxyphenisatin, methyldopa, and nitrofurantoin, but it may rarely be seen with other agents.

Immunological mechanisms are thought to be responsible on the basis of a drug hypersensitivity reaction. A high proportion of cases have smooth muscle or antinuclear antibody and some a positive LE cell phenomenon. Specific lymphocyte transformation to the drug can only rarely be demonstrated, and it is not clear whether these immunological phenomena are causative. The reactions are most likely to occur in women, but this may only represent their greater usage of the drugs. In some cases there is an association with the HLA-B8 haplotype. Progression to cirrhosis may occur, and since regression is usual on stopping the drug, its recognition as the causative agent is important. A chronic persistent hepatitis may also be associated with drugs such as isoniazid, but chronic cholestasis and postnecrotic cirrhosis are rare.

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Clinical, Histologic, and Immunopathologic Features of Primary Biliary Cirrhosis

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1. Introduction

Primary biliary cirrhosis (PBC) was previously considered a rare disease which was diagnosed almost only at autopsy. Liver biopsy, both surgical and by needle, sometimes assisted by laparoscopy and laboratory data, has led to its more frequent recognition, although it remains one of the less common liver diseases. Immunologic parameters have assumed great diagnostic significance. This has also led to the assumption that immunologic processes are the basis of the pathogenesis of PBC. The next two chapters are devoted to the establishment and clarification of this assumption. In addition, there is some basis for this concept in the clinical and particularly in the pathologic evolution of the disease, to which this chapter is devoted, supplemented by limited immunopathologic data.

PBC is about ten times more common in females than in males [13, 83] and almost always becomes apparent only after 30 years of age, most frequently between 40 and 60 years. The prototype starts with an asymptomatic or oligosymptomatic stage recognized by elevation of alkaline phosphatase activity and by the presence of mitochondrial antibodies. This stage may persist for up to 10 years. Usually, manifestations such as pruritus and hepatomegaly develop earlier, associated with slight elevation of aminotransferase activities. Eventually, jaundice and other skin manifestations such as xanthomas and increased pigmentation develop, as well as portal hypertension, and eventually fatal hepatic failure sets in. This sequence is often modified as far as duration of stages, appearance of specific features, and associated symptoms are concerned.

The main obstacle to understanding the pathogenesis of PBC is determining which of its clinical, laboratory, and morphologic expressions are related to the initiation of the disease and which are consequences of other processes.

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Basically, four processes stand out in the evolution of PBC: (1) etiology, including genetic features; (2) immunologic (clinical and histologic) features; (3) disturbance of bile flow, progressing to cholestasis; and (4) hepatic derangement other than biliary and its consequences, culminating in cirrhosis.

2. Clinical Observations

2.1. Etiologic and Genetic Factors

A genetic predisposition to PBC is best supported by the conspicuous female predominance, but it is weakened by the age restriction. Whereas superficial similarities exist in intrauterine of neonatal destruction of bile ducts, including biliary atresia (now best designated as infantile obstructive cholangiopathy [49]), this disease has no relation to PBC in adults and may be the result of a viral infection. The genetic predisposition to PBC is best supported by the immunologic alteration found in relatives and by a few reported familial occurrences [9, 11, 27, 80, 81]; there is one reported instance of a combination of genetic susceptibility with environmental factors [18]. Although PBC was originally recognized mainly in middle-class white women, it is now diagnosed in all races and geographic locations, suggesting that recognition rather than genetic factors accounts for the previously assumed predilection.

The suggestion that drugs produce PBC is based on observations that either jaundice or pruritus with elevated alkaline phosphatase activity sets in after exposure to such drugs as phenothiazines, contraceptives, or anabolic steroids [31, 39, 73]. However, it is now clear that patients with PBC are particularly susceptible to cholestatic drugs, which probably unmask a preexisting disease rather than cause it. The same is probably true for the onset of PBC in pregnancy. While drugs may produce prolonged cholestatic jaundice with cholangitic features and even severe pruritus, the lesion eventually disappears. This is well illustrated by the hepatic reaction to organic arsenicals, probably the first hepatic drug reaction described [35].

A primary role of altered bile acid metabolism has been considered. Lithocholate in animals produces an inflammation of the portal tracts which has no relation to PBC [5]. While high bile acid levels characterize the precholestatic stages of PBC, at this time they have to be considered a secondary effect rather than a cause. The stimulation of biliary epithelium to cell division by lithocholate [5] may account for rare consequences of PBC, including carcinoma [47], but appears more important in sclerosing cholangitis.

Biliary tract diseases such as cholelithiasis and cholecystitis, and also, viral hepatitis may precede the first manifestation of PBC [69], but their connection to PBC may be fortuitous. Gallstone disease may develop during PBC but as a secondary event. Markers of hepatitis B infection are no more frequent in PBC than in control populations.

We are thus left with the vague impression that patients with PBC have genetically "hypersensitive" intrahepatic bile ducts. This is in keeping with the impression that the life span of patients with PBC is shortened by surgical

exploration of the bile ducts [69]; this points to the importance of avoiding this procedure, also discouraged by the modern cholangiographic techniques. The HLA system has been suggested as a marker of this susceptibility, although so far with unconvincing evidence. No association between PBC and HLA-A, B, or C antigens has been found [26]; however, a recent report indicates increased incidence of the DRw3 antigen [22]. An increased incidence of Dw3 antigens has been found in other diseases associated with altered immune responses.

2.2. Immunologic Features

PBC is sometimes associated with diseases designated as collagen diseases and presumed to have an immunologic basis, including scleroderma [62, 63], rheumatoid arthritis [11, 60], thyroid diseases [15], and relatively frequently, Sjögren's syndrome [3, 24]. There is also an occasional association with the CRST syndrome (calcinosis cutis, Raynaud's phenomenon, sclerodactyly, telangiectasis) [75], renal tubular acidosis [24], and celiac disease [54]. The temporal relation between these manifestations and PBC is not clarified and they may precede recognition of PBC. In a sequential study of a large sample [13], scleroderma and fullblown Sjögren's syndrome only occasionally developed during the course of PBC. Possibly, PBC is a manifestation of a generalized "dry gland" syndrome [21].

Anergy to tuberculin and dinitrochlorobenzene has been reported during the course of PBC [24] but it is not related to its clinical evolution, again leaving open the question of whether it is a primary phenomenon. While the nature of PBC is not clear, many observations suggest that at least in some instances it is a systemic rather than solely an hepatic disorder [83] and it has even been designated as a collagen disease [8].

2.3. Disturbance of Biliary Secretion

Conspicuous elevation of activites of alkaline phosphatase, particularly of its hepatic fraction, is an early sign of PBC in the asymptomatic and oligosymptomatic stages. This is accompanied by elevated activities of other enzymes which also reflect disturbed biliary secretion, such as 5-nucleotidase and gamma-glutamyl transpeptidase. Biliary secretion of test dyes is also impaired, although visualization of the gallbladder dyes, while often faint, may succeed in the early stages. The serum bile acid level, more that of chenodeoxycholic than of cholic acid, is conspicuously elevated in the relatively early stages [7]. This explains the early appearance of pruritus, although the relation of the itching to dermal bile acid deposition is still problematic [30]. Hyperbilirubinemia may be absent for long periods, and frank jaundice sets in after a variable duration of the disease, sometimes relatively late and sometimes fluctuating. Jaundice, particularly deepening, is prognostically the most ominous sign, if not explained by administration of cholestatic drugs. Survival after the appearance of conspicuous jaundice is usually short [13, 82]. Hypercholesterolemia, though present in the early stages of PBC, is more frequent in later stages; at levels above 500 mg/dl it may be associated with skin xanthomas, particularly if total lipids are above 800 mg/dl. Hypercholesterolemia and xanthomas may yield to cholestyramine therapy, but they may also disappear as a result of failure of hepatic function. Peripheral neuropathy may also be observed.

Myocardial infarction caused by arteriosclerosis occasionally accounts for death not explained by hepatic failure [80].

Other relatively late manifestations of PBC are related to prolonged impairment of biliary secretion of bile acids. They include steatorrhea and diarrhea, and low vitamin A blood levels, not associated with visual disturbances [38]. Of major importance are bone lesions (osteomalacia and osteoporosis) [74] confirmed by bone biopsy and reflected as pain in the back and over the ribs and spontaneous fractures, particularly collapse of vertebrae. These lesions are induced or aggravated by corticosteroid therapy. They are the result of a relative deficiency of vitamin D substrate on a multifactoral basis in which malabsorption, faulty vitamin D metabolism, and excessive urinary excretion may all play a role [56]. Oral administration of 25-hydroxyvitamin D seems to correct the lesion [48].

2.4. Nonbiliary Hepatic Derangement

Hepatomegaly associated with fatigue and vague abdominal pain may be an early reflection of disturbed hepatic function as well as of impaired biliary secretion. The same is true of the early elevation of the erythrocyte sedimentation rate and of aminotransferase activities, the moderate height of which is out of proportion to the high activities of alkaline phosphatase and gammaglutamyl transpeptidase. In general, however, in contrast to other chronic liver diseases, many patients with PBC feel surprisingly well except for itching. In later stages, hepatocellular damage and fibrosis progress to cirrhosis with aggravation of both clinical and laboratory manifestations.

Portal hypertension and its sequelae are usually late symptoms. In some cases, however, manifestations of portal hypertension may precede not only the cirrhotic transformation but even severe hepatic fibrosis. Bleeding esophageal varices are sometimes an early clinical presentation [25, 43, 51, 55, 92]. The moderate splenomegaly, also occasionally seen in the precirrhotic stages of PBC, is only in part explained by portal hypertension in view of the proliferation of splenic cells and, in some instances, of granulomas. Together with the rare peripheral lymphadenopathy, the frequent enlargement of portal lymph nodes may thus also reflect an altered immune status.

3. Histologic and Immunopathologic Features

The study of many biopsies during the evolution of PBC has led to the description of stages [77, 81] and recently to an attempt to assess response to treatment [17, 57]. Experiences with even more patients and, particularly sequential biopsies of the same patients [13] have demonstrated significant overlapping of the stages with recurrence of early features in subsequent biopsies taken years later. Nevertheless, the framework of stages is retained here to develop a common evolution. However, these stages serve as a diagnosis rather than prognosis or therapeutic indications.

3.1. Stage 1: The Ductal Stage (Chronic Nonsuppurative Destructive Cholangitis)

The characteristic lesion involves interlobular and septal bile ducts, approximately 100 μm in diameter. They often show uniform alterations of the epithelial lining

which is high columnar, eosinophilic, slightly granular, and not vacuolated. Intracytoplasmic filamentous structures grouped into bundles are seen under the electron microscope [10]. Some nuclei are very slender and hyperchromatic, and few lymphocytes lie between the epithelial cells. There are often short papillary extensions of the epithelial lining into the lumen. The basement membrane is broken in part of the circumference and then the lining is heavily infiltrated by lymphocytes and plasma cells while the epithelial cells are swollen and sometimes desquamated. Outside these "ulcerations", lymphocytes and IgM and IgG-containing plasma cells [66] intermixed with a few eosinophils but no segmented leukocytes, accumulate in circumscribed foci in the edematous, slightly enlarged but round portal tracts. Bile ductules do not proliferate and hepatocytes in the intact limiting plate do not show alterations. Complement and immunoglobulins have been identified in the bile ducts [66]. These deposits can be seen focally but, more frequently, they are localized in the entire circumference of the duct. Copper cannot be detected. Sometimes the destruction involves the entire circumference of the duct, which is surrounded by inflammatory cells. Three-dimensional reconstruction [53] demonstrated that the destruction is segmental and bile ducts of equal size without destruction or inflammation but with altered epithelium are seen. Some such bile ducts are surrounded by annular fibrosis. Smaller portal tracts reveal nonspecific, mainly mononuclear, slight inflammation. Particularly in portal tracts with bile ducts showing at least partial destruction, epithelioid cells accumulate, eventually to form granulomas next to the lymphocytic and plasma cellular exudate. They contain giant cells which may have inclusions similar to those seen in sarcoidosis. Periodic Acid Schift (PAS)-positive protein strands extend between the epithelioid cells, sometimes even into the lumen of the eroded bile ducts. The granulomas are also surrounded by a layer of lymphocytes. The portion of the bile ducts being destroyed may itself be adjacent to the granulomas. Granulomas are also seen in the portal (usually enlarged) lymph nodes and, exceptionally, in bone marrow, lungs, and spleen. A recent study [65] based on serial sections suggests that bile ducts surrounded by edema and fibrosis may eventually become compressed and be eliminated by atrophy without significant inflammatory changes. However, it is not clear whether this occurs in the first stage.

The hepatocytes in the lobular parenchyma fail to show significant alterations on light microscopy. On electron microscopy, the Golgi zones often have dilated vesicles containing laminated dense material, presumably excess biliary constituents. Centrolobular cholestasis is usually absent and the microvilli of the bile canaliculi are normal [44]. A recent abstract, however, reports obliteration of bile canaliculi by "soldering" without inflammation and associated with dilatation of neighboring larger bile canaliculi [76]. In this stage rare instances of cholestasis not induced by exposure to drugs may be related to these observations.

In contrast to the normal hepatocytes, various sinusoidal cells are activated throughout the entire lobule. Electron microscopy identified them as lymphoid cells, plasma cells, and macrophages [44]. The histiocytic cells may form small nodules of almost granulomatous character scattered throughout the parenchyma. Focal necroses may be conspicuous near areas of sinusoidal cell activation and have been explained by local disturbance of the microcirculation [69].

Recognition of the ductal stage of PBC may be difficult in needle biopsy specimens when large portal tracts are absent. It is suggested by nonspecific portal inflammation associated with activation of the sinusoidal cells in the absence of hepatocytic lesions in a patient with or without mild jaundice but with elevated alkaline phospatase activity and mitochondrial antibodies.

3.2. Stage 2: The Ductular Stage

Portal tracts of the same size or smaller than those showing bile duct destruction in the first stage exhibit conspicuous proliferation of bile ductules with altered epithelium which contains, besides bile pigment, PAS-positive material which on electron microscopy consists of whorls of presumably phospholipid character. They are surrounded by lymphocytes, plasma cells, and eosinophils and also by neutrophilic leukocytes. These ductular cells exhibit many blebs on their luminal surface as well as increased tonofilaments [10]. Their epithelium is infiltrated by lymphocytes, histiocytes, and few IgM, IgA, and IgG-containing plasma cells. The ductules often seem to undergo destruction and fibrosis develops around them. Bile ducts in ths stage rarely show destruction but are conspicuously decreased. This observation has been placed on a firm basis by histometric analysis of the relation of bile ducts to arteries [65]. While normally, and also usually in stage 1, 70% to 80% of the arteries are accompanied by dile ducts, this percentage is significantly reduced in PBC, particularly for arteries with diameters of 35 to 95 μm. Loss of a bile duct is thus recognized by the presence of an artery. Quantitative measurements indicate that this reduction also involves small portal tracts [65]. On cholangiography, the intrahepatic bile ducts are narrower than normal and occasionally slightly deformed [52]. Throughout the portal tracts, various inflammatory cells are seen, including pigmented and lipid-containing macrophages. Complement and immunoglobulins are not detected in the ducts or ductular cells. Granulomas are present about 25% of the time, some undergoing fibrosis. Moreover, a periportal necrosis and partial collapse may be found in a sleeve of lobular parenchyma. This results in an apparent enlargement of the portal tracts which seem to be stellate. In the periportal fibrosing zone, hepatocytes may persist in acinar arrangement.

Three types of portal and periportal inflammatory reactions can be recognized which vary in character and extent in different tracts and even around the same tract. The most common is predominance of macrophages, often pigmented and vacuolated, over lymphoid cells. The cytoplasm of the hepatocytes adjacent to the lesion and also of those trapped in the zone of periportal necrosis is also vacuolated and may contain copper granules, demonstrated by rhodamine or other copper stains or by Shikata's orcein stain. Orcein does not demonstrate copper but demonstrates its binding proteins [84, 86]. Orcein stain is thus of equal significance as, and slightly more sensitive than, conventional copper stains in demonstrating granular deposits of the metal [32]. Serum ceruloplasmin is, however, charcteristically elevated in contrast to Wilson's disease but urinary copper may be elevated. In about 15% of the specimens, Mallory's hyalin is noted in the periportal, mainly vacuolated, hepatocytes [59]. Electronmicroscopically it is identical to the hyalin in alcoholic liver injury [28] but segmented leukocytes are absent. Some of the hepatocytes in presumably more advanced stages now also have bile-pigmented

granules and surround a few, often bulky, bile plugs. This justifies the designation "peripheral cholestasis" rather than "precholestasis".

In a second form, lymphocytes intermixed with plasma cells predominate conspicuously over macrophages in the portal tract and in the periportal necrosis to resemble chronic active hepatitis. The hepatocytes are in close contact with lymphocytes which also infiltrate the surrounding parenchyma to varying degrees. A third reaction, more pronounced in the next (scarring) stage, is predominance of fibrous tissue with limited inflammatory infiltration and increase of fat storing cells.

Two types of lesions radiate from the portal tracts into the parenchyma: one is septa surrounded mainly by macrophages and a few lymphocytes as well as by precholestatic or cholestatic hepatocytes; and the other is streaks of heptocellular necrosis with predominance of lymphocytes. Both may extend to the hepatic vein tributaries as is characteristic of bridging necrosis. The hepatocytes on the lobular periphery are frequently in two-cell-thick plates. Focal cholestasis may be seen within the lobular parenchyma.

3.3. Stage 3: The Scarring Stage

The periportal and septal reactions undergo scarring. Lymphoid cells accumulate, however, in the enlarged portal tracts, in the form of follicles which often show germinal centers. Bile ductules are absent from circumscribed portions of the fibrotic portal and periportal tissue and few bile ducts are found. Cholestasis and copper distribution are prominent in hepatocytes around portal tracts and septa but vary in degree around different tracts. Copper granules are more numerous in each hepatocyte and more cells contain them. The extent of both cholestasis and copper deposition appears to be related to the degree of periportal fibrosis. Centrolobular cholestasis is often conspicuous. Connecting septa produce parenchymal nodules, usually with plates two or more cells thick. Occasionally, areas of multilobular collapse are noted. Single or groups of pseudoxanthoma cells appear throughout the parenchyma.

3.4. Stage 4: The Cirrhotic Stage

The lobular architecture is obscured by regenerative nodules, some multilobular, and by septa, many of them linking central with portal canals; but most of the hepatic vein tributaries are preserved. The dense septa consist of few inflammatory cells except for lymphoid follicles. Sparse bile ducts with normal lining are surrounded by annular fibrosis, thought a few bile ducts may still undergo destruction. Bile ductules are usually absent except for circumscribed foci of proliferation, sometimes associated with active inflammation and accentuated fibrosis. Immunoglobulins and complement are not visualized in the portal tract or parenchyma. The border between parenchyma and septa is as a rule sharp. The peripheral layer of the parenchyma may show cholestasis associated with copper deposition and sometimes with hyalin. However, the periportal parenchyma may also be normal and piecemeal necrosis is rare. Centrolobular cholestasis is usually, but not necessarily, conspicuous.

In a few instances, however, a cirrhosis of mixed macromicronodular type is seen which exhibits septal and periseptal lymphoid and plasma cellular infiltration,

proliferation of bile ductules, limited cholestasis, and absence of hepatic vein tributaries. This picture cannot be distinguished from cirrhosis after hepatitis nor from the cryptogenic type.

4. Differential Diagnosis

Four possibilities arise in the distinction of PBC from chronic active hepatitis (CAH):

1) True CAH may show some features suggesting PBC [3, 4], including bile duct lesions [72] which in typical cases differ from PBC lesions in that they involve only a few medium-sized interlobular ducts in the center of the portal tracts; their epithelial cells are vacuolated and multilayered, narrowing the lumen. Characteristically, the basement membrane, although infiltrated by lymphocytes, remains intact; granulomas are always absent. Copper deposition and peripheral cholestasis are only found in the rare instances of CAH complicated by prolonged cholestasis. The evolution is that of CAH and corticosteroid therapy should not be avoided if it is otherwise indicated.

2) In patients with PBC established by previous biopsy or clinical and laboratory features (including typical mitochondrial antibodies), features of CAH may become prominent in the later stages of the disease. Copper is usually present.

3) Different types of mitochondrial antibodies (trypsin sensitive CAH-PBC mixed antigen) [45] are found in a distinct group of patients with histologic features of both PBC and CAH, which respond well to steroid therapy.

4) The actual combination of both diseases cannot now be excluded since their etiology and pathogenesis are not fully established.

In the foolwing instances, clinical and laboratory features, including cholangiography and determinations of mitochondrial antibodies as a rule permit the *exclusion* of PBC, even in the face of confusing histologic observations:

a) *Sclerosing cholangitis with extrahepatic and/or intrahepatic involvement* is a disease of unknown etiology characterized by hepatic lesions which reflect either the consequences of extrahepatic biliary obstruction by the disease process or involvement of the intrahepatic bile ducts leading, as does PBC, to "vanishing" intrahepatic bile ducts [23, 49]. Intrahepatic involvement is best established by corkscrew appearance of the bile ducts on cholangiography [88]. Based on this picture as reference, the evolution of the disease is now being recognized. It includes a histologic phase similar to CAH. Subsequently, a peripheral precholestasis with copper deposition, and then, cholestasis develops but bile ducts are not actively destroyed, but replaced by scars.

b) *Sarcoidosis* is similar to PBC because of the granulomas in the latter. This difficulty becomes accentuated in the rare instances of bile duct involvement in sarcoidosis, resulting in long-lasting cholestatic jaundice [78]. The prolonged

persistence of the same histologic picture of fibrosing granulomas, in addition to a positive Siltzbach-Kveim test, facilitates the differential diagnosis. True combinations of sarcoidosis with PBC have been considered [87].

c) *Non-Hodgkin's lymphomas* and, exceptionally, *Hodgkin's disease* may destroy bile ducts, with some resemblance to PBC. Besides cytologic characteristics, the uneven involvement of the portal tracts, only some of which are packed with lymphoma cells, is helpful in the diagnosis.

d) A mild degree of *drug-induced cholangitis* is elicited by many cholestatic drugs, but cospicuous lesions have been seen following chlorpropamide and propyl-N-ajmalin [37], both associated with hepatitic features.

e) In long-term survivors of marrow transplants, sparse bile ducts have been explained as a *graft-host reaction* [6], but destruction of the basement membrane has apparently not been found.

f) *Obstruction of large bile ducts* can, as a rule, be distinguished histologically from PBC in the first and second stages. A problem may arise in the scarring and cirrhotic stages can readily be resolved by cholangiography.

5. Postulated Evolution of PBC (Fig. 1)

Although PBC is disease of potentially very long duration, with survival of up to 20 years, the evolution in some patients, particularly those of older age, is conspicuously shortened [13], with morphologic changes rapidly progressing. Jaundice and histologic cholestasis are, as already mentioned, the ominous indication of this progression. Clinical, laboratory, including immunologic and morphologic observations suggest a hypothesis of the evolution, which may also assist in the differential diagnosis. Similarly, therapeutic observations have provided clues as to the evolution, although the benefits are hard to evaluate in a disease of potentially long duration.

5.1. Histogenesis of Initial Stage

The process seems to begin with diffuse subtle changes in a circumscribed part of the intrahepatic bile duct system, designated conventionally as interlobular and small septal bile ducts with diameters of about 100 μm. This is associated with segmental or focal destruction of these ducts, reflected in breaks of their basement membranes and conspicuous inflammatory infiltration mainly by mononuclear cells. This may involve the entire and sometimes only part of the circumference of the segment. The reported obliteration of these bile ducts by edema, followed by fibrosis leading to atrophy [65], requires confirmation. The destructive lesion is often only detected in serial sections and need not be seen in the needle biopsy specimen.

Genetic and environmental predisposing factors can be incriminated. Since the bile ducts are often sparse, underdevelopment of the biliary system has been considered but is not proven.

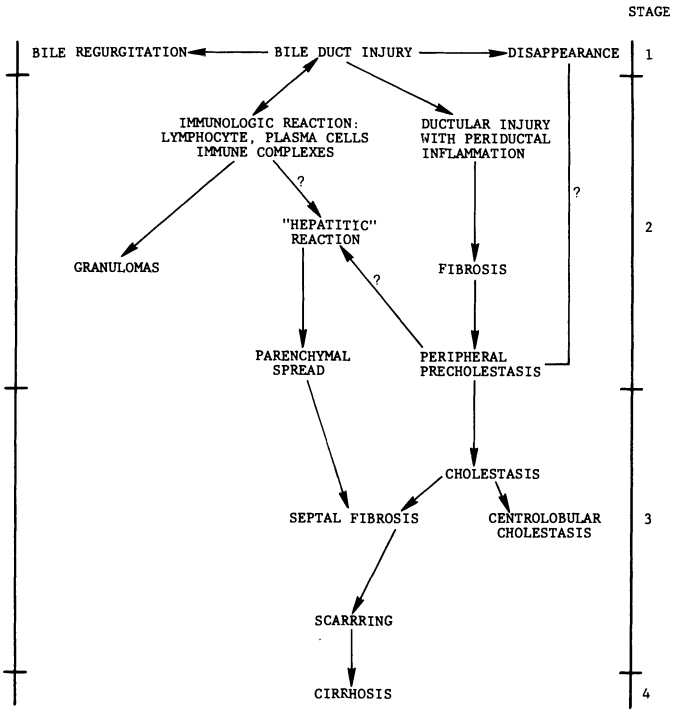

Fig. 1. Postulated evolution of primary biliary cirrhosis

The character of the cellular exudate around the initial bile duct lesion supports the assumption of a presumably lymphocytic immunologic reaction. An immune reaction is further indicated by the immune complexes visualized in the bile ducts, by the granulomas, and by the diffuse activation of the sinusoidal cells not reactive to hepatocytic injury. The occasional granulomas outside the liver and the associated symptoms of collagen disease, possibly preceding the manifestations of PBC, suggest a generalized disorder, at least in some patients.

The pathogenesis of PBC has been tentatively explained [89] by effects of immune complexes, reflected not only in the granulomas, but also in abnormalities in the Kupffer cells, with activation of the complement system [70, 71]. Although reduction of circulating immune complexes by penicillamine is associated with decrease in the elevation of aminotransferase activities, there is only limited histologic evidence of the importance of the granulomas in the evolution, even when they fibrose.

Focal breaks in bile duct continuity would explain regurgitation of biliary material into the blood and may thus account for the elevated serum activities of alkaline phosphatase and other enzymes and also for high bile acid levels. While bilirubin may also regurgitate, its compensatory hypersecretion, reflected in the excess material in the Golgi zone of the hepatocytes, may initially prevent hyperbilirubinemia or result in bilirubin elevation up to 5 mg/dl without evidence of cholestasis.

5.2. *Histogenesis of Progression*

The initial process extends in a ductular stage from the bile ducts to the ductules which also are destroyed and this is associated with spread of the lesion from the medium-sized portal tracts to smaller ones. Their bile ducts now appear, on the basis of morphometric measurement, even more involved. Ductal and, particularly, ductular alterations are associated with inflammation which in turn results in progressive fibrosis. Recent investigations suggest that mediators produced by lymphocytes and macrophages (lymphokines) play a significant role in fibroplasia, and even in chronic active hepatitis these factors have been considered more important than liver cell necrosis [42]. This represents a different immunologic reaction from the one implicated in the initiation. Thus, interference with the bile flow by periductular fibrosis, possibly associated with amputation of the bile-ductular connections, accounts for a peripheral mechanical precholestasis and cholestasis [68]. This was schematically illustrated in 1935 [19]. This process of periductular fibrosis which follows inflammation around proliferated bile ductules is, however, not specific for PBC and is found in other conditions with prolonged cholestasis and accounts for similar evolution and histologic features. The subsequent cytoplasmic retention of detergent bile acids explains the histologic changes of the periportal areas designated as precholestasis. It proceeds subsequently to bile pigment retention (cholestasis). The copper accumulation demonstrated histologically and also by chemical methods can be explained by the impaired bile flow even before cholestasis develops. It seems to reflect the longer duration of the interference with bile flow rather than its acute interruption. This accounts for the frequent discrepancy between bile pigment retention and copper deposition. Small amounts of copper deposition need not be recognized in significant elevation of total hepatic copper content but contents as high as in Wilson's disease have been encountered [32]. The copper is initially located in the cytoplasm but subsequently is concentrated in lysosomes [91].

Whether the copper deposition exerts an additional injury to hepatocytes, as in Wilson's disease and in Indian childhood cirrhosis, is not established. There is no evidence that copper deposition increases hepatic fibrosis in PBC [20], nor that its effective removal by D-penicillamine influences the course of the disease. Copper localization in lysosomes, characterized by the granular appearance, may protect from cellular injury by copper [58, 64, 79, 86]. Thus, while copper deposition assists in the differential diagnosis as a nonspecific marker of precholestasis, it need not be an indication for chelating therapy, but supposedly militates against corticosteroid use [86].

Hyalin is composed of intermediate filaments of prokeratin character [16]. Its accumulation in alcoholic liver disease and in experimental injury from griseofulvin is assumed to result from an antitubulin effect. This is supported by the hydropic appearance of the hepatocytes in the periportal zone. In alcoholic liver injury hydropic hepatocytes have been considered a result of a tubulin alteration with retention of protein normally secreted by the hepatocytes [61]. However, both features, like the copper deposition, are nonspecific features of prolonged precholestasis and cholestasis.

5.3. *Histogenesis of Septa Formation and Cirrhosis*

In most instances of PBC, the cholestasis or precholestasis (both secondary to fibrosis) seem to lead to septa formation which together with some regeneration distorts the lobular architecture to a moderate degree and may terminate in biliary cirrhosis. Some preservation of the architecture is still indicated by the persisting hepatic vein tributaries. While peripheral cholestasis is prominent, a focal and subsequently centrolobular cholestasis gradually sets in even in the absence of drug exposure.

A second, later, histologic evolution may be associated with or even overshadow the biliary fibrosis. This is predominantly lymphoid reaction around hepatocytes extending from portal tracts and periportal zones into the parenchyma. It potentially progresses to bridging necrosis and even to multilobular collapse. These features resemble the processes characteristic of CAH. In an analogy to its postulated pathogenesis, one might assume that a secondary immune reaction against hepatocytes may develop during PBC. The features resembling CAH, which in different patients complicate to various degrees the typical evolution of biliary destruction of the parenchyma, create problems in differential diagnosis. However, the observed crossover from morphologic features of PBC to those of CAH [67] need not suggest overlapping of two independent diseases. In some instances of PBC these hepatitic features may be responsible for destruction of the parenchymal architecture and result in a type of cirrhosis which histologically cannot be distinguished from that induced by the hepatitis viruses. In most instances, however, the "biliary" (predominantly macrophagic) and the "hepatitic" (predominantly lymphocytic) processes are combined to a varying extent. This explains the protean manifestations of PBC.

5.4. *Therapeutic considerations*

There is evidence, although limited, that therapy alters either the survival rate or the accelerated evolution of PBC. Corticosteroids sometimes appear beneficial, judging from the results of hepatic tests, but have no influence on survival, and the same is true for azathioprine [14]. Response to corticosteroid therapy has been claimed to be a diagnostic criterium for CAH but not for PBC [29]. D-penicillamine now appears to be the preferred drug, although diverse side effects are more frequent than in Wilson's disease, possibly because of the presence of collagen disease-like changes in PBC. It may even modify the survival rate. Moreover, this therapy has contributed to the understanding of the pathogenesis of PBC. The resulting modification of the immune status is associated with improved hepatic function. While penicillamine reduces hepatic copper, it does not influence fibroplasia [20], contradicting a role of the metal or of the drug in hepatic fibroplasia at least in this disease. The type of cross-links in the collagen in the hepatic parenchyma is not sensitive to D-penicillamine. Today, symptomatic therapy — for the itching, the bone lesions, and the ascites — are the best established modalities.

6. Conclusions

This review of clinical and morphologic features of PBC deals with four processes in the evolution of the disease: (1) etiologic and predisposing features; (2) injury of bile ducts and secondarily of hepatocytes; (3) precholestasis and cholestasis; and (4) hepatic derangement not of a biliary nature. It further refers to four stages in the evolution of PBC — ductal, ductular, scarring, and cirrhotic stage — with extensive transitions between each other and recurrence of previous stages in later ones. Probably a different immunologic process is responsible for the initial and continuing bile duct injury and for a secondary "hepatitic" component as well as for the periductular fibrosis. The further evolution of PBC, however, appears to be dominated by the cholestatic consequences of mechanical fibrotic interference with biliary secretion, mainly in the periportal area. Assisted by progression of the "hepatitic" process, it results to varying degrees in destruction of the hepatic architecture, potentially terminating in cirrhosis. But PBC is not a primary cholestatic disease; frank cholestasis is clinically and histologically a relatively late phenomenon in a disease still mysterious and with a potentially long evolution. Cirrhosis, which is the conventional name of the disease, is only present or even in development during a short terminal fraction of the total life span of the disease.

Although PBC is a relatively rare disease, even if now more readily recognized, it nevertheless serves as a useful experiment of nature for clarifying the pathology of liver disease in general and its immunologic aspects in particular.

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Serology of Primary Biliary Cirrhosis

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1. Introduction

The aetiology of primary biliary cirrhosis (PBC) still remains unknown. Many features point towards an autoimmune disorder [25]: the female preponderance, the familial aggregation, the association with other autoimmune conditions, the protracted course, the presence of various types of antibodies (smooth muscle (SMA), nuclear (ANA), and especially mitochondrial antibodies (AMA)), and the histological lesions (showing granuloma formation and infiltration of bile ducts by lymphocytes). In addition in a high percentage of patients circulating cryoglobulins have been detected [91] and there is a striking abnormality of the complement system [45] and impairment of cell mediated immunity [62]. Also cellular immunity against biliary antigens [64] and mitochondria [18] has been described, and patients' lymphocytes also seem to interfere in vitro with some mitochondrial functions [16, 24]. AMA apparently play no role in the pathogenesis of PBC but their incidence in almost 90% of all PBC patients led to the speculation that these antibodies could be related to the aetiology of the disease.

One explanation for the formation of AMA in PBC or other diseases would be that antigens very similar to mitochondrial proteins are expressed on the surface of micro-organisms or that phage-like particles infect mitochondria thereby leading to the expression of aberrant mitochondrial proteins [81]. Some mitochondrial proteins may also have a certain affinity to drugs, or proteins of the invading micro-organism thus inducing the formation of neoantigens between the hapten and the mitochondrial autoantigen [93].

For example, cardiolipin F (CLF) antibodies [95] found in sera from patients with syphilis, may have been formed as the consequence of cross-reaction, and the fact that CLF antibodies disappear from the sera after successful therapy would also favour this concept. Indeed, had the spirochaete not been descovered, syphilis could well habe been taken to be an ideal model for an autoimmune disease. AMA have also been observed in various drug-induced diseases and especially in the pseudolupus syndrome (PLE), a systemic LE-like disease induced in response to the drug Venocuran [39].

Further evidence for the association of AMA with the aetiology of a disease is the observation that the antigen specificity varies according to the type of disease. Thus, AMA found in sera from patients with PBC do not cross-react with the cardiolipin antigen or the PLE antigen [66, 76]. Purification of the various types of mitochondrial antigen characteristic of these different diseases could provide not only useful diagnostic markers but also important information about the possible nature of the exogenus causative agent. For example, monoamine oxidase on the outer mitochondrial membrane could be involved in the metabolism of certain drugs, whilst the ATPase of the inner membrane has close structural similarities with that on the plasma membrane of certain micro-organisms [47]. In this chapter the humoral phenomena observed in patients with PBC will be discussed and compared with those found in other AMA-positive hepatic and nonhepatic discorders focusing especially on the unique specificity of the humoral AMA response in PBC and its relationship to a postulated infectious aetiology of PBC.

2. Heterogeneity of the Mitochondrial (M) Fluorescence Pattern

2.1. Detection of AMA

The standard immunofluorescent technique is commonly employed using unfixed cryostat sections from a composite block of human thyroid, stomach, and kidney. To detect all the different types of antibodies reacting with homologous and heterologous tissues, rat liver and stomach should be added.

Screening test are performed with the patients' serum diluted 1:10, since undiluted specimens may give non-specific fluorescence and, occasionally, strongly positive sera show prozone effects and appear negative until diluted. Titres up to 2000 can be seen with this method.

Most of the mitochondrial antibodies so far described also fix complement. Using anti-β 1 C conjugates a typical AMA pattern can thus be obtained giving little non-specific background staining. Antibody activity is mostly found in the IgG fraction but also occurs in the IgM and to a lesser extent in the IgA fractions[1]. Polyvalent anti-immunoglobulin conjugates should therefore be used.

When AMA are present it is difficult to see organ-specific thyroid cytoplasmic and gastric parietal cell antibodies coexisting in the same serum, despite the differences in staining patterns. Passive haemagglutination tests can be used to detect organ specific thyroid antibodies, or else sera have to be absorbed with a large excess of liver mitochondrial protein.

2.2 M-Fluorescence Related to PBC

Sera from these patients show a very typical immunofluorescence. Renal tubules are uniformly stained, distal tubules and the ascending loop of Henle giving the brightest reaction. Thyroid cells show a coarsely granular pattern except for oxyphil

[1] Using a new radioimmunoassay technique it has been shown that in some cases AMA activity is contributed to almost equally by IgG and IgM (F. Meek, manuscript in preparation).

cells which stain more intensely owing to their high content of hypertrophied mitochondria. Gastric parietal cells are uniformly stained and have a compact appearance whereas chief cells look faintly granular. Liver cells also show a weak coarsely granular fluorescence but bright staining is obtained on salivary duct cells, lactating mammary tissue, heart, and red striated muscle fibres. Thus, in general the brightest staining is seen on those tissues with the most active aerobic metabolism.

This non organ- and non species-specific antibody reacts with an antigen localized within the inner mitochondrial membrane [9, 10] and the highest antigen content was found in the thermogenic mitochondria of brown adipose tissue (BAT) from new born animals [12].

2.3. M-Fluorescence not Related to PBC

Cardiolipin Fluorescent Antibody (CLF). The classical "Wassermann antibodies" are directed against cardiolipin but do not normally give rise to fluorescence although complement fixation may be demonstrated with appropriate tissues. This suggests that these antibodies are probably directed against hydrophobic sites that are not accessible to the antibody in the tissue sections.

However when sera of untreated cases of active secondary syphilis were tested they were found to produce a staining pattern resembling that seen with sera from patiens with liver cirrhosis although there are some differences. The cytoplasm of renal distal tubules is diffusely stained but proximal tubules are unstained. In stomach sections the parietal cells are brightly and diffusely stained but chief cells react equally well [95].

Thyrotoxic thyroid glands give a variable staining pattern: Askanasy cells fluoresce quite brightly but normal epithelial cells are only weakly and diffusely stained. Absorbtion with purified cardiolipin or VDRL (Venereal Diseases Research Laboratory) antigen abolishes completely the immune fluorescence but had no effect when sera from patients with PBC were used. Fixation of tissues in 95% ethanol also abolished the reaction.

M-Fluorescence in Chronic BFP Reactors (Chronic Biological False Positive). AMA have been found in up to 56% of these cases [26]. They are of low titre, cannot be absorbed out with VDLR antigen, and belong to the IgM class. They are mostly found in patients suffering from collagen-like disorders such as rheumatoid arthritis, Sjörgren's syndrome, systemic lupus erythematosus (SLE), or a neurological syndrome diagnosed as multiple sclerosis. The complement fixation titres with rat liver mitochondria did not always correlate to the immuno fluorescence-IFL titres when the test was done with whole homogenate of rat kidney.

M – Fluorescence in Atypical Collagen Diseases. Labro et al. [53] recently described a non organ- and non species-specific antibody which was found mostly in patients suffering from collagen-like disorders such as lupus erythematosus (LE) or haemolytic anaemia. There may be similarities to the antibody type found in the BFP reactors. The antibody stains predominantly renal proximal but not distal tubules or gastric parietal cells. The antibody activity could be absorbed with whole

mitochondria or a preparation of mitochondrial inner membranes but not with VDLR antigen. Recent studies, however, indicate that the target antigen is a component of the outer mitochondrial membrane but, unlike the outer membrane antigen of PLE (see below), is sensitive to digitonin treatment [66].

M – Fluorescence in Pseudolupus Syndrome (PLE). The pseudolupus syndrome has been shown to be induced by a drug called Venocuran which was given for vascular disorders mainly in Germany, Switzerland, and Spain [39, 40]. This drug contains phenopyrazone, horse chestnut extract, and glycosides from several plants. High titre antimiochondrial antibodies have been found during the acute stage of the diseases and after rechallenge with the drug or the constituent phenopyrazone [13, 59].

The IFL pattern is quite distinct from that observed with sera from PBC patients or other M positive cases. Distal and proximal tubules are equally brightly stained and hepatocytes show a strong homogenous reaction. Trypsin treatment of the tissue section does not abolish the fluorescence in contrast to the reaction with PBC sera which becomes negative after this procedure.

The antibody belongs to the IgG class and strongly fixes complement. It reacts with an antigen localised on the outer mitochondrial membrane and chaotropic agents or digitonin were not able to dissociate the antigen from the membrane [66, 76]. After withdrawal of the drug the antibody titres fall rapidly but in some instances persistence of AMA at low titre could be observed over many years.

Liver/Kidney Microsomal (LKM) Antibodies These antibodies must be distinguished from the PBC specific ones because they also occur in patients with liver disorders, especially in young patients with chronic active hepatitis. The antibody reacts with a lipoprotein antigen in the membranes of rough and smooth endoplasmic reticulum, and fluorescence is restricted to the third portion of the renal proximal tubules in rat kidney, and to hepatocytes and certain cells in bronchial, oesophageal, and duodenal epithelium. No other organ in the body was found to be reactive [71]. The antibody could not be absorbed with purified mitochondrial preparations but could be absorbed with a microsomal preparation.

In the immunofluorescence the best distinction is seen in the border between inner cortex, whose tubules stain with LKM, and the medulla, where the ascending limbs of the loop of Henle stain most clearly with AMA of the PBC type, and not a all with sera positive for LKM. However, the LKM pattern may be confused with the PBC pattern when organ specific antibodies are also present against thyroid and gastric-specific microsomal antigens. The presence of ANA and SMA, on the other hand, would exclude LKM since LKM have not been found in association with any other antibodies. LKM antibodies also strongly fix complement with liver microsomes, and follow-up studies should be done using the CF (complement fixation) test since antibody titres may fall at remission.

In view of the practical diagnostic importance of AMA, and in the light of the heterogeneity of AMA detected by immunofluorescence [27], the serological diagnosis of PBC should not rely on the immunofluorescence test alone. Complement fixation tests (CFT) should be performed in addition, using defined antigen preparations which can be obtained by various standardised methods.

3. Preparations of Complement Fixing Mitochondrial Antigens (CF-Antigens)

For routine testing it is sufficient to use sonicated whole mitochondria from rat kidney, or submitochondrial particles (SMPs) from bovine heart [88]. The latter provides a more sensitive test since the major PBC antigen is probably associated with the inner face of the inner mitochondrial membrane and preparations of SMPs consist of vesicular fragments of inner membrane with an "inside out" orientation. Crude preparations of SMPs also contain fragments of outer membrane and some "right side out" inner membrane vesicles so that all potential membranous antigens are exposed (Fig. 1). SMPs also have very little matrix or inter-membrane soluble proteins and microsomal contamination is very low. CF titres of 256 to 32,000 can be obtained with this antigen preparation. *For differential testing* three approaches have been successfully applied. One involves separation of antigens by equilibrium density gradient centrifugation. This has been done with sonicated rat kidney mitochondria layered on sucrose gradients at densities between 1.08 and 1.24 [7].

A second approach has been to fractionate, by methods such as gel filtration, the supernatant fraction of a rat liver homogenate obtained after centrifugation for 1 h at 105,000 *g* ("Supernatant 40"). This procedure which follows the method described by McFarlaine et al. [65] yields not only fragments from mitochondria broken in the process of homogenization but also true cytoplasmic (soluble) proteins including the so-called "LP 2" fraction.

Good discrimination between different types of AMA-positive sera has been achieved by these two methods. The third approach has been to prepare biochemically defined fractions from mitochondria or submitochondrial particles, which were subjected to treatments with chemicals whose effects are known. Thus, the inner membrane, outer membrane, intermembrane proteins, and matrix proteins can be separated from rat liver mitochondria by a graded osmotic lysis and gentle sonication or treatment with digitonin [80, 84]. Detachable proteins can be removed from bovine heart SMPs by controlled treatment with trypsin, chaotropic agents, or chloroform. Chemical modification of specific proteins can also be carried out (for example the blocking of thiol groups with mercurials) prior to such extractions. At all stages, fractions can be characterised, and their purity assessed, by reference to marker enzyme activity.

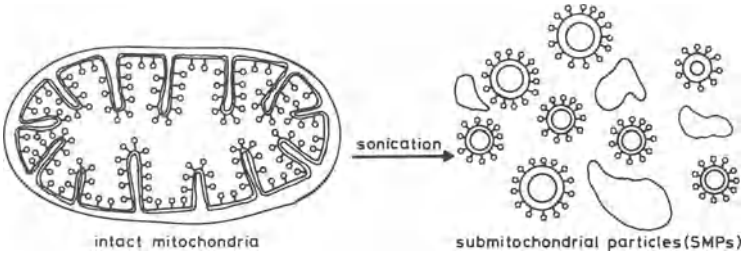

Fig. 1 Diagrammatic representation of the preparation of submitochondrial particles (SMPs) by sonication of beef heart mitochondria. SMPs are inner membrane vesicles exposing "knobs" containing the F_1 sector of the ATPase-complex (Fig 3). This fraction is still contaminated with outer membrane vesicles, and usually (not shown) "right side out" particles with the knobs orientated inwards.

Over the past few years these methods have not only been useful in defining the different types of AMA and correlating these with IFL patterns [66], but they have been the basis for attempts precisely to identify the PBC antigen. Although the antigen has not yet in fact been unambiguosly identified, all data clearly indicate that it is not associated with proteins involved in electron transport or uncoupled ATP hydrolysis. It may, however, be a marker for a distinct mitochondrial function (present in BAT mitochondria) concerned with the control of proton fluxes across the coupling membrane (see below) [12, 55, 78].

4. Purification of the PBC Specific Antigen

Initial investigations characterised the antigen as a lipoprotein [11] and localised it to the inner mitochondrial membrane – a finding that was confirmed by the electron microscopic studies of Bianchi et al. [14].

Using affinity chromatography, with inner membrane preparations applied as immunoabsorbants, Ben Yoseph et al. [3] substantially purified the PBC antigen. The purified antigen was passed through Sephadex G 200 in 6 M urea and the estimated molecular weight was found to be approximately 180,000. Polyacrylamide electrophoresis of this purified mitochondrial inner membrane protein, solubilised in 8 M urea, gave a major protein band and two minor bands.

Strong evidence for an association of the PBC antigen with the mitochondrial ATPase came from studies by Sayers and Baum [75] who found that ATPase prepared according to the method of Beechy et al. [2] was very antigenic in the complement fixation test. Furthermore elution of the ATPase band from 5% polyacrylamide gels gave rise to an antigen fraction which reacted strongly with PBC sera in the CFT. This finding suggested that the PBC antigen might be a subunit of mitochondrial F_1 ATPase complex (see Section 7) [1]. However it soon was recognised that brown fat mitochondria, although very rich in PBC antigen, exhibited very low ATPase activity and possessed a low absolute content of F_1 [20]; and pure F_1 ATPase fractions prepared by other methods hardly reacted with PBC sera [55]. Furthermore, when a chloroform extract from brown fat mitochondria or when the chloroform-released ATPase of bovine heart SMP were further purified by gel filtration or ion exchange chromatography a clear-cut dissociation between ATPase and antigen activity could be achieved. From these observations one must conclude that the PBC specific antigen is not part of the F_1 ATPase complex, although it is released from the inner membrane by similar treatments [76], and does tend to co-purify with F_1. Further speculation on the actual identity of the antigen will be deferred until Section 7. Nevertheless, certain properties of the antigen should be mentioned. Firstly its antigenicity is very sensitive to treatment with mercurials [1, 66], implying a specific involvement of a thiol group(s) at the antigenic site. Also, isofixation curves indicate an exceptionally limited number of antigenic determinants [1], possibly accounting for technical difficulties sometimes encountered in demonstrating antigen-antibody binding, particularly with purified antigenic fractions (Joshi, Sanadi, and Baum, unpublished observations).

5. ATPase Associated Antigen – a PBC Specific Marker Antigen

Partially purified mitochondrial antigen fractions prepared by treating SMPs with chloroform, which released the antigen together with the ATPase complex from inner mitochondrial membranes, were used in the CF test to re-evaluate the diagnostic specificity of the antigen preparation. Sera were screened from 120 patients with AMA positive hepatic and nonhepatic disease. Only sera from patients with cholestatic liver diseases reacted with this ATPase-associated antigen and none of 40 patients with PLE, syphilis II, or undefined collagen diseases [77].

AMA have been reported in patients with extrahepatic biliary obstructions [49, 54] but these early reports were not confirmed [61, 70]. From all the serological data obtained to date one can confidently state that PBC-specific antimitochondrial antibodies do not occur in proven cases of extrahepatic jaundice – although both conditions can coexist – as well as in primary sclerosing cholangitis, biliary atresia, alcoholic liver disease, or Wilson's disease.

M fluorescence was found in population studies in female and male patients [25] and also in relatives from patients with PBC [73, 89]. Feizi et al. [33] noted a significant increase in antimitochondrial antibodies among the relatives of 26 patients with PBC and in another study comprising patients with PBC and CAH the prevalence of AMA was not higher among the PBC relatives than in relatives with CAH [36]. The reason for this familial incidence is uncertain. We also have been testing relatives and twins from patients with PBC but were unable to detect complement fixing antibodies reacting with the PBC specific antigen.

However, there may be a genetic predisposition for the formation of autoantibodies in these families [21, 43]. Thus the histocompatibility antigen DW3 – frequently associated with autoimmune disorders – was also more frequently found in PBC patients than in normal controls [21]. It seems therefore that the demonstration of CF (complement fixing) antibodies against the ATPase-associated antigen has a high diagnostic relevance with respect to the PBC specific bile duct lesions. In the absence of these antibodies the diagnosis of PBC should be only established after an appropriate period of clinical observation.

5.1. Association of the PBC Specific Antibody with Two Other Complement Fixing Antibodies: The "Mixed Form" Type

In 1965 [90] and 1967 [68] ist was already recognised that AMA occur not only in sera from PBC patients but also in about 20% patients with chronic active hepatitis or cryptogenic cirrhosis. There is considerable overlap between the hepatic findings in PBC and chronic active hepatitis (CAH) and in certain instances multiple liver biopsy sections show the classical features of CAH in one part and PBC in another [23, 38, 58, 67, 72, 83, 96]. Popper [69] has delineated two groups of PBC patients with distinct histological differences: in one situation the fibrotic septa are seen as a consequential effect of the bile, being a result of hepatocellular accumulation of detergent bile acids and copper; in the second group cirrhosis seems to develop in a fashion similar to CAH. Piecemeal necrosis is prominent and immunological factors could be responsible for this type of lesion. Klatskin and Kantor [51] reported on 22 patients – all M antibody positive – who had atypical PBC and in 19 of whom the

histological pattern showed an established cirrhosis which they described as postnecrotic. They concluded that the syndrome of postnecrotic necrosis with evidence of cholestasis represented a late manifestation of PBC.

Recently AMA of different specificities were demonstrated in sera from patients with cholestatic chronic liver diseases [5, 6] and the liver histology of these cases also showed alterations of a heterogeneous nature [7, 52].

In view of the heterogeneity of AMA in hepatic and non-hepatic disorders – as previously described, AMA positive sera were re-examined using purified outer and inner membrane fractions, a soluble cytoplasmic fraction (LP 2), and sub-mitochondrial particles from beef heart stripped of the PBC specific antigen [79, 85, 92]. Three different reaction patterns were obtained: (a) sera reacted exclusively only with the ATPase-associated antigen (inner membrane antigen), (b) sera reacted simultaneously with the inner membrane antigen and a second, outer membrane, antigen which was resistant to trypsin treatment ("trypsin insensitive mixed form"), (c) sera reacted simultaneously with the inner membrane antigen and a second, soluble antigen (LP 2 fraction) ("trypsin sensitive mixed form"). Among the 59 AMA-positive patients with cholestatic liver disease, all of whom had been positive with the ATPase-associated antigen, there were 29 who reacted only with the PBC antigen, whilst 17 were of the "trypsin insensitive" and 13 of the "trypsin sensitive" mixed form types respectively. Histological analysis of 140 liver biopsies taken from these patients over a period from 2–14 years showed that the serologically classified PBC cases all had the classical features of PBC (Klöppel, Kirchhof, Berg, in preparation). Among the 30 patients with a second complement-binding antibody only nine were clear-cut PBC cases.

Trypsin Insensitive Mixed Form. The 17 patients belonging to this group have been followed for 3–14 years and 44 liver biopsies were taken during this time. Only three patients revealed stage I and stage II features of PBC. The other had histologically chronic active hepatitis. In addition granulomatous changes and/or augmented bile duct proliferations were observed. In three of these patients follow-up biopsies over 4–10 years revealed the development of CAH into postnecrotic cirrhosis. In one of the two patients who died, postnecrotic cirrhosis was confirmed at autopsy, lacking signs of primary biliary cirrhosis.

Biochemically the most striking features in this group were the increased immunoglobulin levels which were due to the simultaneous elevation of both IgM and IgG as compared to PBC patients who never showed any significant rise of the IgG globulins even in the late stage of the disease. The other discriminating parameter was the lack of pronounced bilirubin elevation which normally accompanies the late phase of PBC. All patients so far analysed (with one exception) did not exceed bilirubin levels above 2–3 mg%. Alkaline phosphatase levels can be quite high independently of the stage of the disease and no dramatic change in transaminase activity was observed, as normally found in other forms of CAH. Pruritus was less frequently found and some patients complained about athralgia. There is some evidence that this group of patients respond well to treatment with prednisolone and azathioprin. A decrease in alkaline phosphatase and immunoglobulin levels and the disappearance of histological features of CAH was noticed in response to this type of therapy (Klöppel, personal communication). The immuno-

fluorescence pattern allows no distinction between this subgroup and PBC, but liver may stain brighter than in the case of classical PBC. However, prior treatment of cryostat section with trypsin does not abolish the fluorescence as would be the case with PBC sera, although a decrease of the intensity of the staining pattern is obvious.

The Trypsin Sensitive Mixed Form. All 13 patients have been followed for 2–10 years and 24 liver biopsies were obtained. Histologically this group is very similar to PBC and features suggestive of an overlap between PBC and CAH could be found only in seven patients. However, clinically atypical courses have been observed with sudden elevation of bilirubin and transaminases followed by complete remission. The level of CF antibodies reacting with the soluble protein LP 2 seems to vary according to the stage of the disease [85]. Features of CAH may be more prominent in the early stages of the disease and can disappear at late stages. IgM and IgG globulins may be elevated as in the insensitive group and immunosuppressive treatment seemed to be effective in some cases.

Possible Aetiology of Mixed Forms. The demonstration of a group of patients whose sera react in the CFT simultaneously against the PBC specific antigen and at least one other complement fixing antigen could indicate that PBC does not represent a single aetiology but may be initiated by a variety of factors. Some cases have been ascribed to drugs [35] and this speculation is of interest in respect of the observation that the PLE antigen and the trypsin insensitive mixed from antigen have many features in common: both are localised on the outer membrane, cannot be solubilised by various treatments, and are not destroyed by trypsin [76]. One would be inclined therefore to speculate that the trypsin insensitive mixed form might reflect a superimposed response to a drug – at least in some instances – or a second pathogen. Alternatively these various types of CF antibodies could arise as a consequence of a genetically determined hyperimmune response which is exhibited by a group of PBC patients. However, recent observations that a few histologically proven, but AMA-negative, PBC cases have antibodies either against the LP 2 or the trypsin insensitive antigen of the outer membrane (unpublished observation) would indicate an immune response to antigens different from those involved in the aetiology of PBC. On this basis it is, however, difficult to explain those PBC cases, about 10%, which remain AMA negative. Klöppel (personal communication) has followed 80 PBC patients over 12 years and found 13 patients in whom no AMA was detected when tested by immunofluorescence and complement fixation. Ten patients exhibited histologically features of PBC stage I, in the other three patients the disease progressed and they developed cirrhosis.

One is inclined to speculate that this group of PBC patients may either have an aetiology different from the AMA positive cases or respond immunologically in a different way. Asymptomatic PBC cases have been described in whom the histological stage remained static for many years, even when symptoms had developed, but all of them had strongly positive antimitochondrial antibodies [57]. Although the prognosis of the AMA negative cases may be better it should be stressed that these patients appear in no way different from those reacting with the PBC specific antigen.

5.2. Association of the PBC Specific Antibody with Other Serum Autoantibodies Detected by Immunofluorescence.

In a prospective study of the clinical pattern and course of PBC, based on the data of 236 patients in an international randomised trial, 91% had AMA, 33% SMA, 31% ANA, and 26% thyroid antibodies [22]. Mac Sween found SMA and ANA to occur no more frequently than in age and sex matched controls [61] and in our series of 29 PBC patients followed over the last 10 years SMA were found in 17% in low titres and ANA only transiently in a few cases. However, in the trypsin insensitive group SMA predominated with 77% [92] and were found to react specifically with actin, a protein which seems to be one of the target antigens in chronic active hepatitis [31, 87].

Although reports on the frequency of autoantibodies other than AMA vary considerably in PBC patients [28], there is little evidence for an association with high titre autoantibodies of any kind. Bile canalicular antibodies have also been observed [60] but the exact significance, if any, of these antibodies is not yet clear.

Antibodies to ribosomes, using a Farr-type radioimmunoassay, were found in 36% among 14 PBC patients, always in association with AMA [37]. The diagnostic usefulness of these antibodies, however, is limited owing to their low incidence and overlap with other liver diseases like HBsAg-positive and negative chronic active hepatitis. Antibodies against vascular endothelium and sarcolemma frequently observed in patients with viral hepatitis [8] or myocarditis, are not present in sera from patients with PBC or any autoimmune disorders again indicating that the antibody pattern must have some relevance to the aetiology of the disease.

Swana et al. [86] described a human specific mitochondrial antibody (AHMA) whose prevalence is increased in PBC where it may be associated with the standard non species-specific AMA. The importance of AHMA is mainly in possible confusion with organ specific reactions in submaxillary duct and parathyroid oxyphil cells but has little diagnostic relevance. Among 13 PBC cases who had repeatedly given negative results with the standard AMA test only two were found to have the human specific antibodies in titres of 1 in 80 and 1 in 20 respectively.

5.3. Serological Evidence for an Overlap of PBC and Lupoid Hepatitis

Primary biliary cirrhosis has been shown to be associated with rheumatoid arthritis, the sicca complex and Sjögren syndrome, with systemic sclerosis, and other components of the CRST syndrome (calcinosis cutis, Raynaud's phenomenon, sclerodactyly, and telangiectasis [15]. An overlap of two autoimmune conditions within the liver, PBC on the one hand and lupoid hepatitis on the other, seems also to exist in a few cases. This can be postulated on histological and clinical grounds but also in view of the simultaneous appearance of high titre antibodies against smooth muscle, nuclear, and mitochondrial antigens [29]. Changes in the autoantibody patterns expressing either strong ANA or AMA reactions in the immunofluorescence test, lack of transition into classical PBC, high immunoglobulin levels, and signs of multisystemic involvement seem to be characteristic features in this rare condition. Macronodular cirrhosis may develop in the late stage but there is no manifestation of severe jaundice as seen at this stage in PBC cases [4].

6. AMA – a Clue to the Aetiology of PBC?

All data obtained until now point towards an association of the PBC antigen with a mitochondrial protein localised on the inner side of the inner membrane, i. e. this antigen is extremely inaccessible for the induction of an immune response. It also seems unlikely that an organism should loose its tolerance to such an essential organelle during the course of a hepatic infection or as consequence of an alteration of the immunoregulatory system. Perhaps the most likely explanation for the formation of these antibodies is that they reflect a cross-reaction with some micro-organisms. Certainly, it is well established that the plasma membranes of some aerobic bacteria have many components in common with those of mammalian mitochondria [44]. Furthermore, many primitive eukaryotes possess mitochondria, some of which do react with PBC sera. The spectrum of such cross-reacting species is currently under investigation and includes wild and mutand forms of yeast and *Neurospora* as well as some Trypanosoma (Baum and Leoutsakos, unpublished observations).

Cross-reactivity between bacterial and hepatic antigens has been demonstrated for certain strains of β hemolytic streptococci, and rabbits immunised with the appropriate strains of streptococci produced antibodies capable of reacting with liver antigens, the epithelium of interlobular bile ducts, and Kupffer cells [50]. Antigens isolated from bile have also been shown to produce migration inhibition factor (MIF) in the presence of lymphocytes from patients with PBC and microbial antigens may have been responsible for this reaction [64].

It also would seem possible that parasitic antigens which are released during the immune response could bind to host cells rendering them susceptible to destruction by the host's own immune response against infection [45]. If parasitic antigen production and subsequent host cell death was sustained over a sufficiently long period then the chronic release of self components might elicit an autoimmune response for which there might be a genetic predisposition.

Alternatively, activation of the complement system [45] may be responsible for the chronic inflammatory reaction in the liver due to large circulating immune complexes which may act via the alternate pathway. The elevation of the IgM globulins and especially the presence of 7 S IgM monomers in sera from patients with PBC could be also interpreted in favour of an ongoing parasitic infection (32).

In parasitic infections like leprosy, syphilis, or trypanosomiasis specific cellular immunity was also observed and serum factors were found which suppressed the reactivity of normal and patients' lymphocytes to mitogens in vitro [19, 30, 63].

In order to test whether similar mechanisms were operative in PBC we tested lymphocytes from PBC patients against mitochondria isolated from *Neurospora crassa* in autologous and homologous serum. The reactivity of these lymphocytes was compared with those from normal controls [17]. Patients' lymphocytes responded less to this fungal antigen than did the normal controls, and sera from patients with PBC markedly inhibited the proliferative response to the antigen. This phenomenon was especially pronounced when lymphocytes from normal individuals were used (Fig. 2).

There is another interesting aspect which may relate PBC with some fungal infection. *Neurospora crassa* accumulates copper with the concomitant synthesis of

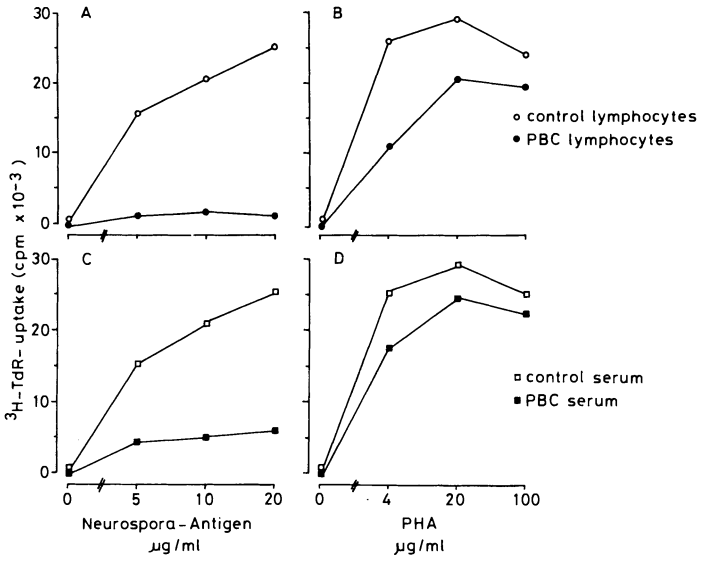

Fig. 2. **A** and **B** compares cellular immune response of PBC patients' lymphocytes and control lymphocytes in the presence of submitochondrial particles prepared from *Neurospora crassa* and the mitogen PHA. In **C** and **D** the effect of patients' serum on the antigen and mitogen induced proliferation of normal lymphocytes is shown. It can be seen that a *Neurospora* specific immune defect may exist in PBC patients.

a small molecular weight copper-binding protein of the metallothionein class [56]. This protein has an exceptionally high cysteine content and seems to fulfil several important physiological functions such as copper storage, copper detoxification, and provision of copper for tyrosinase. The accumulation of copper in the liver of patients with PBC [34, 94] and the increased copper serum level – an early and constant symptom in PBC – could be explained by the presence of such an organism in the liver. Alternatively, these high copper levels might reflect an abnormal turnover of mitochondrial respiratory chains, since copper is a constituent of cytochrome oxidase.

7. Speculation on the Identity and Biochemical Function of the PBC Antigen

The complex enzyme that catalyses the reversible proton-translocating ATPase of the inner mitochondrial membrane consists of two major sectors. One sector (Fo) is intrinsic to the membrane and contains the specific oligomycin-sensitive, proton-conducting channel. The second sector (F_1) is the site of actual ATP hydrolysis. (It is also the site of ATP synthesis, when the system is intact and a proton-motive force is applied across the membrane via the channel in Fo.)

F_1 is identical with the well-known 90 A particles that stud the inner surface of the inner membrane. It is detachable and can be isolated as a water-soluble enzyme of mol. wt. around 390,000. Various, apparently homogeneous preparations of F_1 have been made by different procedures. Most (except for the so-called Factor A

[74]) exhibit ATPase activity, but kinetics are different, ascribable in part to a variable content of a specific, allosterically controlled inhibitor protein. The subunit composition of F_1 is known, but subunit stoichiometry is not clearly established [47]. A possible arrangement is $\alpha_3\beta_3\gamma\delta\varepsilon$ (Fig. 3), the respective mol. wts. being around 54,000, 50,000, 33,000, 17,300, and 11,000. Only subunits α, β, and γ are believed to be essential for ATPase activity, but δ and ε are thought to be necessary for attachment to Fo, and δ may in some cases be related to the inhibitor protein.

The PBC antigen is released from the inner membrane by all procedures that detach F_1, and it co-purifies with the form of the enzyme prepared by the chloroform-release method. However, as was mentioned previously, we can now confidently state that it is not an integral part of F_1. Antigenicity can be separated, albeit with difficulty, from ATPase activity. Highly purified F_1 prepared by other methods and exhibiting all subunit bands on electrophoresis is not antigenic. The non-catalytic subunit δ contains no cysteine residues [82], whilst the antigen must be presumed (because of sensitivity to mercurials) to contain at least one thiol group. Finally, mitochondria and chloroform-released protein from brown adipose tissue (BAT) contain negligible ATPase but are very antigenic [20].

Is the association entirely accidental, or is the antigen another component of the exceedingly complex intact ATP synthetase system? Such other components are known, for example F_6 [48] (possibly a detachable part of Fo concerned with association with it of F_1), and Factor B (F_B) [46, 74] a small peptide, existing in many oligomeric forms, which is very mercury-sensitive and which enhances the coupling of energy-transduction between F_1 and F_1-depleted membranes. (This property need not imply a direct association with the ATPase itself; control of an energy-dissipating ion channel at another membrane locus might account for effects of F_B (Fig. 3), although more recent work does implicate F_B directly in the proton-translocating activity of the intact ATPase complex [42]). Interestingly, crude preparations of Factor B are antigenic against PBC sera, and the chloroform-released ATPase has been shown, immunologically, to contain Factor B [55]. The peptide migrating in SDS gels between the α and β subunits in the crude,

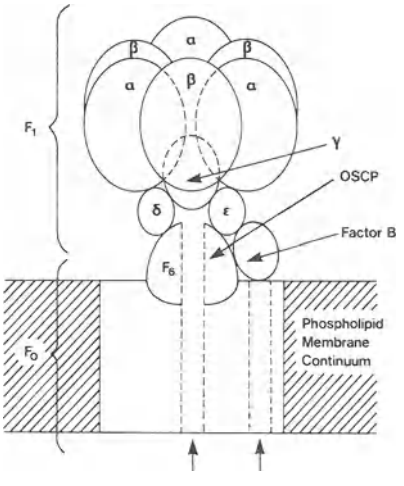

Fig. 3. Speculative model of the structure of the ATP-synthetase complex (H^+-ATPase). OSCP-oligomycin sensitivity conferring protein. *Arrows* indicate possible proteolipid proton-conducting channels.

chloroform-released preparation (and that seems to be absent in very pure preparations of F_1 [78]) might thus be a Factor B oligomer. This possibility is strengthened by recent observations that purified antigenic fractions from BAT mitochondria, free of ATPase activity, react against a specific anti-F_B antiserum; and that PBC sera react against a high molecular weight subfraction from a crude F_B preparation that also reacts against the anti-F_B serum (Joshi, Sanadi, and Baum, unpublished observations).

An argument against direct involvement of the antigen in ATP synthesis is its presence in large amounts in mitochondria from brown adipose tissue. This finding, as well as the general correlation between intensity of IFL and respiratory activity of corresponding tissues, might implicate an unusually readily extractable substrate permease or respiratory carrier. However the lack of F_1 in BAT mitochondria need not indicate a deficiency of Fo components such as F_B that might conceivably play a regulatory role in thermogenesis.

Incubation of mitochondria with respiratory substrates or inhibitors increases their antigenicity, and pretreatment of animals with adrenalin decreases the antigenicity of subsequently-isolated liver mitochondria (Berg, unpublished observations). Now, it is known that there are thiols on the inner membrane (apparently concerned with the binding of ADP and the maintenance of membrane integrity) that can only be kept in a fully reduced state if substrates are available to reduce endogenous NAD^+, and if its subsequent re-oxidation by the respiratory chain is inhibited [41]. Is this a further clue to the identity and role of the antigen?

Clearly, speculation can only be resolved by further experimentation. Methods are now being developed for the extensive purification of the antigen, when it is hoped that its chemistry will be more precisely defined, for example its lipid content, its spectral characteristics, and in particular its relationship, if any, with F_B.

8. Conclusions

The testing of antigens isolated from mitochondria with AMA positive sera from patients with defined clinical entities has provided useful diagnostic markers which can be used in the complement fixation test. In five different clinical diseases or syndromes, antimitochondrial antibodies of different specificities could be found (Table 1).

The exact nature of the PBC specific antigen remains to be established but some immunological similarities between PBC and parasite infections and the demonstration of a *Neurospora* specific cellular immune defect raise the possibility that cross-reaction with microbial antigens may have triggered the antimitochondrial response.

The serological definition of two subgroups of cholestatic liver diseases, which may be relevant to the occasionally observed overlap between PBC and CAH, may be taken as evidence for a separate clinical entity where sensitisation occurs against the PBC specific and another yet undefined antigen. The observation that abnormalities of the complement system occur only in patients with PBC but not in HBsAg negative chronic active hepatitis also clearly shows a fundamentally different pathophysiological process in this disease.

Table 1. Heterogeneity of antimitochondrial antibodies

Classification	Disease occurence	Nature of antigen
M 1	Syphilis II	Cardiolipin
M 2	PBC (primary biliary cirrhosis)	Trypsin sensitive inner membrane, ATPase associated loosely bound
M 3	PLE (pseudolupus syndrome)	Trypsin intensitive outer membrane, digitonin insensitive, tightly membrane bound
M 4	Cholestatic CAH; postnecrotic; liver cirrhosis	Trypsin insensitive outer membrane, digitonin insensitive, tightly membrane bound
M 5	Various atypical collagen diseases	Trypsin insensitive outer membrane, digitonin sensitive

Drugs may play some role in these mixed form cases and the biochemical similarities between the PLE and trypsin insensitive antigens could be taken as a strong argument in favour of this hypothesis. Although some patients in this group present with a long history of drug abuses there is no convincing evidence for such a relationship. However it would be interesting to study the role of mitochondrial antigens in drug metabolism.

PBC becomes recognised more and more at early stages and there seems to exist great variations as far as clinical, histological and immunological parameters are concerned. One would, therefore, not be surprised to find that PBC consists of a group of aetiologically different diseases similar to that seen in chronic active liver diseases.

The biochemical identification of mitochondrial antigens like the PBC or PLE specific antigens are not only diagnostically relevant. They might also provide a valuable tool for mitochondrial research. The high antigenic activity in brown fat tissue and the modulation of antigenicity with metabolic state could indicate that the PBC specific antigen can be correlated to metabolic function, and might perhaps give clearer insight into the role of Factor B.

Since the PBC specific antigen is present in all mitochondria so far tested – with the possible exception of certain plant mitochondria – it must be a vital constituent and also a useful phylogenetic marker helping, en passant, to delineate the range of organisms that could be considered as putative causative agent for PBC.

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Pathogenic Mechanisms in Primary Biliary Cirrhosis

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Introduction

Primary biliary cirrhosis (PBC) is a granulomatous destructive disease involving the epithelium of the proximal intrahepatic biliary tree to the level of the interlobular ducts [34, 48]. More recently the polyglandular nature of the disease has become evident by the demonstration that 70–100% of patients also have lacrimal and salivary gland lesions [1, 17], and pancreatic dysfunction is not uncommon [11, 26]. It is predominantly the ductular components of these glands which are destroyed by an inflammatory or immunological process while the acinar elements are spared. In addition, dermal thickening (scleroderma-like) and pigmentation are prominent features, and the syndrome is accompanied by severe abnormalities of both cell-mediated and humoral immunity. Patients exhibit a generalised state of immune hyporesponsiveness being anergic to DNCB [15] and producing a diminished antibody response to parenteral antigenic challenge [16, 57]. They also exhibit failure of the immune response to convert from IgM to IgG antibody synthesis [57].

The biliary lesions contain multinucleate giant cells and epithelial cells — presumably of monocyte/mactophage origin — and numerous activated T lymphocytes [47, 56]. This composition suggests the involvement of a cell-mediated immune response presumably directed to either a native biliary antigen, one derived from the ductular epithelium, or an extrinsic antigen deposited in the portal tract. In addition, B lymphocytes of IgM class are found in the peripheral zones of the lymphoid aggregates, and granular interstitial deposits of immunoglobulin and complement (C3) are seen in areas of ductular damage [39, 56]. This raises the possibility of the additional involvement of cytotoxic antibody dependent or immune complex mechanisms of tissue damage.

Following the finding of complement activation products (C3b) in the plasma [54] and increased Clq and C3 catabolism in these patients [42, 43], circulating immune complexes were demonstrated by Clq binding [58] and C3b receptor adherence [64] techniques. It is noteworthy that the liver has a major role in the clearance of complement fixing immune complexes [63] and thus the plasma clearance of immune complexes might be expected to be diminished in any form of

chronic liver disease in which there is shunting of blood away from the liver [59]. The significance of the Clq binding [58] and C3b receptor adherent [64] moieties in PBC plasma was apparent when it was established that in this disease the immune complexes are associated with a much greater degree of complement activation than is found in other forms of liver disease [60, 61] with a similar level of Clq binding activity [58]. This appears to be related to the greater size of the complexes [58] partly because they contain IgM [64], but also because of the presence of antiglobulins and immunoconglutinins (personal observation). Furthermore, complement activation products (C3d) are present in greatest concentration in the earliest stages and fall as the disease progresses to the fibrotic and cirrhotic stages [56]. This is in contrast to the plasma Clq binding activity which remains constant or rises as the disease progresses [10]. These data probably reflect a change in the composition of the complexes and hence a charge in their ability to activate complement. Alternatively, plasma C3d concentrations will reflect both intra- and extra-hepatic activation of complement, whereas Clq binding will only reflect the presence of circulating immune complexes. Thus in the early stages of the disease there may be deposition of disease specific complement activating complexes in the tissues with "spillover" into the circulation, whereas in the later stages, the presence of circulating complexes may merely reflect impairment of the liver's ability to clear gut derived complexes, a phenomenon also seen in other forms of chronic liver disease [58, 59].

The search for the antigen contained in these complexes has been unrewarding and it now seems possible that the majority are the results of globulin-antiglobulin, C3b-antiC3b, and idiotype-anti-idiotype reactions. Complexes containing food proteins or intestinal bacterial antigens may accumulate later in the disease as a result of impairment of hepatic reticuloendothelial function.

The presence of large quantities of circulating immune complexes, whatever their cause, will have a profound effect on the immune response. IgM containing complexes readily activate C3, bind C3b, and attach to hepatic C3b receptor bearing macrophages and B-lymphocytes. The former have the capacity to phagocytose these complexes thus clearing them from the extracellular space. The overload of this system [22] may be one factor contributing to the accumulation of complexes in the circulation. This may have a significant effect on the ability of C3b receptor bearing monocytes and macrophages to cooperate with the lymphoid cells and may contribute to the anergic state that these patients exhibit on intradermal skin testing [15]. The accumulation of C3b bound to large immune complexes may also influence the humoral immune system in a manner somewhat similar to that seen when cobra venom, a toxin which activates C3 and binds C3b, is injected into an animal [35]. In these animals, conversion from IgM to IgG antibody production in response to a soluble or particulate protein antigen does not occur and protracted macroglobulin antibody response is seen. A similar response to the bacteriophage ØX174 has been described in primary biliary cirrhosis [57] and may be related to blockage of C3b receptor bearing cells by C3b bearing complexes. The continued presence of circulating C3b complexes may also result in a more rapid turnover of C3b receptors and possibly other B-cell membrane components and may explain the findings of large quantities of monomeric IgM [12] and beta 2-microglobulin (personal observation), B-cell membrane proteins, in the serum of these patients.

The proportion of C3b receptors occupied at any point in time is dependent on the concentration of C3b complexes and the rate of clearance of the receptor. The latter is dependent on the cleavage of C3b to C3c and C3d by C3b inactivator (KAF), C3b inactivator accelerator (BIH), and on the solubilization function of the alternative pathway on large immune complexes [36]. The former process appears to function adequately in that large amounts of C3d are formed in these patients sera [56] and KAF and BIH concentrations are normal or increased (personal observation). The solubilization function of the alternative pathway has not been tested but haemolytic (personal observation) and opsonic functions of this pathway are normal [33].

Thus the presence of circulating large immune complexes containing IgM antibody may contribute to the causation of many of the immunological phenomena, including the macroglobulinaemia and anergy, that PBC patients exhibit.

Once bile ductule damage has progressed to the stage of cholestasis, additional changes in the immune system might be anticipated. Plasma cholesterol concentrations increase during cholestasis and an alteration in cell membrane lipid composition follows [38]. This involves an increase in total cholesterol and in the cholesterol to phospholipid ratio. Such changes have been demonstrated in PBC patients and result in a generalised increase in membrane viscosity (personal observations with J. Owen). The activation of lymphocytes by antigens and lectins, such as concanavalin-A (Con-A) or phytohaemagglutinin (PHA), is dependent on the movement of receptor molecules in the plasma lipid bilayer. This occurs less efficiently when the membrane viscosity is increased by increasing the cholesterol content [2]. Such an increase in membrane free cholesterol and viscosity in patients with primary biliary cirrhosis may therefore contribute to the diminished response to antigens and lectins seen in this disease [30]. In addition, normal lipoproteins appear to regulate a number of specific cellular biochemical reactions by attachment to the cell surface via their apoprotein moieties. For example, cellular binding of normal low-density lipoproteins (LDL), at physiological concentrations, inhibits mitogen-induced lymphocyte proliferation [6]. Furthermore, abnormal high density lipoprotein (HDL) from patients with PBC, inhibits binding of LDL to the high affinity receptor on cultured skin fibroblasts (and presumably also lymphocytes) because of its increased content of apoprotein E. This abnormal HDL in the serum of patients with PBC has been shown to inhibit mitogen transformation of lymphocytes [37] and may be an additional factor contributing to the anergy that these patients manifest. These changes progress as the severity of the disease increases and are also seen in other forms of liver disease [4, 62] in which similar lipid changes are found [38].

Cholestasis may also result in abnormalities of immunoglobulin metabolism. In normal rats dimeric IgA is actively transported from the portal venous blood, across the hepatocyte, into the canalicular bile. This transport mechanism is selective for dimeric IgA (and possibly also pentameric IgM) but does not function with monomeric IgA or dimeric IgA which is complexed to the secretory piece [14]. This data has led to the hypothesis that dimeric IgA (and perhaps pentameric IgM) is cleared from the portal blood by a receptor specific active transport system, which involves binding of the polymerised immunoglobulin, by J-chain, to secretory piece

embedded in the hepatocyte sinusoidal membrane. In cholestatic and perhaps in other forms of hepatocellular liver disease, this mechanism might be impaired. The rise in serum IgM and IgA concentrations seen in cholestatic liver disease [5], including PBC and extrahepatic obstructive diseases, are circumstantial evidence in favour of this suggestion. The greater increases in IgM seen in PBC compared with other forms of cholestasis may in part relate to the protracted course of the disease and also to the presence of monomeric IgM in these patients [12].

Pathogenic Mechanisms

Any hypothesis of the pathogenesis of PBC must explain the prominent ductular damage, the extraglandular features of the disease, and the severe perturbation of the immune system including macroglobulinaemia and immune complex formation. Two hypotheses have been proposed:

A) A Primary Defect in Immunoregulation. This is said to permit an aberrant immune attack on ductular tissues which share a common antigenic determinant.

The presence of macroglobulinaemia suggests a deficiency of suppressor T cells capable of controlling immunoglobulin synthesis. Such a defect has been demonstrated by functional assay [23] but is in apparent conflict with the observation that suppressor: helper T lymphocyte subpopulation ratios are increased in PBC rather than reduced [45]. These observations suggest that there is either a functional defect in the suppressor cell population or that there is a defect in a population of T cells required by suppressor T cells to mediate their regulating effect on the B lymphocyte. Whether these changes in the regulation of immunoglobulin synthesis are primary or secondary events is unknown. It is, however, noteworthy that they are not seen in other types of cholestasis [23, 45].

Abnormalities of suppressor T cell control of the proliferative response of cytotoxic T cells have been claimed [7, 67]. Mitogen stimulated cells from these patients fail to show the usual increase in ability to suppress the proliferative response of effector T cells to concanavalin-A and HLA antigens. Such a state of affairs would be manifest if the suppressor cells were deficient, defective, or already maximally operative. The observation that the proportion of peripheral T cells which are suppressor T cells is increased in these patients [45] and that the patients exhibit severe anergy to DNCB and recall skin testing [15], would lend support to the later possibility, although a defect in the effector limb of the delayed hypersensitvity response is an alternative explanation. Further studies are needed to differentiate between these possibilities, and to determine whether they are primary or secondary events.

The involvement of an autoimmune process in the destruction of the ductular epithelium is suggested by the observation that the peripheral blood lymphocytes of patients with primary biliary cirrhosis are sensitised to a biliary protein [29]. It is proposed that it is the immune response to this antigen which is responsible for immune damage to the biliary tree. This thesis is weakened by the demonstration of a similar level of sensitization to the same biliary protein in sclerosing cholangitis [29], a disease which does not exhibit similar features to PBC. It seems more likely

that the sensitization to this biliary antigen is a secondary manifestation of the disease occurring as a result of denaturation of epithelial cell antigens. The biliary protein which has stimulated most interest (BP III) has a molecular weight of 790,000 and is present on biliary and submaxillary duct, spleen, and thyroid cells [66]. It is of interest that these tissues also display a high density of histocompatibility complex proteins [40, 50] and it is possible that the biliary lipoprotein antigen (BP III) contains the proteins of the histocompatibility complex.

B) A Graft-Versus-Host-Like Process. Many of the clinical and immunological features of PBC can also be seen in patients with chronic graft-versus-host (GVH) disease following bone marrow transplantation [9] (Table 1). This raises the possibility that an abnormal immune response to the antigens of the histocompatibility complex may underlie the pathogenesis of primary biliary cirrhosis.

In chronic graft-versus-host disease, the grafted lymphoid cells mount an immune response to the recipients tissue antigens. This response is primarily directed to the histocompatibility antigens and characteristically damages the epithelium of the small intrahepatic bile ducts [28, 41, 46, 55] which are known to display a high density of histocompatibility complex antigens [40, 50]. These patients also develop a sicca syndrome with histological similarity to the salivary lesion seen in primary biliary cirrhosis. The ducts of these glands also display a high density of histocompatibility antigens [50]. Thus is chronic GVH, ductular damage

Table 1. Similarities between primary biliary cirrhosis and chronic graft-versus-host disease

Clinical features	PBC	Chronic GVH
Liver disease	Cholestatic	Cholestatic
"Sicca" complex	+	+
Skin pigmentation	+	+
Sclerodermatous skin	+	+
Raynaud's phenomenon and calcinosis	+	+
Liver histology		
Small bile duct damage	+	+
Portal fibrosis and inflammation	+	+
Piecemeal/parenchymal necrosis	±	±
Immunology		
Autoantibodies	+ (especially AMA)	+ (probably not AMA)
Hyperglobulinaemia	IgM, IgG	IgM, IgG
Immune complexes	+	+
Failure of conversion from IgM to IgG synthesis	+	+
DNCB anergy	+	+

Epstein, Thomas, and Sherlock (1980)

leads to development; of a "dry gland syndrome", similar to that seen in PBC [9]. In addition to cholestasis and the sicca syndrome, patients with chronic GVH develop extraglandular features similar to PBC, including skin pigmentation [18, 19, 51], sclerodermatous skin lesions [18, 19, 27, 31, 51, 52], Raynaud's phenomenon [19], and calcinosis [18].

Furthermore, abnormalities of cell-mediated and humoral immunity similar to those seen in PBC occur in chronic GVH disease. These patients also exhibit a generalised state of immune hyporesponsiveness, being anergic to DNCB and producing diminished antibody responses to antigenic challenges [55, 65]. Lioke patients with PBC, they exhibit failure of the immune response to convert normally from IgM to IgG antibody synthesis [65]. In both diseases there is an increase in the ratio of suppressor to helper T lymphocyte [44, 45] which is presumably contributary to the state of anergy. The small intrahepatic bile ducts are damaged and portal tracts are infiltrated with mononuclear cells [46] and in animal models of chronic GVH disease, multinucleate giant cells and epitheloid cells are present in the tissues [46]. Patients develop hyperglobulinaemia (IgM and IgG) [65] and markedly increased levels of circulating immune complexes are detected in the serum [19]. Various autoantibodies are also produced [51].

Is PBC a Form of Chronic GVH Disease?

In both diseases there is an immune assault on the ductular epithelial tissues and the skin and this is associated with severe perturbation of the humoral and cellular immune system. In the case of GVH disease the immune reaction is directed towards the histocompatibility antigens. In the case of PBC, if these antigens are the basis for the immune assault, there must be either an alteration in their antigenicity or in the capacity of the lymphoid cells to maintain a state of tolerance to these antigens. The evidence for the existence of these two mechanisms of induction of a GVH-like disease is reviewed.

A) Alteration of the Antigenicity of the HC Complex. In normal mice, GVH-like disease has been described following chronic viral infection [53], and in an HLA-matched bone marrow transplant recipient, after acute measles infection [13]. In both circumstances it seems probable that viral infection produced a change in the antigenicity of the histocompatibility complex [8, 13]. In addition, cross-reactivity between human leucocyte antigens (HLA) and bacterial lipopolysaccharides [21] and the M protein of the streptococcus has been described [20]. Thus, both viruses and bacteria may alter the antigenicity, or cross-react with, the HLA determinants. In PBC, the GVH-like process may be triggered by a similar mechanism involving either an alteration of the antigenicity of the histocompatibility complex or partial cross-reactivity between these antigens and exogenous antigen, possibly microbial. This would result in damage to tissues bearing histocompatibility antigens in high density and, in addition, result in polyclonal activation of lymphoid cells resulting in hyperglobulinaemia, autoantibody synthesis, and immune complex formation.

B) Alteration in the Lymphoid Cells Involved in HC Antigen Recognition. An alternative explanation for the occurrence of a GVH reaction in PBC is the possibility that the disease represents true GVH disease resulting from the

transplacental passage of maternal lymphocytes to the foetus. Such an event has been shown to cause acute neonatal GVH disease [20], and it has been suggested that low dose materno-foetal lymphocyte transfusion may result in clinically detectable disease in man after a prolonged latent period [49]. The persistence of maternal cells in the neonate may depend on induction of tolerance in utero, or failure of the immature foetal surveillance mechanism to recognise antigenically similar maternal lymphocytes as "foreign". In order to test this hypothesis we have examined the peripheral lymphoid cells of five male PBC patients for male/female chimerism by chromosome analysis. Preliminary data has failed to establish such a state, but it remains possible that the level of chimerism is very low.

Finally, a GVH-like reaction might also occur if there was a deficiency of the lymphoid cell population responsible for recognition of self-histocompatibility complex antigens. Such a defect would explain the multisystemic nature of PBC.

Summary

The clinical, histological, and immunological similarities between PBC and chronic GVH disease suggest that an immune response to a native or altered HC antigen might underlie the pathogenesis of both diseases. The insidious onset and slow rate of progression of PBC would suggest the involvement of a minor, rather than a major, antigen within the HC complex. An immune response to such an antigen might occur as a result of alteration of the antigenicity of the HC antigens, partial cross-reactivity between HC and environmental antigens, or changes in the lymphoid system including the existence of maternally derived lymphocytes or a deficiency of the cell population required to maintain tolerance to the native self HC antigens. Such mechanisms would explain the glandular and extraglandular features of the disease and also the marked aberration of the humoral and cellular immune systems. The latter would not be expected if the disease were the result of a specific defect in regulation of the immune response to a non-HC antigen displayed on the biliary ductular epithelium and cross-reacting with determinants on other glandular, dermal, and renal tissues.

Finally, the specific association of the mitochondrial antibody with primary biliary cirrhosis, must be accounted for by any hypothesis dealing with the pathogenesis of the disease. Various autoantibodies are produced in patients with chronic GVH disease but the mitochondrial antibody is not amongst them (personal observation). It is possible that an extrinsic event precipitates or induces failure of self recognition by T cells either by alteration of the HC antigens or of the regulation of the lymphoid system and that this event also initiates the production of antibody cross-reacting with the mitochondrial antigens. In this sphere it is interesting that many micro-organisms show antigenic cross-reactivity with the mitochondrial [24] and histocompatibility complex antigens [20, 21].

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Hepatic Transplantation

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Introduction

Clinical transplantation of the liver began in Denver in 1963 and since that time the Denver series has now reached some 200 cases. The Cambridge/Kings College Hospital series, dating from 1968, has now reached 95 in number. The practice of liver transplantation elsewhere has been limited, although a number of series in other centres are now under way. The lack of enthusiasm for liver transplantation has been due to the poor overall results, the difficulty in management of the patients, and particularly the hazards of the operation itself. For transplantation of the kidney, haemodialysis can keep the patient in excellent condition whilst awaiting the operation and can maintain the patient should the organ be slow to develop full function or suffer functional impairment from irreversible rejection. In liver transplantation there is no such comparable luxury of artificial liver support. For such a dangerous and unproven procedure, referring physicians tend to think of liver transplantation late in the course of the patient's illness and sometimes when the patient is in a moribund state. To operate on such a case can do the patient no good and failure to operate implies a lack of interest on the part of the Transplant Centre. Only improved results will generate confidence in referring patients for operation before they are too sick for the procedure.

Indications

In the course of our experience, we have transplanted patients with many different diagnoses but we feel now that certain cases are totally unsuitable and others seldom good candidates. Thus, patients with metastatic cancer, although withstanding the operation well, have all developed recurrent growth very quickly. Patients with cholangio-carcinoma have also done badly with early recurrence. The only primary malignancy in which long-term survival has occurred has been primary liver cell cancer with or without accompanying cirrhosis. Of the cirrhotic processes alcoholic cirrhosis tends to be unsuitable because of the nature of the

patients who develop the disease. They are frequently unreliable and do not respond well to the rigorous post-operative management that is required. The truly reformed alcoholic would be considered. Children with biliary atresia have not figured prominently in our series because of the difficulty of obtaining donor livers and also the extremely unpleasant side-effects of steroid drugs that may occur in children. With the advent of non-steroidal immunosuppression this latter contra-indication would be removed. In the Denver series, excellent results have been obtained in children with primary biliary atresia and most of Starzl's patients have been in the paediatric age group. Co-existing infection is a contra-indication to liver transplantation, unless this is confined solely to the liver. Even in cases of secondary biliary cirrhosis with recurrent cholangitis, there is a danger of sepsis developing post-operatively. We are reluctant to transplant patients over 60 years, because of the poor results in older patients.

Assessment

If a patient presents with primary hepatoma involving both lobes of the liver and is therefore unsuitable for partial hepatic resection, liver transplantation may be considered. Great care is taken to try to exclude metastatic deposits from the growth, especially in the lungs, bone, and lymph nodes. Angiography of the hepatic arterial system, portal venous tree, and inferior vena cava, whole lung tomography, and computerised axial tomography are performed. Even when such precautions have been taken, metastatic growth has occurred following transplantation in 60% of our patients with primary liver cell cancer. Some patients appear to have been cured of their disease. It is also important to ensure that the diagnosis is correct, since secondary tumours, especially from colon, pancreas, and kidney can mascarade as primary hepatoma. Intravenous urography, barium studies of the gastro-intestinal tract, and the computerised axial tomography should exclude such occult primaries.

For patients suffering from a cirrhotic process, which include the Budd-Chiari syndrome, it is important that liver transplantation should be considered before the patient has taken the final downhill course towards a moribund state. Ideally, the clinicians should think of liver transplantation the first day it is apparent that the patient cannot leave hospital or can only lead a severely incapacitated life outside of hospital. For instance, a patient who has developed porto-systemic encephalopathy with or without a shunt having been performed; or a patient who has bled from oesophageal varices and has insufficient liver function to withstand a shunt operation. It is desirable for the patient to be referred to the Liver Transplant Centre at an early stage, even prior to definite consideration for liver transplantation, so that he can meet the nursing and medical staff including the Anaesthetist and Intensive Care nurses and discuss the pros and cons of operation. It is very important that the patient should really want the operation and his family should be supporting him in this since the psychological aspects post-operatively may well determine survival. Once the patient has been accepted on the transplant programme, he must be within easy access to the hospital where the operation will be performed, since seldom is there more than six hours' notice available.

The Operation

The donor must be a case of complete and irreversible cerebral death with an intact circulation, maintained on a ventilator, free of sepsis, and systemic malignancy. The criteria are the same as for donors for renal transplantation.

We now have confidence in ten hours of preservation of a liver by simple cool flushing and storage in ice [10] so we are prepared to travel by car, plane, or helicopter to any institution where there is a suitable donor, where the distances will allow transportation of the liver back to Cambridge, so that the liver can be revascularised within ten hours of removal from the donor. The minimum team required to remove the liver is two surgeons, a scrub nurse, and a technician for the perfusion. We always remove the two kidneys as well as the liver and have also removed the pancreas for transplantation purposes.

The donor operation takes between one and two hours and requires careful dissection under optimal conditions so that the liver is not damaged. Anomalous blood vessels in the liver, particularly of the arterial supply, make it necessary to dissect with great care so that all arterial branches are preserved. The most common abnormalities encountered are a right hepatic arising from the superior mesenteric artery and coursing posterior to the portal vein, which occurs in 17% of cases, and the branch supplying the left lobe arising from the left gastric which occurs in 23% of cases. When the liver is fully skeletonised and all peritoneal attachments have been divided, and only the vascular connections remain, the ventilator is stopped and blood is removed from the aorta via a cannula in the right external iliac artery and from the inferior vena cava (IVC) by similar cannula in the venous system, and the portal vein is perfused through the superior mesenteric by gravity drainage from one metre with one litre of Hartmann's solution at 4° C. Whilst this is in progress, the two kidneys are removed and preserved in the normal way. The perfusate is then changed to plasma protein fraction with additives giving approximately intracellular constitution [10]. Then the liver is removed, placed in a bowl containing iced cold saline. One hundred and fifty ml of the same solution are perfused through the hepatic artery and a similar volume through the main hepatic ducts and the gallbladder, after two incisions have been made in the gall-bladder, one in Hartmann's pouch and one in the fundus. With the liver cold the common bile duct is cut obliquely and anastomosed to the incision in the fundus of the gall-bladder with a 4/0 running catgut stitch reinforced with seramuscular prolene stitches. The liver in its bowl of saline is then packed in two sterile polythene bags containing iced saline, the outer one being surrounded by non-sterile ice in a polystyrene box.

The Recipient Operation

Skilled and experienced anaesthesia is essential with adequate technical assistance for monitoring of the arterial and central venous pressure, the ECG, and frequent estimations of the acid base balance and blood gases. Urinary output is measured by an indwelling catheter. Under light general anaesthesia with full relaxation an incision is made across the abdomen with a vertical extension upwards to the xiphoid. The chest is not entered. Skeletonisation of the diseased liver may be

extremely difficult if there is portal hypertension with huge venous collaterals under high pressure and impaired clotting. Prior to surgery an attempt is made to improve impaired clotting by administration of fresh frozen plasma, and vitamin K. Further fresh frozen plasma, platelet packs, and blood as fresh as possible is kept in reserve for the moment of revascularisation of the new liver. Bank blood is used during the removal of the patient's own liver. Despite the difficulties of hepatectomy both Starzl and ourselves believe that the orthotopic operation is better than inserting an extra or an accessory liver, because techniques for such a procedure have not yet become reliable and safe. Prior to removal of the patient's own liver, the vena cava is temporarily clamped to determine whether the patient can accept this fall in cardiac output. Some patients with severe liver disease have a toxic myocarditis, arteriovenous pulmonary shunts, and their poor general condition will not enable them to withstand caval clamping. If the blood pressure continues to fall the patient will succumb even when extra fluids are infused into the superior caval system, because the heart cannot respond to this load of banked blood or other fluids in the right side of the circulation. Under these circumstances we perform partial cardiopulmonary bypass, draining blood from the inferior vena cava via the right femoral vein; warming, oxygenating, and pumping blood under pressure into the right common femoral artery. The use of bypass gives excellent control of the circulation but the patient requires full heparinisation and the blood receives further damage during the bypass procedure. Therefore, if it is possible, bypass is avoided and it is preferable to operate on the patient before it is needed. The preoperative blood pressure is a useful guide. If it is below a systolic of 100 then the patient is probably too sick for liver transplantation.

Following clamping of the vena cava, portal vein, hepatic artery, and vena cava above the liver, great attention is paid by the anaesthetist to maintaining an adequate circulation. The new liver is removed from ice and the vena cava above the liver is anastomosed end-to-end donor and recipient. Then the portal vein similarly an end-to-end anastomosis. Prior to completion of this anastomosis a cannula is inserted into the portal vein and the liver perfused with 500 ml of plasma protein fraction at room temperature, to start warming the liver, and to remove potassium and acidic ions that have accumulated in the hepatic veins from the liver cells and preservation solution. The effluent escapes through the inferior vena cava, below the liver which is left open, and when the portal venous anastomosis is completed, the first 100 ml of blood are allowed also to escape from the inferior vena cava below the liver as this will contain stagnant blood from the visceral circulation. The vena cava below the liver is then clamped and the clamp removed from the vena cava above the liver. The vena caval anastomosis below the liver is then constructed end-to-end and a Carrel patch of aorta, containing the orifice of the coeliac artery, is anastomosed to the recipient common hepatic artery. The fundus of the gall-bladder is now anastomosed to the recipient common bile duct and the two biliary anastomoses are splinted by a guttered Latex T-tube brought out via the body of the gall-bladder, and a stab incision in the abdominal wall. This T-tube is left in place for three months. For the first ten days it is allowed to drain freely; then, if a cholangiogram is satisfactory, the T-tube is spigotted and left in place until three months have elapsed when another cholangiogram is performed. If again, the picture is satisfactory, the T-tube is removed. The T-tube provides access to

the biliary tree should the patient become jaundiced, when satisfactory drainage of the extra-hepatic biliary system can be demonstrated, to exclude an extra-hepatic cause of the jaundice.

The wound area is drained with four drains and the patient is nursed in the Intensive Care Unit till he has been extubated and is able to be moved to the general ward where barrier nursing is carried out for the first ten days.

Immunosuppression

Standard immunosuppression has been Imuran and steroids in roughly the same dose as those given to kidney transplant recipients. Acute rejection, which commonly occurs at about a week, is treated by increasing the steroid dose either orally or with intravenous doses of Solumedrone.

In the past year we have been investigating Cyclosporin A as an immunosuppressant. This drug can be hepato-toxic and is certainly nephrotoxic. Since it is fat soluble absorption probably depends on biliary secretion, and excretion of the drug is also via the bile. Thus liver metabolism must be central in the pharmakinetics of Cyclosporin A. We have therefore given a low dose of Cyclosporin A 10 mg/kg/day instead of 17 mg which is the starting dose in renal transplant patients. Some patients have done very well but nephrotoxicity has made management difficult in two patients who required post-operative dialysis. Both these patients had been in renal failure from hepato-renal syndrome prior to liver transplantation and both recovered renal function. The place of Cyclosporin A in liver transplantation is still being investigated.

Diagnosis of Rejection

Fortunately rejection of the liver is frequently mild and less severe than rejection of kidney or heart transplants. The diagnosis can be difficult but rejection is always accompanied by a rise in the serum bilirubin and alkaline phosphatase levels and later a rise in transaminase levels.

Leucocyte migration may indicate an active immune process. Cholangiography is performed and, if the biliary drainage is satisfactory, a liver biopsy is carried out, provided the patient's clotting factors and platelet count are not impaired. The histological features of rejection are similar to those in the kidney but less specific. There is round cell infiltration in the portal tract and in more severe cases accompanying hepatic necrosis and infiltration of mononuclear cells into the hepatic parenchyma. An incidious form of rejection which is extremely difficult to treat may be manifest by progressive jaundice with good residual liver cell function until a terminal state has been reached. Biopsy shows destruction and eventual disappearance of small intra-hepatic bile ducts. The histological picture is similar to primary biliary cirrhosis but it occurs in patients who have not had primary biliary cirrhosis and rejection is not accompanied by a rise in anti-mitochondrial antibodies.

Other Complications

Apart from rejection, the other main complications are sepsis, either in the abdomen or lungs. In the past this was often preceded by leakage of bile and cholangitis but since the gall-bladder has been used as a biliary conduit and bile has been carefully flushed out of the biliary system prior to preservation of the liver, complications of biliary drainage have been few. To date, the patient's own disease has not recurred with the exception of malignancy referred to above.

Results

The Denver series were reported in detail in 1979 [9]. In our series 95 orthotopic liver allografts have been performed since May 1968 to May 1980. Sixty patients were operated on up until the end of December 1976 [1]. Since 1976 management has changed by a process of evolution based on past experience. We have moved towards an earlier selection of patients and modifications of technique have resulted in the current methods described above. Patients have returned to normal active life with long-term survival. In the past year 10 patients received liver transplants and five are currently alive between two weeks and nine months. Nineteen have now lived more than one year, eleven survived over two years, eight more than three years, three more than four years, and eight patients are at present alive more than one year after operation. Of those patients surviving less than three months eight died from uncontrollable haemorrhage in the peri-operative phase or from cardiac arrest at the time of revascularisation of the new liver. Another three patients died from uncontrollable haemorrhage shortly after surgery and one patient died from unexplained cardiac arrest the third day after operation. Three patients had progressive hepatic necrosis and two of these also had thrombosis of the hepatic artery. One patient, transplanted for a Budd-Chiari syndrome, developed thrombosis of the hepatic veins, hepatic artery, and inferior vena cava three weeks after operation. She had not been treated with anticoagulants. Since that time we give continuous anticoagulation to Budd-Chiari patients. Most deaths are due to sepsis caused by biliary leaks or sludge formation before the introduction of current techniques. Recurrent malignancy occurred mainly between three months and one year after operation and occurred in nine of 15 patients with malignant disease. Chronic rejection caused death in seven patients.

In addition to the eight current survivors of the 19 patients who lived a year or more, five other patients are alive who have undergone operation during the past year. These are all well currently. One of these patients is alive eight months after operation. He is a 25-year-old man who received a segmental pancreatic graft for severe insulin-dependent diabetes in addition to a liver transplant for his cirrhosis following hepatitis. He is receiving only Cyclosporin A as an immunosuppressant and no insulin.

Long-Term Survivors

The mean age of those surviving a year or more was 40, the eldest being 59 years. Nine had primary malignancy, eight of these have died subsequently between one and six years later, five from recurrent tumour. Of the 10 patients with non-maligant disease only three deaths have occurred, from septicaemic cholangitis; at 16 months, 22 months, and more than two years after operation. All patients in this group of 19 one year survivors have been able to lead active lives after initial recovery from operation and, with the exception of two patients, all were able to return to their original occupations. The three longest survivors, two of whom lived for more than five years, had originally suffered from primary liver cell cancer. The longest survivor (5 years and 10 months) died from variceal haemorrhage and cirrhosis of the transplanted liver, presumed to have resulted from non-A, non-B hepatitis. She had led an active life which included swimming and cycling. Another patient, who lived for more than five years, returned to work as a Domestic Supervisor, and died in 1974 from cholangitis and septicaemia. There was inspissated sludge throughout the biliary tract and in neither this patient or the other five year survivor was a recurrent tumour found. The third long survivor had a familial hepatoma and is well with no tumour recurrence four years after transplantation. His serum had been positive for hepatitis B antigen and during surgery he was given specific anti-HBs immunoglobulin. Since that time he has been HBs Ag-negative [3].

Two 33-year-old patients have been very active after transplantation, both patients more than three years after operation can walk long distances and one runs five miles cross country every day. The other has been on a long camping holiday carrying a back-pack across the Yorkshire Dales.

Conclusions

The perioperative mortality of cirrhotic patients having a liver transplant has been high because the patients have often been referred too late. Circulatory collapse during surgery following clamping of the vena cava or excessive potassium and hydrogen ion flux into the systemic circulation when the portal venous, and sub-hepatic vena caval anastomoses have been opened, have been responsible for some of the fatalities. It is to be hoped that earlier operation and the use of cardio-pulmonary bypass in selected cases should reduce these operative deaths.

McMaster has shown that sloughing of the biliary mucosa is probably a consequent of damage of the mucosa by bile left in the biliary tract when the liver is being cooled and the normal protective mechanisms in the mucosa have paralysed. Careful washing of bile has reduced the incidence of sludging and this, together with the gall-bladder conduit technique [2, 5] have greatly reduced the previously common biliary complications.

There has been no attempt to select donors for HLA A and B antigens and the data analysed by ourselves and the Denver group have failed to show significant correlation with donor and recipient match. Transplantation has even been carried out in the presence of cytotoxic antibodies in the serum that destroyed 100% of donor lymphocytes, and in the Denver series seven patients have received ABO

incompatible transplants without evidence of rejection [7]. Despite the low incidence of rejection corticosteroids and Azathioprine have resulted in depressed defences against infection which has been the main cause of death. Four of the six patients treated with Cyclosporin A are alive and well. Although tumour recurrence occurred in 60% of our patients, our three longest survivors were in this group and worthwhile palliation has been achieved in a significant proportion of cases [4]. Our criteria of selection has become stricter and we only consider now young patients who are alpha-fetoprotein negative since in these patients tumour growth is probably slower [6]. In such patients and also cases with end stage cirrhosis or chronic irreversible liver damage from other causes such as the Budd-Chiari syndrome, the best results have been obtained. Primary biliary cirrhosis has been a particularly good indication for liver transplantation probably because the disease tends to run a predictable course, the final phase being more easy to recognise than in other forms of cirrhosis. Many of the cirrhotic patients have had osteoporosis and this is exacerbated by corticosteroids used in immunosuppression. We give microcrystalline calcium hydroxyapatite to patients with bone pain if they have received steroids.

In patients with primary biliary cirrhosis antimitochondrial antibody has fluctuated in the serum after operation without any evidence of recurrence of the disease in the transplanted liver. An initial drop post-operatively is probably due to the dilution effect of blood transfusion occurring during the operation [8].

Although there have been many failures, liver transplantation has restored to normal life for a number of years patients who were dying of untreatable liver disease. The longest survivor, a patient in Denver, is now more than 10 years after operation and well. Two patients in Denver have given birth to normal children after liver transplantation.

Improvements in operative technique and earlier and more stringent selection of cases have, in recent years, caused an improvement in the results. The operation remains formidable and can only be seriously undertaken by a dedicated team with ongoing experience of successful orthotopic transplantation in large animals and preferably in the environment of a programme of kidney transplantation to provide the familiarity with organ donation, organ preservation, immunosuppression, and the immunology of transplantation.

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The Nature of the Hepatitis B Virus and its Mode of Replication

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1. Introduction

Many viruses infect the liver of man and may produce severe disease. Although cytomegalovirus, Epstein Barr, and yellow fever viruses are frequently associated wiht inflammation and necrosis of the liver, these are frequently excluded by common usage from the term viral hepatitis. This term is generally used to describe infection by one of four agents, all of which are primarily hepatotropic and represent the more important viruses associated with human liver disease. Infection with hepatitis B virus is a major public health problem in all parts of the world and constitutes a major hazard of the transfusion of blood and plasma derivatives. In addition to the acute illness, hepatitis B may progress to chronic liver disease including chronic active hepatitis, cirrhosis of the liver, and probably primary liver cancer. Furthermore, persistent infection may be established and it is estimated that there are about 176 million carriers of this virus throughout the world (WHO 1977). The immune response to infection with hepatitis B virus is elicited by at least three antigenic systems – hepatitis B surface antigen (HBsAg), the core antigen (HBcAg), and hepatitis B e antigen (HBeAg) – resulting from replication of the virus in the liver. Persistence of HBsAg in the circulation is a characteristic feature of chronic infection with this virus, whereas the presence of antibody to this antigen (anti-HBs) is associated with convalescence and protection from re-infection.

Despite the repeated failure of attempts to isolate hepatitis B virus in vitro, the role of individual antigens in the immunopathogenesis of hepatitis B can be evaluated in the light of independent lines of experimental investigation. These include (a) the use of sensitive serological tests for detecting virus-specific antigens and antibodies in acutely and chronically infected persons, (b) the development of the susceptible chimpanzee as an experimental model of infection, (c) the study of liver hepatoma cells maintained as continuous cell cultures, and (d) the application of DNA cloning techniques for analysis of hepatitis B genome structure. It is remarkable that despite the lack of conventional tissue culture systems for the study of the virus, the possible mode of replication of hepatitis B virus within the infected hepatocyte and the strategy of its genome can be predicted. Indeed, the wealth of information available has resulted in the present evaluation of purified HBsAg-reactive material as a potential hepatitis B vaccine in man.

This review outlines the nature and properties of hepatitis B gene products, the possible replication strategy of the hepatitis B virus, and indicates areas of future investigation. For additional information, the interested reader is directed to Vyas et al. [87] and Zuckerman and Howard [92].

2. The Properties of Hepatitis B Virus Gene Products

a) The Surface Antigen (HBsAg)

The overwhelming mass of HBsAg-reactive material in the sera of hepatitis B infected individuals is present as small 22 nm lipoprotein particles (Fig. 1), and is distinct from the hepatitis B virus particle which has a larger diameter of 42 nm with a defined inner core component. Filamentous form of HBsAg also occur, although the relationship of these structures to either whole virus or 22 nm particles remains unclear. Purified 22 nm particles are readily obtained by ultracentrifugation, the relatively slowly sedimenting small particles having a mean sedimentation coefficient ($S_{20,w}$) in the range 33–45 S. The mol. wt. of these structures is estimated at 2.5×10^6, of which up to 30% may be accounted for by the lipid moiety. Further details of the physicochemical properties of HBsAg 22 nm particles may be found in Howard and Burrell [25] and Zuckerman and Howard [92].

The protein moiety of HBsAg-bearing particles has been extensively analysed. Early amino acid analysis showed that the 22 nm particles are rich in tryptophan and hydrophobic amino acids, particularly leucine. These early studies are in close agreement with the predicted amino-acid sequence obtained by nucleotide sequencing of the surface antigen gene. Purified HBsAg is remarkably stable in the presence of compounds promoting dissociation and denaturation, including urea, sodium dodecyl sulphate (SDS), various proteolytic enzymes,and is stable for many hours at an acid pH. Reduction of disulphide bonds, however, results in the complete loss of HBsAg reactivity, although considerable antigen activity may be recovered by the alkylation of free sulphydryl groups with iodoacetamide. Serological analysis has shown that HBsAg particles share a common group specific antigen *a* and generally carry at least two mutually exclusive subdeterminants *d* or *y*, or *w* or *r*.

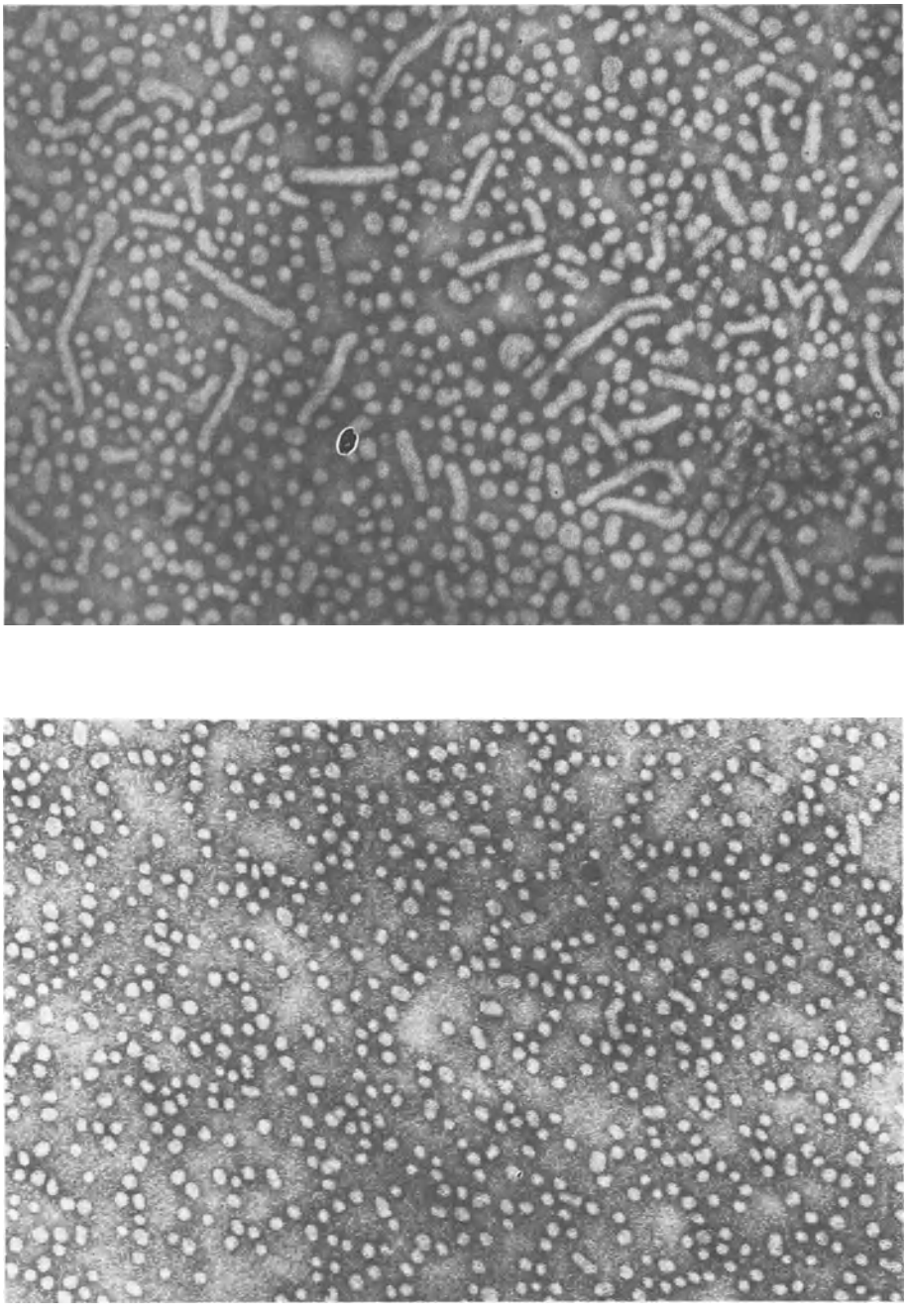

Fig. 1 a. Negative staining electron microscopy of hepatitis B antigens. Small 22 nm HBsAg spherical particles and filaments are abundant in the sera of infected individuals. The hepatitis B virus is occasionally seen as a larger 42 nm particle with a distinct 27 nm core. **b.** Purified preparation of 22 nm HBsAg particles. Similar preparations are currently under investigation as suitable vaccines for the immunoprophylaxis of hepatitis B

The group determinant *a* is destroyed by exposure to dithiothreitol at concentrations below 10 mM. At higher concentrations antigenic reactivity, which is resistant to reduction and unrelated to the *d*, *y*, *w* and *r* subdeterminants, exists on the same antigen particles [31]. It has also been demonstrated that the *a* group-specific determinant is stable at 60° C for periods of up to 21 h, whereas the *d* and *y* subtype reactivities are markedly decreased after 3 h at the same temperature.

The polypeptide composition of the major HBsAg subtypes has recently been compared by Shih and Gerin [68]. Up to seven polypeptides were resolved in all the preparations examined. Two polypeptides with molecular weights of 23 000 and 29 500 respectively were consistently found as the major component. The remaining polypeptides were present in variable amounts, with the number and relative concentrations varying both between heterologous subtype preparations and within preparations of the same subtype obtained from different sources. The most notable of the minor components was a 72 000 molecular weight polypeptide which was particularly prominent in some gels.

Skelly et al. [75] have demonstrated the presence of host protein in purified preparations of 22 nm small particles by treatment of HBsAg with the non-ionic detergent Triton X-100 and subsequent affinity chromatography. Passage of the solubilized material through a column of concanavalin A-Sepharose 4B in the presence of the detergent separated the components of HBsAg particles. The first fraction, which did not bind to the lectin, contained exclusively a 64 000 mol. wt. component which was identified chemically and serologically as serum albumin. The second fraction, recovered by elution with α-methyl-D-mannoside, contained a glycosylated 28 000 mol. wt. polypeptide and a non-glycosylated 23 000 mol. wt. polypeptide. Both these polypeptides were major components of the intact 22 nm HBsAg particles (Fig. 2) and these components may possibly be joined by protein-protein linkage. Mishiro et al. [43] have also shown that a 49 000 mol. wt. sub-unit can be prepared in the absence of reducing agents by treatment of HBsAg particles with 1% SDS. The reactivity of these sub-unit preparations with anti-HBs and the absence of interaction with antisera to serum proteins make these suitable candidate preparations for prophylactic use. Such a "second generation" HBsAg preparation may be prepared as a vaccine in micellular form: Skelly et al. [76] have shown that HBsAg micelles are considerably more potent inducers of anti-HBs in mice when compared to intact 22 nm particles. Chemical analysis showed only the presence of the 28 000 and 23 000 mol. wt. HBsAg polypeptides.

The similarly sized 23 000 and 29 500 mol wt. components reported by Shih and Gerin [68] as major components of HBsAg particles were subsequently shown to have a similar amino-acid composition [69]. Peterson et al. [59] independently obtained sufficient quantities of each polypeptide to enable the identification of amino-terminal and carboxy-terminal sequences by chemical analysis. Both polypeptides contained the same NH_2- and -COOH terminal amino acids as follows:

Met -Glu-Asn-Ile-Thr-Ser-Gly-Phe-Leu-Gly-Pro-Leu-Leu-Val-Leu*-Glx-Ala-Gly-
(NH_2) Val-Tyr-Ile
(COOH)

* Originally tabulated as Ser by Peterson et al. [59]

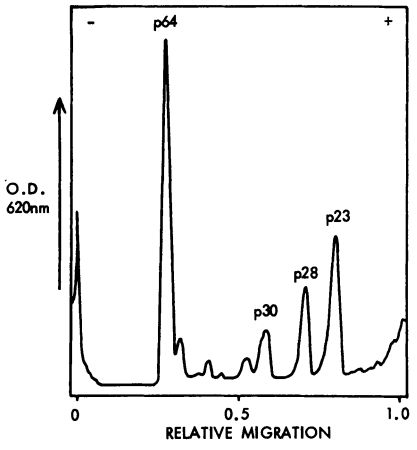

Fig. 2. SDS-polyacrylamide gel electrophoresis of purified HBsAg 22 nm particles. Major components migrate with estimated molecular weights of 23 000, 28 000, 30 000, and 64 000 respectively. The 64 000 mol. wt. protein has been shown to be chemically and immunologically indistinguishable from serum albumin [75]

The common amino-terminal and carboxy-terminal sequences of the amino acids in both polypeptides strongly suggest that the HBsAg preparation consisted either of a single major polypeptide existing in both glycosylated and non-glycosylated forms or two nearly homologous polypeptides with minor differences in limited areas of their structure. The former possibility now seems the most likely now that nucleotide sequence data is available for the hepatitis B virus genome. Valanzuela et al. [86] using cloned DNA fragments have identified an 892-base pair region along the full length DNA strand as coding for the small 23–25 000 mol. wt. polypeptide of HBsAg particles. The translation of this sequence predicts precisely the previously published limited sequence of amino acids at the amino and carboxyl ends of this protein. The full amino acid sequence predicted by these studies is shown in Fig. 3. The 226 amino acid long polypeptide contains a relatively high content of aromatic and hydrophobic amino acids, in accord with the published amino acid content of HBsAg particles. The sequence also predicts a globular configuration with a 19-amino acid long hydrophobic region. Galibert et al. [17] have reported the corresponding sequence for the *ayw* subtype HBsAg gene which suggests a total of 16 amino-acid changes, six of which are clustered in a region immediately on the carboxyl side of the conserved hydrophobic region.

From the evidence discussed so far, it would therefore appear that the HBsAg particles contain at least two virus-specific polypeptides resulting from transcription of the same gene, one of which is glycosylated, and together form a sub-unit structure via disulphide bonds. Within the infected cell, therefore, a proportion of the "S" gene products become glycosylated through the action of the (host-specified?) glycosylation pathways. The carbohydrate moiety of the 28 000 mol. wt. component contains terminal sialic acid [47, 74]. In the latter study, it was found that radiolabel was introduced into sub-terminal galactose residues following the sequential removal of sialic acid, oxidation with galactose oxidase, and reduction with tritiated sodium borohydride. Subterminal galactose was also identified independently by Neurath et al. [48] who found that desialyated HBsAg was retained on columns of immobilized peanut lectin specific for D-galactose. Skelly et al. [75] using radiolabelled concanavalin A identified two glycoproteins with

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                                                                  10
H2N.met glu asn ile thr ser gly phe leu gly pro leu leu val leu gln ala gly phe
20                                                                30
phe leu leu thr arg ile leu thr ile pro gln ser leu asp ser trp trp thr ser leu
40                                                                50
asn phe leu gly gly ser pro val cys leu gly gln asn ser gln ser pro thr ser asn
60                                                                70
his ser pro thr ser cys pro pro ile cys pro gly tyr arg trp met cys leu arg arg
                                    thr
80                                                                90
phe ile ile phe leu phe ile leu leu leu cys leu ile phe leu leu val leu leu asp
100                                                              110
tyr gln gly met leu pro val cys pro leu ile pro gly ser thr thr thr ser thr gly
                                                          ser
120                                                              130
pro cys lys thr cys thr thr pro ala gln gly asn ser met phe pro ser cys cys cys
    arg         met     thr                 thr         tyr
140                                                              150
thr lys pro thr asp gly asn cys thr cys ile pro ile pro ser ser trp ala phe ala
                                                                      gly
160                                                              170
lys tyr leu trp glu trp ala ser val arg phe ser trp leu ser leu leu val pro phe
180 phe     ser                 ala
val gln trp phe val gly leu ser pro thr val trp leu ser ala ile trp met met trp
                                                      val
200                                                              210
tyr trp gly pro ser leu tyr ser ile val ser pro phe ile pro leu leu pro ile phe
                                    leu             leu
220                                 226
phe cys leu trp val tyr ile.COOH
```

Fig. 3. Predicted amino acid sequence of the HBsAg 23–25 000 mol. wt. polypeptide, subtype *adw* as reported by Valenzuela et al. [86]. The corresponding sequence for the *ayw* subtype gene suggests a total of 16 amino acid changes (*in italics*). Carbohydrate side chains may be attached via asparagine (*asn*) residues at position 3, 59, and 146. There is a long hydrophobic sequence of 19 amino-acids between positions 80 and 98 inclusive

molecular weights of 30 000 and 32 000 in addition to the galactose-containing carbohydrate chain present in the 28 000 mol. wt. component. This suggests that the additional glycopeptides contain carbohydrate chains consisting only of mannose and glucosamine. A carbohydrate moiety was not found to be associated with the 23 000 mol. wt. component by either this technique or by the galactose oxidase method.

Shiraishi et al. [71] have suggested that in the absence of N-acetyl-galactosamine the linkage between sugar and protein probably occurs via N-acetylglucosamine to asparagine. There are three possible sites, therefore, of glycosylation at asparagine residues: positions 3, 59, and 146 [86] (Fig. 3). The possible role of carbohydrate in preserving the structural integrity of adjacent antigenic sites or carrying a novel haptenic specificity has yet to be clearly distinguished. Burrell et al. [6] found that combined treatment of HBsAg particles with SDS and trypsin resulted in the release of a small 5–15 000 mol. wt. glycoprotein bearing the group-specific *a* determinant. Although the serological reactivity of this peptide resisted heat at 100° C, the antigen was destroyed by exposure to reducing agents indicating the presence of disulphide bonds. Neurath et al. [49] obtained slightly different results after treatment of HBsAg with SDS and chymotrypsin. The released material from the *ad* subtype contained both *a* and *d* antigenic determinants and was found to consist of several polypeptides with mol. wts. of 8500 and 7800 respectively. However, when trypsin was substituted for chymotrypsin, serologically reactive material was not released. There was no evidence of interpeptide disulphide bonds in the serologically active SDS-chymotrypsin cleavage fragments obtained from the *ad* subtype. In this connection,

Neurath et al. [48] reported that delipidation was insufficient to release any protein components. This is a further indication of the importance of protein-protein interactions in maintaining both the structural and serological integrity of HBsAg.

The establishment of a HBsAg-producing PLC/PRF/5 cell line from a human hepatoma has provided an alternative source of HBsAg particles with the added advantage that both the production and quality of the antigen may be more precisely controlled [3, 36]. A study by Copeland et al. [11] has shown that the rate of HBsAg production in the PLC/PRF/5 cell line was minimal during logarithmic cell growth and maximal towards the end of a 7-day maintenance period. Titres of up to 1.3 $\mu g/cm^3$ of HBsAg were obtained by incubating confluent cultures of maintenance medium for seven days. The HBsAg particles produced by the PLC/PRF/5 line were found to be very similar in size, morphology, and buoyant density to the 22 nm particles found in the sera of infected individuals [75]. In the same study, Skelly et al. found affinity-purified HBsAg particles consisted of four polypeptides with mol. wts. of 47 000, 34 000, 28 000, and 23 000 respectively. The latter two polypeptides co-migrated with the major virus-specific polypeptides of serum HBsAg: the smallest 23 000 mol. wt. component was the predominant species in material prepared from hepatoma cell cultures and there was a notable absence of a component in the mol. wt. range of serum albumin which has been identified as a constituent of purified preparations of serum HBsAg [75]. Essentially, the same results have been reported by Monjardino and Crawford [44] and Marion et al. [39] using metabolically-labelled infected cell supernatants as starting material. In addition, Marion et al. found that the pattern of glycosylation in HBsAg produced by PLC/PRF/5 cells is similar to that of HBsAg from infectious serum.

Little attention has been paid so far to either the biochemical or immunochemical nature of the HBsAg material which is found in the outer coat of the hepatitis B virus particle. Immune electron microscopy suggests the presence of at least some common antigenic determinants between the complete virus particles and HBsAg 22 nm particles. Hess et al. [20] demonstrated the specific immunoprecipitation of virus particles with monospecific antisera to subdeterminant *d* or *y*. In addition, Alberti et al. [1] have reported the existence of a determinant unique to the surface of the hepatitis B virion. Confirmation of this finding will be of paramount importance for the future design of experimental hepatitis B vaccines. As yet, there is little biochemical evidence to suggest the existence of correspondingly unique polypeptides in the outer surface coat. Shih et al. [70] reported the presence of a 48 000 mol. wt. component in purified hepatitis B virus particles, but no information was presented as to the possible serological reactivity of this component.

b) The Core Component

The existence of various subpopulations of hepatitis B virus particles was revealed by buoyant density analysis of sera containing large numbers of the 42 nm spherical forms. Gerin et al. [18] found two populations of these particles by equilibrium centrifugation in caesium chloride with respective buoyant density values of 1.19–1.20 g/cm^3 and 1.25 g/cm^3. Somewhat higher values of 1.24 and 1.27 g/cm^3 were reported by Hruska and Robinson [27], but this discrepancy was almost certainly due to the different centrifugation times used by the two laboratories. In both

studies a close correlation was demonstrated between the presence of DNA, DNA polymerase activity, and particles banding at the higher density. The remaining particles by these criteria may represent incomplete or defective virus particles with little or no genome content.

Removal of the outer surface antigen coat with non-ionic detergents such as Triton X-100 or Nonidet P40 results in the release of serologically reactive core antigen. Moritsugu et al. [46] demonstrated the presence of two density populations of core particles released in this manner from circulating virus particles; the heavier particles banded at 1.35–1.36 g/cm^3 in caesium chloride corresponding to the 110 S DNA polymerase-positive core component described by Kaplan et al. [33]. A more heterogeneous population was also recovered in the lighter density range of 1.28–1.32 g/cm^3, probably containing free cores without DNA polymerase in addition to cores containing DNA polymerase complexed with core antibody. It thus seems that hepatitis B virus of higher buoyant density gives rise after detergent treatment to DNA containing core particles with DNA polymerase activity whereas the lighter band of virus particles contains predominantly low density core components with little or no DNA content. This is consistent with the enhanced degree of penetration by negative stain observed with particles of lower density.

Liver-derived hepatitis B core antigen particles were described in earlier reports as consisting of a homogeneous population of particles without DNA polymerase and with a density of 1.30 g/cm^3, but more recent studies suggest some heterogeneity in material prepared from infected hepatocytes. Fields et al. [15] found that a core preparation derived from a liver had more DNA polymerase activity than a similar preparation obtained from circulating hepatitis B virus particles assayed at the same concentration per ml. Gel chromatography revealed that the population of cores with DNA polymerase activity derived from infected liver had a slightly higher molecular weight compared with the bulk of the core antigen. Similarly, Onda et al. [55] demonstrated the presence of DNA polymerase-positive core particles in infected liver which were subsequently shown to have a buoyant density of 1.34 g/cm^3 in caesium chloride.

Isoelectric focusing is a widely used technique for separating macromolecules by surface charge. Using this method, Howard and Zuckerman [26] found that the subpopulation of core antigen with DNA polymerase had an isoelectric point of 4.4. Further studies by other laboratories demonstrated the heterogeneous nature of the core antigen. Fields et al. [16] found that partially purified core antigen derived from an infected liver focused at a pH of 3.7. Radioiodination in the presence of chloramine-T increased this value slightly to 4.0 corresponding closely with the value obtained with radioiodinated core antigen derived from circulating virus particles. Neurath et al. [48] found that core antigen prepared from serum yielded two peaks with isoelectric points of 4.40 and 4.86 respectively. Similar results were obtained with core antigen derived from sera with or without *e* antigen, despite the finding that core antigen prepared from serum containing *e* antigen produced two peaks by equilibrium centrifugation in contrast to only one peak from serum without detectable *e* antigen. This suggests that core antigen may exist in different conformations independent of whether or not DNA is present.

Although much attention has been devoted by many laboratories to the structure and nature of surface antigen polypeptides, fewer attempts have been

made to characterize in detail the protein composition of the virus core. In the past this has been due partly to the scarcity of suitable assays but also to the considerable difficulty of either separating sufficient quantity of the antigen from circulating virus particles or preventing the formation of immune complexes during the process of extracting core antigen from infected livers.

Hruska and Robinson [27] used plasma which contained particularly high numbers of virus particles. The core antigen component was released from partially purified 42 nm particles by treatment with Nonidet P40 and separated further by equilibrium centrifugation in caesium chloride gradients. The material which banded at a density of 1.38 g/cm^3 was analysed by acrylamide gel electrophoresis after disruption with sodium dodecyl sulphate and 2-mercaptoethanol. The staining of separated components with Coomassie Blue showed the presence of a major 19 000 mol. wt. component, together with two minor components of 70 000 and 80 000 mol. wt., respectively. Similar polypeptides were obtained with core antigen material recovered at the higher densities of 1.33 g/cm^3 and 1.30 g/cm^3, suggesting that the association of DNA with the core antigen does not appear to require a gross change in polypeptide composition. In the same study, core antigen prepared from the liver of an infected chimpanzee also contained the same polypeptides; in addition, a larger species of mol. wt. in excess of 200 000 was also observed. This species was only occasionally seen in core antigen preparations derived from circulating virus particles and may represent either a contaminant or a precursor structure. Periodate-Schiff staining of separated components did not reveal the presence of glycoproteins at any stage.

Fields et al. [16] similarly compared core antigen prepared either from plasma or infected liver. Analysis of polypeptides after radioiodination revealed a considerably greater number of components than reported by Hruska and Robinson [27]. Core antigen released from circulating 42 nm particles banded predominantly at an average buoyant density of 1.33 g/cm^3 and was found to contain a major 79 000 mol. wt. component together with 88 000 and 59 000 mol. wt. polypeptides as minor components. A similar polypeptide profile was obtained with a preparation of core antigen extracted from an infected human liver. In both instances, however, additional protein was present which comigrated with surface antigen preparations run in parallel as a control. Although cell preparations were serologically negative for surface antigen, contamination with polypeptides characteristically found in purified surface antigen remained a possibility. Budkowska et al. [5] also analyzed radioiodinated core antigen prepared from infected liver and in addition they examined the polypeptides present in immunoprecipitates of core antibody and the labelled antigen. Two major polypeptide species were resolved with mol. wts. of 35 000 and 17 000. Furthermore, identical polypeptide profiles were obtained regardless of whether or not the solubilized material was subsequently alkylated with iodoacetamide prior to electrophoresis. This finding suggested that the 35 000 mol. wt. component did not represent a simple dimer of the smaller 17 000 mol. wt. structure found as a result of random reoxidation of sulphydryl groups.

Neurath et al. [48] compared the distribution of polypeptides in core antigen separated by equilibrium centrifugation into two bands. Acrylamide gel electrophoresis of radiolabelled core antigen with a density of 1.28 g/cm^3 to 1.30 g/cm^3

in caesium chloride showed two major components with mol. wts. of 16 000 and 5000 respectively. Minor components with mol. wts. of 68 000, 39 000, 22 000, and 20 000 were also resolved. Of these, the 68 000 mol. wt. was much more prominent in core antigen with buoyant density 1.36 g/cm^3. The finding that some of the radiolabelled core antigen particles were removed on passage through a column of immobilized surface antibody was noteworthy. Subsequent analysis of the unbound fraction confirmed that the major 16 000 and 5000 mol. wt. polypeptides, together with the minor 68 000 mol. wt. component were specific to the core antigen.

A protein kinase activity has recently been identified in association with hepatitis B core particles obtained either from infected livers or complete virus particles [2]. A proportion of the major core polypeptides was identified as the primary phosphorylated product migrating with a slightly increased mol. wt. of 20 600 as estimated by gel electrophoresis. During the course of these studies, it was noted that this phosphorylated component underwent cleavage to give polypeptides with respective mol. wts. of 14 700 and 6000. This process was accelerated by the specific addition of anti-HBc, suggesting that immune complex formation activated a protease activity, possibly located within the core component. The presence of a hepatitis B-specific protein kinase has been confirmed (K. von der Helm, personal communication) and the presence of trace amounts of virus particles may have accounted for a similar activity being found in HBsAg preparations by Howard [23]. It remains to be established as to what role this enzyme plays in hepatitis B virus replication, although it should be noted that many enveloped viruses possess protein kinase activities for modification of virus proteins closely associated with viral nucleic acids.

Information is now emerging as to the immunochemical properties of core component proteins. Budkowska [4] studied the effect on the core antigen of a variety of physical and chemical agents using both immunoelectrophoresis and electron microscopy. The immunoreactivity of core antigen particles prepared from the liver of a patient who died from chronic hepatitis was affected by a variety of proteolytic enzymes. Somewhat unexpectedly, both the immunoreactivity and morphology of the core particles were also affected by solvents such as 50% methanol, ethanol, and butanol. In addition, the reactivity of core antigen with specific antibody was also found to be dependent on the presence of intact sulphydryl groups and possibly internal hydrophobic bonding. The morphology and immunoreactivity of the core antigen was also modified by exposure to pH below 3.0 or by heating at 100° C.

Recent evidence concerning the nature of hepatitis B *e* antigen has led to the suggestion that this antigenic specificity may be closely associated with the core antigen. Although the two antigens do not give lines of identity by immunodiffusion, several recent studies have clearly shown that solubilization of either core components from infected liver or intact hepatitis B virus particles results in the release of material which reacts with *e* antibody. Yoshizawa et al. [90] found that treatment of hepatitis B core particles with 0.1% SDS and 0.1% 2-mercaptoethanol destroyed reactivity for anti-HBc but resulted in the appearance of HBeAg. Similarly, Takahashi et al. [83] found that similar treatment of cores purified from circulating hepatitis B virus resulted in loss of HBcAg activity but a dramatic increase in HBeAg titre.

The core component polypeptides with mol. wts. of 45 000 and 19 000 respectively were found to react with anti-HBe. In further studies, the same laboratory has shown that solubilized virus particles elicit an anti-HBe response in immunized rabbits [84]. Also in this later study, it was shown that core components of varying density could be released by protease treatment in the presence of nonidet P40. The action of the proteolytic enzyme resulted in the loss of the 45 000 and 19 000 mol. wt. components and the appearance of two smaller polypeptides of mol. wt. 31 000 and 15 000 respectively. The 31 000 mol. wt. component probably represented a dimer of the smaller protein and both were reactive for *e* antigen.

HBcAg activity has been detected in *E. coli* cells after transfection with plasmids or phage vectors containing the whole, or fragments of, HBV-DNA [7, 57]. Although detailed amino acid analyses for purified HBcAg polypeptides are not available, Pasek et al. suggested that HBcAg activity resides in a 183 amino acid long polypeptide. Arginine-rich regions are predicted towards the carboxyl-terminus of the 21 000 mol. wt. polypeptide, indicating that the carboxyl terminal region binds with the DNA genome within the virus particle while the remaining regions fulfil a structural role. In this context, it is interesting to note that Ohori et al. [54] found that *e* antigen released from disrupted virions was maximum for fractions containing DNA polymerase and the DNA genome; the authors therefore suggested that polypeptides bearing this antigen may function during virus assembly as a maturation protein. Clarification as to the exact nature of the *e* antigen, HBcAg, and their corresponding relationship to core component polypeptides will require more thorough investigation of the properties of these proteins.

3. The Hepatitis B Virus Genome

The discovery of an endogenously-primed DNA polymerase activity within hepatitis B virions provided the initial step in characterizing the genome and has ultimately led to a description of its complete nucleotide sequence.

Kaplan et al. [33] demonstrated that incorporation of deoxynucleotides into an acid insoluble product follows the removal of the outer surface antigen coat by treatment with a nonionic detergent. The reaction proceeds in the absence of an exogenous template and it is stimulated by the presence of magnesium and ammonium ions. The monovalent ions sodium and potassium also stimulate the reaction, and the activity of the enzyme at high salt concentrations has been used as the basis for differentiating core antigen-associated DNA polymerase from other similar enzyme reactivities [21].

Although it is by no means clear that hepatitis B DNA polymerase is specified by the viral genome, a comparison of the ionic requirements of the enzyme and its sensitivity to N-ethylmaleimide suggest that this polymerase is unique to hepatitis B infection [92, Chapter 11].

Despite the somewhat specialised nature of the assay, the detection of DNA polymerase is a useful marker of virus replication. The precise conditions required for the assay of the enzyme for diagnosis of hepatitis B have been reviewed by Howard [24]. Cappel et al. [8, 9] demonstrated that the presence of DNA polymerase activity is often the only marker of hepatitis B virus. Hepatitis B surface

antigen was detected in 88% of 178 individuals exposed to hepatitis B and another 11% developed core antigen-associated polymerase in the absence of the surface antigen. Hepatitis B was later confirmed in almost all of these patients after further serological examination. It has also been noted that there is frequently a close association between DNA polymerase activity and the titre of the surface antigen.

The finding of an apparently virus-specific nucleic acid polymerase with unique properties stimulated laboratory and clinical investigation of a number of antiviral compounds which might be of value in the treatment of chronic hepatitis B infection. These include leucocyte and fibroblast interferon, adenine arabinoside, and others. Another advantage of complementing the current serological methods is that the assay of DNA polymerase activity may discriminate between complete and defective virus particles. It has been suggested that the production of defective particles may be necessary for the establishment and maintenance of virus persistence [18]. The frequent fluctuation in the enzyme levels often observed in serial samples from persistently infected patients and the finding of apparently "empty" virus particles is consistent with this hypothesis. It is interesting that consistently high levels of DNA polymerase activity have often been noted in the serum of surface antigen-positive patients treated by maintenance haemodialysis. Possible variations occurring during the natural course of infection should therefore be considered during the monitoring of patients involved in clinical trials of antiviral agents.

The product of the endogeneously primed DNA polymerase reaction has been characterized as DNA with an approximate sedimentation coefficient of 15 S. The observation that the reaction was not stimulated by the addition of a number of synthetic or natural DNA structures indicates that the reaction template was sequestered within the core of the 42 nm virus particle [33]. Further studies have since confirmed that at least a proportion of the separated core components contains DNA. Robinson et al. [65] visualized circular molecules with a mean length of 0.78 μm after extraction of core particles with sodium dodecyl sulphate and mercaptoethanol. All the circular forms seen by electron microscopy were abolished by prior incubation with DNase, although pre-treatment of isolated core particles with the same nuclease did not affect the recovery of intact DNA. This latter observation provided further evidence for the sequestered nature of the DNA genome.

The size of the circular forms recovered by Robinson et al. [65] was compatible with an estimated mol. wt. of $1.6–2.0 \times 10^6$, which is considerably smaller than the genome of any other known animal DNA virus. It also appears to be smaller than the total amount of nucleic acid required to code directly for the many virus-specific antigenic determinants found in the sera of acutely and persistently infected individuals. It has been estimated that full expression of this structure would permit the synthesis of unique polypeptides with an approximate total mol. wt. of 125 000 [64].

The apparently unique structure of hepatitis B virus DNA and the associated polymerase prompted Summers et al. [80] to apply restriction enzyme cleavage techniques to synthetic DNA prepared by the reaction of *E. coli* DNA polymerase I with denatured core antigen DNA as the template. The high specific activity product was resolved by polyacrylamide gel electrophoresis and found to consist

largely of a homogeneous 3600 nucleotide component, together with small quantities of a more rapidly migrating heterogeneous component containing a total of 3132 nucleotides. The cleavage of the major component with the restriction enzyme endo. *R. Hae* III generated 13 further fragments. The smaller heterogeneous band was found to contain between four and seven of these structures. Confirmation that the synthetic DNA product arose by the duplication in equimolar amounts of each denatured strand present in the core particle was obtained since unequal lengths of DNA were formed. Extracted DNA was incubated without prior denaturation with the polymerase of avian myeloblastosis virus.

This enzyme is a nucleotydyl transferase without exonuclease activity. The incorporation of new radiolabel further confirmed the existence of substantial single-stranded regions in the native circular DNA molecule. On denaturation the native DNA molecule therefore produced two main components which behaved as linear and circular single-stranded DNA respectively. Cleavage analysis showed that the avian myeloblastosis virus polymerase reaction progressively added the remaining six fragments present on the complimentary circular DNA strand by the addition of nucleotides at one or more exposed 3′-OH groups. The DNA polymerase activity associated with the intact 42 nm virus particle may therefore function as a repair enzyme by closing single-stranded regions in the otherwise double-stranded DNA structure.

By direct examination, Hruska et al. [28] estimated the frequency and size of different DNA molecular forms before and after the DNA polymerase reaction. A 27% increase in the mean length of the circles spread in an aqueous solution was observed after the enzyme reaction, which is consistent with the synthesis of complimentary strands in single-stranded regions. A similar increase in the mean length of the visualized molecules was obtained by spreading unreacted DNA in formamide. The significance of circular forms with short tails in less than 10% of the observed structures was not clear, although Overby et al. [56] suggested that these forms may represent structures undergoing replication by the rolling circle model for DNA replication.

Undoubtedly the biggest advance in our understanding of the hepatitis B virus genome has resulted from the application of gene cloning technology. At least four groups have recently reported the successful introduction of hepatitis B DNA fragments into *E. coli* using plasmid or phage vectors [7, 77, 86, 94]. The restriction enzyme maps obtained by these workers is summarized in Fig. 4. Although there are

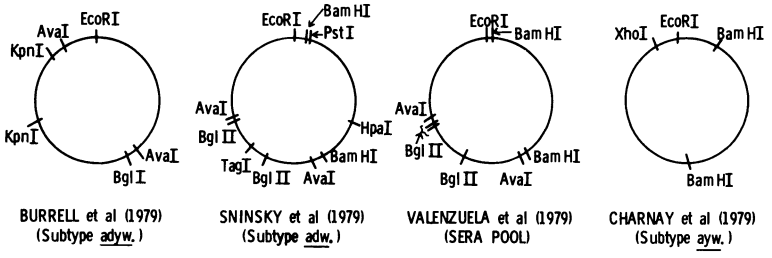

Fig. 4. Diagrammatic representation of restriction enzyme cleavage sites within the circular hepatitis B virus genome

some similarities and differences between DNA cloned from sources containing different HBsAg subtypes, differences between preparations expressing HBsAg of the same subtype have also been reported [39]. Important findings of these studies include (a) confirmation that the circular nature of the genome is due to base pairing of the 5′ ends of each strand, (b) the location of the single-stranded region in one strand and a nick in the other larger DNA strand, (c) the location of the gene coding for HBsAg determinants on the fully replicated strand, extending into the single-strand region, (Fig. 5), and (d) *E. coli* transfected with plasmids containing HBV DNA sequences synthesize HBcAg. Although this approach provides a potentially excellent source of HBsAg for the development of a hepatitis B vaccine, attempts to demonstrate the synthesis of this HBV gene product in transfected *E. coli* have proved unsuccessful to date.

Despite the experimental difficulties involved, several attempts have been made to determine whether the DNA of hepatitis B virus contains either unique, virus-specific sequences or heterogeneous DNA of host origin which is incorporated at random during some stage of virus replication. Hung et al. [30] demonstrated that specific hybridization could be achieved between extracted DNA and the exogenous linear DNA found free in the plasma of persistently infected carriers. No reaction was obtained with similar preparations of linear DNA made either from the plasma of healthy individuals or with DNA extracted from uninfected human liver. Lutwick and Robinson [35] extended these observations by demonstrating the specific hybridization of viral DNA to cellular DNA extracted from the liver of patients infected with hepatitis B virus. The DNA probe reannealed with a Cot $\frac{1}{2}$ value compatible with previous observations that 25 to 50% of the circular molecule is used as a template during the DNA polymerase reaction thereby providing further evidence on the uniformity of the template molecules. Also addition of unlabelled DNA from six patients with hepatitis B infection decreased the rate of reassociation by a factor of 2 or more when compared with the control reactions; thus DNA from all the infected livers appeared to contain virus specific base sequences. It was estimated that at least 12 copies of the core-associated DNA polymerase product was present per cell DNA equivalent. This may be a conservative estimate since not every cell within the infected tissue may have been infected.

The high frequency of hepatitis B surface antigen and other markers of hepatitis B virus infection in patients with primary hepatocellular carcinoma suggests that the viral genome may play some role in the oncogenic transformation of hepatocytes. Summers et al. [81] investigated this further by examining DNA

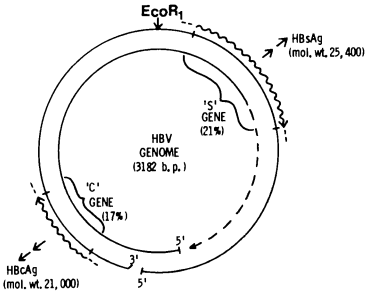

Fig. 5. Location along the hepatitis B virus genome of the "S" and "C" genes coding for HBsAg and HBcAg proteins respectively. The *E. coR1* restriction enzyme cleavage site is shown for orientation and the DNA synthesized by the endogenous DNA polymerase shown by a dashed line. Hepatitis B gene products may be direct translation products of virus mRNA that arise by cleavage of larger precursor molecules from transcription of larger sequences encompassing the relevant genes

extracted from tumour, cirrhotic, and metastatic tissues from four patients with primary hepatocellular carcinoma. Two of the tumours were found to contain approximately one to two genome equivalents per cell. Tumour tissue from a third patient with hepatitis B surface antigen was found to contain considerably fewer specific DNA sequences, and material from the fourth patient with hepatitis B surface antibody did not contain detectable hepatitis B viral DNA. It is possible that the positive findings may have represented the reaction of the ^{32}P-DNA probe with DNA present in the form of core or complete virus particles rather than with host cell DNA, thereby suggesting that integration of the genome of hepatitis B virus into every cell is not required for the maintenance of the neoplastic transformation of hepatocytes.

Marion et al. [39] recently demonstrated the presence of hepatitis B viral DNA in the PLC/PRF/5 human hepatoma cell line. DNA extracted from these cells showed a marked enhancement in the reassociation of the ^{32}P-HBV DNA probe prepared by nick translation of virion DNA. It was further established that up to four copies of sequences representative of the entire HBV genome were present in high molecular weight DNA extracts. Furthermore, virus-specific RNA transcripts from all parts of the virus genome were present; suggesting full genome expression in these transformed cells.

The above studies suggest that the mode of replication of this virus may be unique relative to the more familiar strategies of viral genomes. A consideration of the findings by Sanger et al. [66] that the genome of ϕX174 virus may code for different polypeptide species by selective initiation at different points along the virus genome may help to overcome the objection that the coding capacity of the virus genome does not allow for the coding of the multiple antigenic determinants expressed during infection. An inspection of the nucleotide sequence data published so far indicates that at least eight open reading frames may exist on the viral genome with frequent overlapping of possible transcription sequences [17]. In addition, posttranscriptional splicing of viral RNA may occur prior to synthesis of virus-specific polypeptides. Although the hepatitis B virus may therefore possess the smallest genome amongst human pathogens, much remains to be learnt as to the strategy of the viral nucleic acid within the infected cell.

4. Possible Strategy of Hepatitis B Virus Replication

Hepatitis B surface antigen is abundantly present in the sera of individuals during acute hepatitis B infection. Its appearance coincides with the onset of liver damage and it has been suggested that hepatitis results from the host's immunological response to viral antigens on the plasma membrane of infected hepatocytes [51]. Evidence of maximum active virus replication precedes this phase of the disease and coincides with high levels of circulating core and e antigens. Antibody to the core antigen may develop during the acute illness soon after the appearance of HBsAg, and is frequently detected for a limited period after the onset of recovery. Core antibody is found at high titre in chronically infected individuals concomitantly with either e antigen or e antibody. The continuing presence of HBeAg in chronic active hepatitis is frequently regarded as an important prognostic marker of disease

severity [89]. In contrast, a serological response to HBsAg in acute infection remains undetected until the clearance of the antigen from the circulation during convalescence. In fulminant hepatitis, there is some evidence to suggest that an unusually strong and rapid immune clearance of HBsAg associated with the early appearance of surface antibody during the peak of liver damage may be involved in the pathogenesis of this severe form of infection.

Both quantitative and qualitative differences in response to hepatitis B antigen may account for the observed variation as to the outcome of infection. Several studies have indicated the presence of immune complexes in patients with acute and chronic hepatitis B [53, 72], and in a recent study it was shown that the highest frequency (90%) of HBsAg immune complexes occurred in patients with acute hepatitis [37]. In the same study, a much lower frequency of 20% was observed in both patients with chronic persistent hepatitis and asymptomatic carriers of the virus. An intermediate value of 40% was found for those patients with histologically-proven chronic aggressive hepatitis. The level and properties of the anti-HBs response in these categories of patients may therefore define the extent of virus replication and eventual outcome of the infection. Eddleston and Williams [13] proposed a hypothesis whereby individuals with chronic active, HBsAg positive hepatitis produce antibody to HBsAg which is either low in titre and/or of low affinity. This inadequate response would limit the spread of virus into adjacent, non-infected areas but fail to adequately prevent re-infection. Such conditions are known to stimulate the appearance of defective interfering particles in other virus systems and ultimately result in the establishment of virus persistence [29]. In order to confirm this hypothesis, it requires to be shown that a proportion of the particles with the morphology characteristic of hepatitis B virus contain defective genomes which require the simultaneous presence of complete standard virus for replication and that this proportion increases in the virus yields from cells co-infected with the standard virus. This situation is much more likely to arise if the rate of diffusion of virus away from the infectious centre is restricted by the presence of a limiting antibody response, thereby increasing the number of virus particles which adsorb to adjacent cells. By inhibiting the production of standard virus, interfering virus particle production is eventually restricted, leading to the cyclical production of standard virus with time. Sinarachatanant and Huang [73] have shown that such a modulation of vesicular stomatitis virus production occurs in the presence of low antibody concentrations and precedes the eventual establishment of persistently infected cells in vitro. Although the study of virus persistence in hepatitis B clearly requires an in vitro culture system, it is interesting to note that cyclical production of hepatitis B virus in the serum does occur in chronically infected chimpanzees [24].

The role of interferon in the development of chronic hepatitis B infection remains unclear. It should be noted, however, that defective interfering particles, if present, may induce interferon. Passive administration of human interferon both in human and chimpanzees has an inhibitory effect on replication of hepatitis B virus. Although serum markers of hepatitis B virus replication fall markedly after the onset of treatment, the effects are transient; prolonged treatment has been shown to lead to clinical improvement in a proportion of chronically infected patients [40] and chimpanzees [93]. Scullard et al. [67] using leucocyte interferon found a similar response but a marked decrease in T lymphocyte-mediated cytotoxicity towards

HBsAg-coated target cells indicating that the antiviral effect of interferon was offset by its suppressent effect on the cell-mediated immune response to infected hepatocytes. Leucocyte interferon has been shown to be considerably more immunosuppressive than fibroblast interferon [79] and augments natural killer cell activity [85, 91]. At present, there is a noticeable lack of information regarding the relative level of circulating interferon in acute and chronically infected patients; the interferon natural killer cell system may be of paramount importance in recovery from acute disease with persistent virus infection arising as a result of a deficiency in either interferon production or natural killer cell activity [42].

Perhaps the most remarkable epidemiological feature of hepatitis B infection is the incubation period which may extend to six months before the development of disease. Clinical hepatitis represents the host's response to infected hepatocytes at a time when virus replication is already in decline; the question therefore arises as to alternative sites of replication prior to hepatitis. One possible site is the reticuloendothelial system, particularly the differentiated monocytes which line the liver sinusoids. These Kupffer cells form a barrier across which it is reasonable to assume either the virus is passively transferred as in rift valley fever, or may indeed replicate, as is believed to occur in yellow fever and pox virus infections [41]. For example, the differential ability of two strains of ectromelia virus to induce hepatitis in mice was directly related to the extent of replication in Kupffer cells [63]. Proliferation and enlargement of Kupffer cells are a characteristic feature of hepatitis [32] and HBsAg antibody complexes have been found in these cells [52]. Passive uptake of HBsAg-reactive material has also been reported for mouse macrophages [58]. Taken together, these findings indicate that the macrophage may play an important role in the immunopathogenesis of hepatitis B.

From the aforementioned studies, a picture is now emerging as to the structure of the hepatitis B virus, the total number of genes required for the synthesis of virus-specified proteins, and some indication as to the precise mechanism of virus maturation within the nucleus of the infected hepatocyte. A close relationship between the appearance of e antigen and HBcAg, both in the serum and the infected nucleus, indicate that the expression of these antigens may represent different stages in the assembly of the virus. This can be expressed simply in Fig. 6 where a 15–19 000 mol. wt. polypeptide dimerizes to form the basic "building block" of the inner core component which is *e* antigen reactive. In contrast, the assembled icosahedral core component is reactive only for HBcAg. It seems unlikely that a single protein of this size is alone responsible for the ultimate structure of the core. Other proteins have been described, in particular a 45 000 mol. wt. protein which is reactive for HBeAg [83]. In addition, a 68 000 mol. wt. protein is a minor component of intact cores [27, 49]. It is interesting that this closely resembles in size the newly-described hepatitis B 'δ' antigen. This new antigen is found in the hepatocyte nucleus, where by immunofluorescence it gives rise to a staining pattern similar, but unrelated, to HBcAg. High titres of antibody to δ antigen occur simultaneously in the circulation of those chronically infected patients that exhibit hepatic antigen [60]. Extraction of infected liver with 6M guanidine-HCl was successful in providing a partially purified antigen preparation which was characterized as having a mol. wt. of 68 000 [61]. Prolonged exposure to chaotropic salts or low pH led to a rapid loss of antigenicity, however, it is possible that δ antigen may therefore represent an

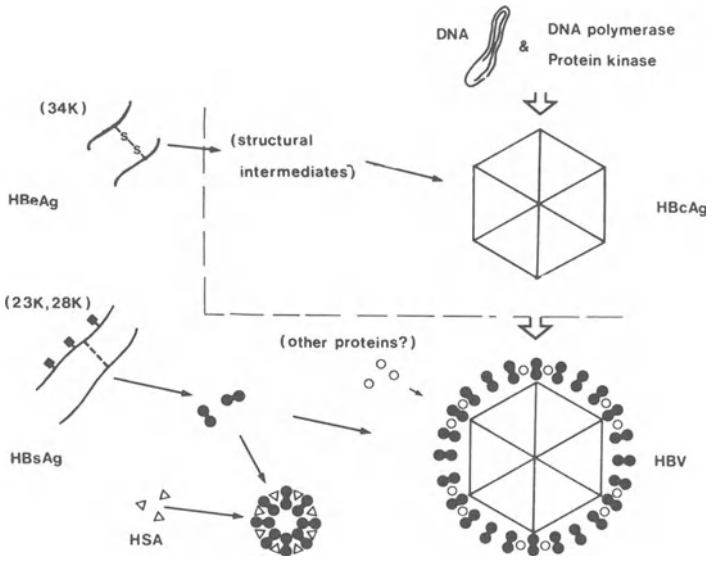

Fig. 6. Assembly of hepatitis B virus and antigenically-reactive particles. It is proposed that assembly of the virus core proceeds via the formation of as yet undescribed intermediate structures that may express HBeAg, HBcAg, and/or new antigenic specificities. The *dashed line* represents an arbitrary division of events between the nucleus (core assembly) and cytoplasm (virus maturation) within the infected cell

intermediate component of hepatitis B virus assembly. Other alternatives yet to be excluded include suggestions that δ antigen is a component of a hepatitis B variant or represents an agent which is defective and interferes with hepatitis B virus replication [62].

It is possible that other intermediate components of core assembly exist which express both HBeAg and HBcAg determinants, with the latter being essentially a conformationally-dependent antigen. Although structures morphologically resembling cores are never seen free in HBsAg-positive sera, proteins bearing these determinants may exist as immune complexes. Indeed the particularly high titres of anti-HBc in persistently-infected individuals may be explained by a continual release of excess core components from infected cells. The heterogeneity of the HBeAg system as found by the immunodiffusion analysis of positive sera may also be explained in terms of free and bound antigen [82].

The apparent instability of the virus core in experimental studies together with the absence of free 27 nm particles in the circulation of infected individuals strongly suggests that the outer surface coat is required for the stability of the hepatitis B virus particle. This HBsAg-reactive coat is acquired in the cytoplasm or perinuclear area of the infected hepatocyte, possibly via the assembly of filamentous forms of HBsAg which are so prominent in infected cells [50]. The suggestion that additional proteins unique to both complete virus particles and HBsAg filaments awaits clarification [1, 45]. Excess HBsAg polypeptides made during virus maturation then combines with other material, notably serum albumin [75] to result in the familar 22 nm HBsAg particle. It is worth noting that autoantibodies to albumin are

a frequent feature of hepatitis B infection. For example, Lenkei et al. [34] have shown that these autoantibodies occur in as many as 60% of all chronic active hepatitis cases as opposed to an average of 20% in cases of chronic persistent hepatitis. It is possible, therefore, that the effectiveness of the anti-HBs response discussed above in terms of affinity and titre may be mediated by the relative binding of serum albumin in close association with virus-specific HBsAg polypeptides.

Interestingly, serum albumin is absent from the HBsAg 22 nm particle produced by the PLC/PRF/5 hepatoma cell line. These cells, containing complete multiple copies of the hepatitis B virus genome, provide an ideal substrate for the study of HBsAg production. Although it is unclear if the major HBsAg polypeptide is a direct translation product of a single mRNA or arises by means of post-translational cleavage, two recent studies indicate either the latter possibility or that the mRNA arises from the processing of a larger transcript. Edman et al. [14] found a large, polyadenylated RNA species composed of about 2000 nucleotides (18 S) which specifically combined to a fragment of virus DNA containing the "S" gene and bases on either side. Chakraborty et al. [10] using similar techniques found two potential mRNA species in the cytoplasm with sizes of 3050 nucleotides (21.5 S) and 2500 nucleotides (19.5 S) respectively. If these RNA structures were translated, virus-specific proteins in the mol. wt. range of 50–75 000 would represent the primary translation products with HBsAg reactivity.

The repeated failure to isolate hepatitis B virus in generally available cell lines may reflect the incapacity of certain cells to support virus replication at any stage from genome penetration to virus maturation and release. A particularly exciting finding, however, was the successful production of HBsAg in mouse cells by introducing a plasmid containing two tandemly arranged virus genomes [12]. By using the technique of cotransformation with the cloned herpes simplex virus thymidine kinase gene, several copies of the plasmid were integrated into high mol. wt. cellular DNA. Detectable amounts of HBsAg were found in the cell culture medium 20 days following transformation and electron microscopy showed the presence of typical 22 nm HBsAg particles. This study demonstrates that at least the mouse L cell line supports transcription and translation of hepatitis B-specific gene products. A similar approach has also been reported by Hirschman et al. [22] who found expression of both HBsAg and HBcAg in cells of non-hepatic origin receiving cloned hepatitis B virus DNA of the *ayw* genotype.

5. Conclusion

Hepatitis B research until recently was confined to a further understanding of virus immunopathology and sequelae. The application of molecular biology techniques to the hepatitis B virus genome has added a new and important dimension to the investigation of the processes accompanying replication of the virus, as has been outlined in this review. It is anticipated that the availability of monoclonal antibodies to hepatitis B antigens will complement this newly acquired information allowing a clearer picture of hepatitis B virus replication to emerge whereby accurate predictions from the genome sequence will be combined with the identification of virus specific products using sera of single specificity. It has often

been stressed that the virology of hepatitis B is unique; this concept has now been swept aside to reveal the hepatitis B virus as one of a family of variously related viruses with similar morphology, proteins, and genome structure. These newly described agents are pathogens of widely differing animal species including American woodchucks [88], prairie dogs [78], ducks [95] and squirrels [96]. There is also a preliminary report that one agent of non-A, non-B hepatitis of man is related to hepatitis B virus [19] but this remains to be confirmed. An expansion of research into the pathogenesis and properties of these similar viruses will inevitably further our understanding of human hepatitis B virus infection.

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The Immunopathology of Acute Type B Hepatitis

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Introduction

Although the identification of the hepatitis B virus (HBV) and the availability of immunologic markers of infection have greatly enhanced our knowledge about hepatitis B, the natural history of the different forms of the infection, acute and chronic, is far from understood.

The aim of this chapter is to summarize currently available findings of morphology, HBV antigen expression, and humoral as well as cellular immune reactions operative in acute hepatitis B, and to speculate on mechanisms responsible for the pathogenesis of liver cell damage.

Morphologic Events in Acute Hepatitis

Acute Hepatitis B in Chimpanzees

The knowledge of the sequence of events in hepatic morphology of acute hepatitis is derived mostly from chimpanzees experimentally infected with the HBV. Recent publications describe in detail the liver pathology in serial biopsies of chimpanzees [29, 89, 90]. The first histologic lesion to occur simultaneously with a rise in transaminases 12–16 weeks after inoculation consists of an activation of sinusoidal lining cells and small foci of hepatocellular necrosis scattered throughout the liver lobule. Portal tracts are densely infiltrated with mainly lymphocytes. This is followed by anisocytosis of hepatocytes with variable staining quality of their cytoplasm and accumulation of lymphocytes in close contact with these altered hepatocytes (peripolesis). In later stages the mesenchymal changes dominate the hepatocellular lesions. Significant biochemical and histologic abnormalities persist for up to 7–15 weeks [89].

Acute Hepatitis in Humans

The basic morphologic features in human acute hepatitis B are the same, although considerable variation in severity and duration of the disease may be recognized.

The course and prognosis of acute HBV infections correlate with type and extension of liver cell necrosis; three histologic forms of acute hepatitis B are thus distinguished [11, 14].

1) Acute Hepatitis with Spotty Necrosis (Classical Lobular Hepatitis). As in chimpanzees, this is the most common reaction to HBV infection in humans. In the *early stage* scattered focal liver cell necrosis is accompanied by accumulation of lymphocytes and histiocytes in portal tracts and in a cord-like fashion along sinusoids. The *fully developed stage* is characterized by a predominance of lymphocytes over macrophages. Interestingly, plasma cells are rare in all stages of acute spotty necrotic hepatitis B. Spotty liver cell necrosis is conspicuous; acidophilic type of necrosis is scattered throughout the lobules whereas the lytic type of necrosis prefers a centrilobular location (Fig. 1 a). A close contact of lymphocytes/macrophages with the membrane of individual hepatocytes (peripolesis) has been interpreted as an immunologic interaction of lymphocytes with altered hepatocytes. Mitoses indicate regeneration.

Although liver cell necrosis appears to be spotty at a given moment, the whole population of liver cells will be renewed with recovery from acute hepatitis. This is demonstrated by cases of Dubin-Johnson's syndrome in which the characteristic pigments disappeared completely from liver cells during the course and in the immediately posthepatitic phase of acute hepatitis while the excretory function of the hepatocytes remained defective [116, 120].

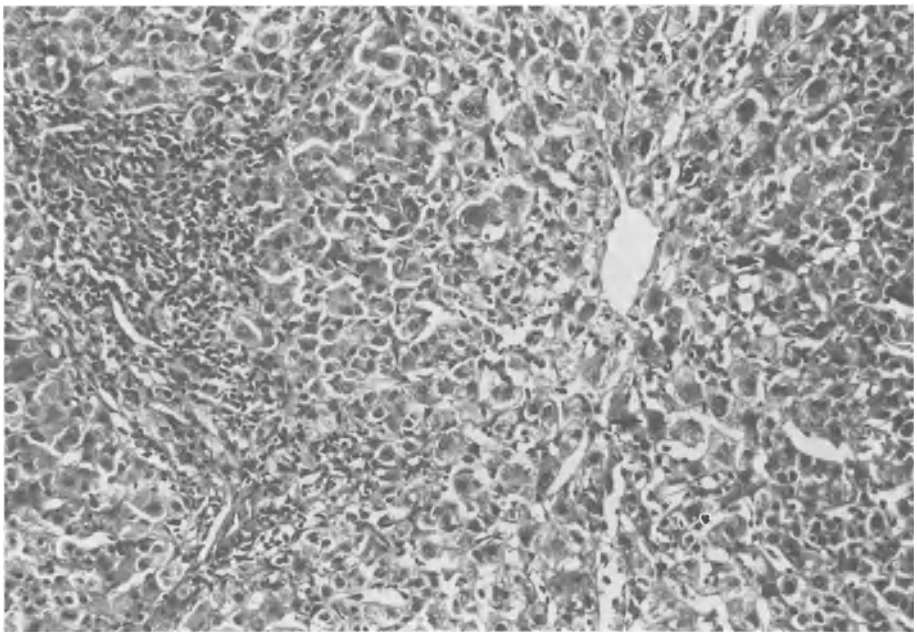
In the *late and residual phases* liver cell degeneration gradually subsides while mesenchymal reaction persists. Macrophages predominate more and more over lymphocytes. Complete restoration of the parenchyma is the rule.

2) Acute Hepatitis with Confluent (Bridging) Necrosis. In more severe forms of hepatitis, in addition to the changes of acute spotty necrotic hepatitis substantial groups of adjacent liver cells undergo necrosis of the lytic type (confluent necrosis; Fig. 1 b) and these necroses may be so extensive as to build necrotic bridges between two central veins or central vein and portal tract (bridging hepatic necrosis; [11, 14]). Necrosis may even affect several complete adjacent acini in a substantial part of the liver (massive necrosis; fulminant hepatitis). Neutrophilic infiltration may be a striking feature. Confluent necrosis may readily heal or may (especially if accompanied by piecemeal necrosis) progress to chronic liver disease [11, 14].

3) Acute Hepatitis with Piecemeal Necrosis. Piecemeal necrosis, the hallmark of chronic aggressive hepatitis, may be added to spotty (Fig. 1 c) or confluent necrosis, particularly in intravenous drug users [11, 14]. In contrast to spotty necrotic hepatitis, plasma cell infiltration may be conspicuous. The course of this variant is prolonged and a substantial proportion of cases progress to chronicity [14].

Characterization of Hepatitis B Virus Antigens

A large body of evidence indicates that the 42 nm Dane particle [25] is the complete virion of hepatitis B. This complex spherical structure is composed of at least two



a

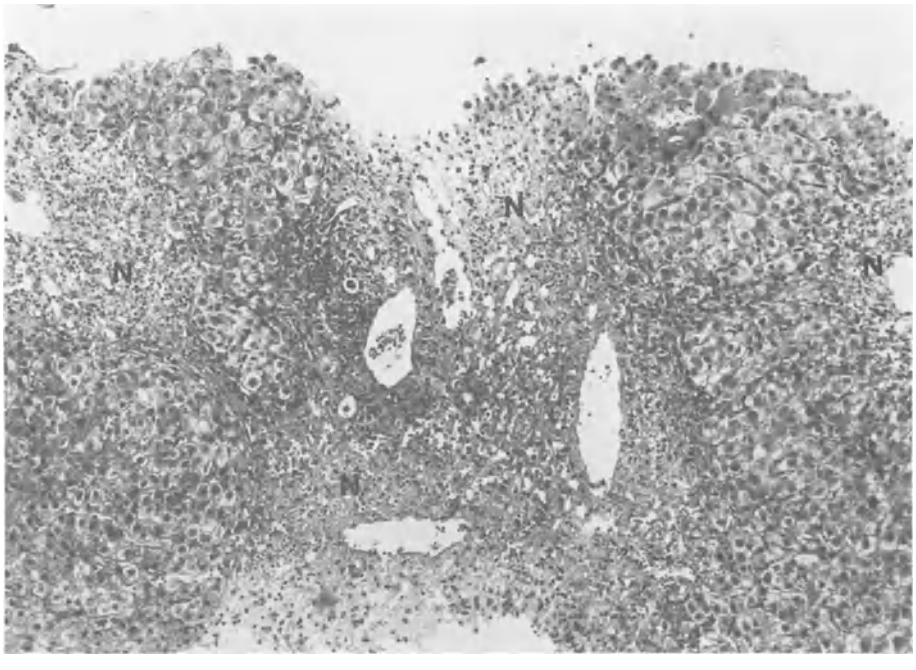


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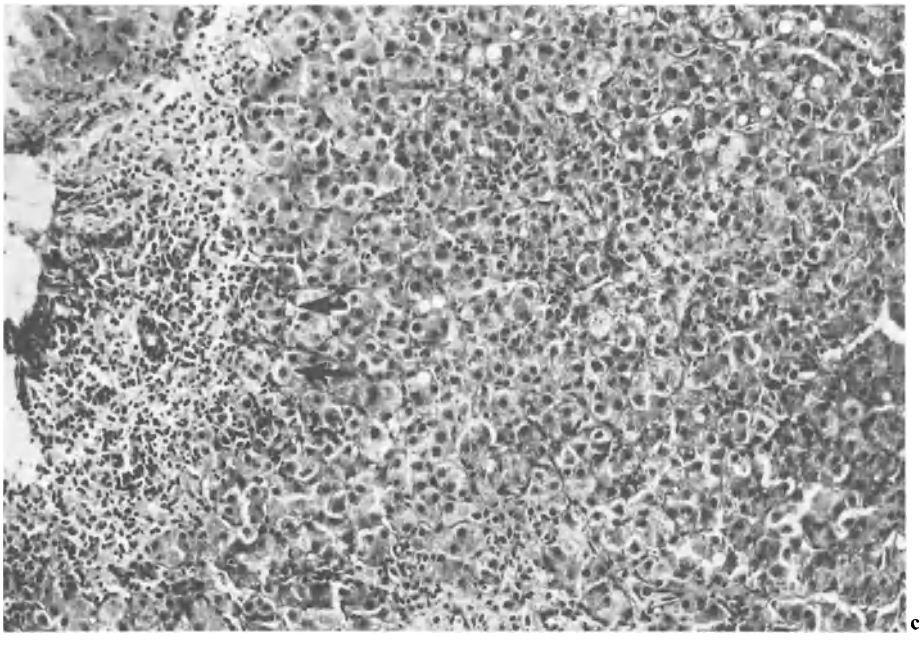
Fig. 1 a–c. Types of necrosis in acute viral hepatitis B. **a** Acute hepatitis with spotty necrosis (classical lobular hepatitis): Eosinophilic degeneration and necrosis (→). Necrosis of the lytic type in centrilobular location. **b** Acute hepatitis with confluent (bridging) necrosis. Area of necrosis (*N*). **c** Acute hepatitis with piecemeal necrosis (→)



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serologically and morphologically distinct antigens [5]: A central core (hepatitis B core antigen: HBcAg) is coated by an envelope containing the hepatitis B surface antigen (HBsAg) including several subdeterminants [69, 122]. The complete Dane particle may be visualized in blood by immune electron microscopy.

HBsAg

HBsAg not only appears as the envelope of core particles in the Dane particle but is produced in excess and circulates as 22 nm spherical particles and tubular structures of the same diameter and variable length. The HBsAg particles are quite heterogeneous in size, molecular weight (2.4–4.6 million daltons) [30, 65], as well as polypeptide and carbohydrate composition [30, 55]. The major immunogenic polypeptide appears to be a 23–26 000 dalton protein [56, 104, for detailed biochemical and biophysical characterization see 42, 51].

In *liver tissue*, HBsAg is exclusively localized in the cytoplasm [47–49, 59] and appears as filamentous material within a proliferated and often distorted smooth endoplasmic reticulum (SER) [107]. By light microscopy, HBsAg-containing cells may readily be recognized as ground-glass hepatocytes [48]. They stain dark brown with orcein [105]. HBsAg may also be detected by immunofluorescence at the cell membrane in certain forms of hepatitis [4, 45, 93]. This membrane-associated HBsAg is linked to nuclear and/or cytoplasmic (submembraneous) core expression in 70–100% of cases [45].

HBcAg

By detergent treatment of Dane particles very regular 28 nm core particles can be prepared, containing the HBcAg on their surface and carrying viral DNA [101] and

DNA polymerase [64, 102]. The major structural component of the core particle is a 19 000 dalton polypeptide and minor larger polypeptides have also been detected [57]. Most of the core particles recovered from liver tissue, however, have a lower buoyant density than cores from Dane particles and do not contain DNA and DNA polymerase activity [100].

In *liver tissue*, HBcAg is preferentially but not exclusively localized in nuclei [9, 47, 92] and appears as non-coated spherical 24–27 nm structures [47, 58, 85]. Excess formation of core particles can be recognized in rare instances even in conventional hematoxylin and eosin stained slides by finely granular eosinophilic inclusions [60], the so-called sanded nuclei [12]. For the formation of complete Dane particles, HBcAg seems to be released from the nucleus through nuclear pores into the extracisternal perinuclear cytoplasm. In some instances of chronic hepatitis B, wavelike sequences occur in nuclear HBcAg formation and cell membrane-directed flow of core particles [44] leading to different distribution patterns, including a submembraneous HBcAg accumulation. The dynamics and the definitive site of Dane particle formation, however, are not known.

HBeAg

The structural nature and the localization of HBeAg are not yet established. The HBeAg/anti-HBe system has been identified as heterogenous HB-associated antigen/antibody system unrelated to HBsAg precipitin lines [72], although anti-HBe-positive sera have been claimed to be capable of agglutinating Dane particles and tubules [83]. This finding, however, could not be confirmed by others [110].

Speculations have considered HBeAg to be a virus-specific antigen [68, 115], the DNA polymerase itself [50], a host-specific immunoglobulin being anti-idiotypic [82] or an extraband of LDH_5 isoenzyme [117]. Electrophoretic studies have defined two species of HBeAg: (a) a fraction smaller than IgG, moving in the alpha-globulin region [109], termed "e_3" by Trepo et al. [114], and (b) a fraction larger than IgG, moving with beta and gamma globulins ("e_1" and "e_2": [114]) which is bound to IgG [109]. Correlation to Dane particles and DNA polymerase seems to be best with "e_3".

In *liver tissue*, HBeAg is claimed to localize in the cytoplasm by some [115, 123] and in nuclei by others [8, 124]. Our own preliminary studies were unable to confirm cytoplasmic localization but provided evidence for nuclear localization, although interference by the nuclear HBcAg system is difficult to exclude.

A recent interesting observation [108, 124] is that the 19 000 dalton polypeptide released from the core particles by dodecylsulfate gel electrophoresis reacts with anti-HBe and not with anti-HBc, whereas the intact core particles exclusively react with anti-HBc. This observation, if confirmed, might establish the nature of HBeAg as part of the HBV core [100].

Dane Particle-Related Antigen/Antibody System

Reports from different laboratories have suggested the existence of antigenic sites distinct from HBsAg on the surface of Dane particles and probably also on tubular HBsAg [81, 83] which might be involved in the formation of immune complexes predominantly consisting of Dane particles and tubular HBsAg [45, 46]. By a radioimmunoprecipitation assay using ^3H-labeled Dane particles, Alberti [1, 2] supported this assumption by presenting evidence for the anti-Dane particle

antibody not to correlate with anti-HBc and anti-HBe activity. Moreover, anti-Dane particle antibodies were present in half of the DNA polymerase-negative cases tested but only in one out of 29 DNA polymerase-positive sera, suggesting an inverse relationship between active virus replication and presence of anti-Dane particle antibodies. More information is needed to better define such a system which may play an important role in the termination of HBV infection.

Delta/Anti-Delta System

By immunofluorescence, an antigen/antibody system different from the known determinants of HBV has been detected in serum and liver of HBV carriers in Italy [97]. Delta antigen exhibits a nuclear fluorescence of hepatocytes similar to HBcAg [97], and sometimes in addition cytoplasmic staining (F. Gudat, L. Bianchi: unpublished observation) as is occasionally seen for HBcAg [44]. This similarity to HBcAg expression suggested a relationship to HBcAg but ultrastructural studies failed to detect core particles or the like in livers positive for delta by immunofluorescence [17]. However, patients expressing delta antigen in the liver regularly proved to be positive for anti-delta in blood [99].

HBsAg- and delta-positive human sera have been inoculated into chimpanzees [98]: (1) Animals susceptible to HBV infection developed acute hepatitis with detal markers. (2) Animals with positive anti-HBs titer before inoculation did not acquire disease or delta markers. (3) A chronic HBsAg carrier chimpanzee without evidence of delta markers responded to the inoculation with the appearance of delta markers coincident with a bout of hepatitis.

In pellets of serum from this carrier chimpanzee, delta antigen could transiently be detected after detergent treatment: 35–37 nm particles, resembling large HBsAg particles could be precipitated with anti-HBs of the subtype of the host chimpanzee but only partially with anti-HBs of the subtype of the donor patient's serum [96].

The nature of the delta/anti-delta system remains to be clarified. It appears to be associated with a transmissible agent that might be either a modified HBV antigen or, perhaps a defective non-A, non-B hepatitis agent that requires the presence of active HBV synthesis for its own replication [96].

Viral Antigens in Liver Tissue in Acute Hepatitis B

It is interesting to note that at the height of classical acute "spotty necrotic" hepatitis no viral components are found in liver tissue despite HBsAg seropositivity, intense lobular inflammation, spotty hepatocellular necrosis, and biochemical signs of liver cell damage. This reaction pattern has been termed the "elimination type" [13, 47]. Consistent with experimental HBV infection in chimpanzees [9], HBc and HBsAg may be demonstrated in the prenecrotic, preclinical (incubation) period [7, 29, 34]. The rare demonstration of some nuclear HBcAg and of hepatocyte membrane-associated HBsAg, in the early phase of acute hepatitis [4] may therefore reflect ongoing elimination. At this stage, virus expression does not necessarily indicate elimination insufficiency and impending persistence. In the late stages, however, this finding may be taken as a marker of possible persistence, even when histologic signs suggestive of chronicity [11] are not present at biopsy.

Viral Antigens in Blood and Humoral Immune Response in Acute Hepatitis B

The first evidence of HBV infection is the appearance of serum HBsAg followed by Dane particles, DNA polymerase, and HBeAg [67]. HBV antigen containing immune complexes may be detected [6]. After the decline of DNA polymerase activity and coincident with the rise of serum aminotransferases, anti-Dane particle antibodies [1] and anti-HBc [15, 27, 52, 67] (Fig. 2) are usually detected as the first markers of immunologic response. Seroconversion from HBeAg to anti-HBe appears to be anticipated by a shift in the amount of small HBeAg ("e_3") towards the IgG-bound ("e_1" and "e_2") form [109]. Little information is available at present on anti-delta: seven out of 135 patients with acute hepatitis tested by Rizzetto [99] had low titers of anti-delta which in three patients became undetectable within two months. Anti-HBs appears at variable times, sometimes as early as in the acute phase of infection, coincidently with the rise in aminotransferases, or as late as several months after the disappearance of serum HBsAg [15, 27, 52, 67]; during this interval, anti-HBc and anti-HBe may be the only markers of HBV infection.

The different biologic implications of the various humoral antibodies should be appreciated:

1) Seroconversion from HBsAg to *anti-HBs* reflects eradication of virus-associated particles, termination of the HBV infection, and in most cases cessation of hepatic inflammation. In some instances, however, inflammation may persist as chronic persistent or aggressive hepatitis despite seroconversion (L. Bianchi, F. Gudat, unpublished observation). Anti-HBs is assumed to have protective properties.
2) The appearance of *anti-HBe* and *anti-Dane particle antibodies* seems to reflect specific immunologic suppression (or elimination) of active core replication in the tissues and of Dane particles and HBeAg in blood but not necessarily of 22 nm spherical and tubular HBsAg particles. Anti-HBe may therefore persist in the HBcAg- and Dane particle-free HBsAg carrier state ([47]; see also Chapter 5 in this issue).
3) By contrast, *anti-HBc*, consistently detectable in nearly all forms of chronic HBV infection, regardless of Dane particle formation, including asymptomatic HBcAg-free carriers, has no eliminating or

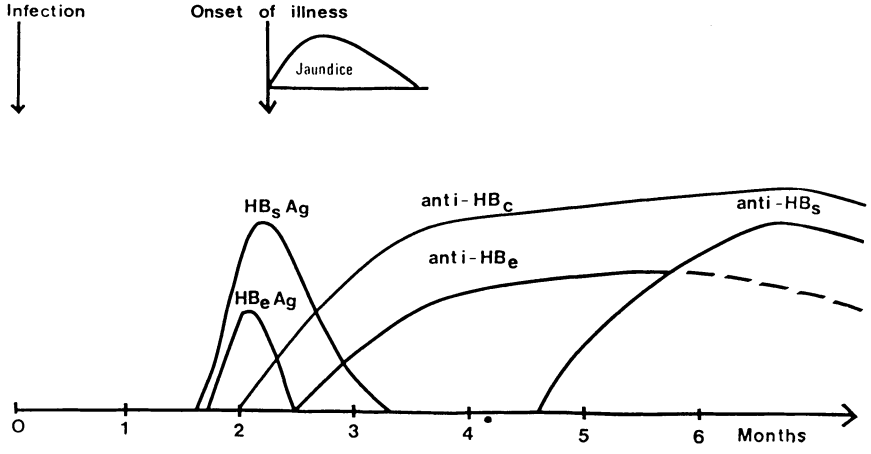

Fig. 2. Serologic events in acute hepatitis B. (Figure kindly supplied by F. Deinhardt and G. G. Froesner, Max von Pettenkofer Institute, Munich)

protective effect. Furthermore, posthepatitic anti-HBc may be short-lived, but anti-HBc may also persist in HBsAg-negative persons without detectable liver disease. Anti-HBc may provide a marker of continued HBV replication in the latter case [34]; see Chapter 5). Transfusion of anti-HBc-positive, HBsAg-negative blood has been followed by either HBsAg-positive [53, 91] or by non-A, non-B acute hepatitis [91].

Cellular Immune Response to HBV Antigens

Cellular Immunity

Three test systems of lymphocyte sensitization to HBV have been used: lymphocyte stimulation and transformation, leukocyte migration inhibition, and production of a migration inhibition factor by lymphocytes exposed to HBsAg. HBsAg-positive sera, and partially or highly purified HBsAg were used as antigens.

In the early stages of acute hepatitis B, sensitization to *HBsAg* has been found in about half the cases by some groups of investigators [31, 32, 62, 70, 113, 121]. Others, however, have found the highest frequency (mean about 70%) in later, convalescent stages [38, 40, 61, 75, 95]. High levels of serum HBsAg or circulating HBsAg/anti-HBs immune complexes might interfere with cell-mediated immunity in vitro. Cellular sensitization to *HBcAg* has been reported in acute and chronic forms of hepatitis B [87] but cellular immunity to *HBeAg* has not been tested so far [35].

T-cell cytotoxicity

T-cell cytotoxicity to *HBsAg*-coated, ^{51}Cr-labeled target erythrocytes was demonstrated in two out of three sera from patients tested 3–5 weeks after the onset of jaundice [3]; the highest cytotoxic indices were found in patients who had just cleared the virus [119]. The reaction regularly remained positive until three months after the onset of jaundice.

Besides evidence of sensitization and cytotoxicity reactions against hepatitis B viral antigens, *Host Antigens* have been considered as a possible target.

Cellular Immune Response to Liver-Specific Proteins

The importance of a liver-specific protein as a possible target in viral hepatitis has emerged from experimental studies of Meyer zum Bueschenfelde et al. They induced chronic aggressive hepatitis in rabbits after repeated immunization with a crude liver-specific but not species-specific protein (LSP) [74, 76] and could demonstrate a whole gamut of humoral and cellular immune reactions against this protein. These observations have stimulated extensive testing of sensitization to LSP in human viral and non-viral diseases [70, 78, 80, 105 and others].

Results from different laboratories about *cellular sensitization to LSP* in viral hepatitis are conflicting [28] and this might be ascribed at least in part to different degrees of purification of LSP as well as its instability. Antibody to LSP could not be detected in sera of patients with acute hepatitis B [54]. It should be noted, however, that IgG bound to the liver cell membrane has been detected on isolated hepatocytes from patients with acute hepatitis B [4]. It remains to be established

whether this represents anti-LSP or an antibody directed against other antigens, e. g., virus-induced neo-antigens [4]. Positive sensitization of lymphocytes from patients towards isolated autologous human or rabbit hepatocytes in vitro may be found in a high percentage of cases of untreated chronic aggressive hepatitis, irrespective of positivity for HBsAg [73, 111], and also in up to 50% of acute hepatitis B, interestingly with disappearance with recovery [70, 75, 111].

Cytotoxicity for (enzymatically) isolated hepatocytes has been demonstrated in sera from patients with both, HBsAg-positive and -negative acute hepatitis [22]. In the first two weeks of clinical illness, 90% of cases were positive and cytotoxicity tended to last longer (more than six weeks) in the HBsAg-positive group. It is of special interest that cytotoxicity to LSP in this system disappeared at the time hepatitis was resolved [19]. The positive cytotoxicity reaction was obtained with a fraction of patient's lymphocytes enriched in B-cells but not with a population rich in T-cells. There was no correlation of cytotoxicity with the degree of liver cell necrosis, however [22]. The cytotoxicity reaction could be blocked with either purified LSP or anti-LSP [23]. This lends further support to the assumption that LSP indeed seems to be the target for the observed cytotoxicity, mediated by an antibody-dependent non-T-population of lymphocytes. The question remains open, however, whether a causal link does exist between HBV and LSP [19].

Another, soluble *liver cell membrane antigen* (LM-Ag) has been identified which appears to be different from LSP [54]. The antibody to this liver membrane antigen (*LMA*) was detected in autoimmune types of chronic aggressive hepatitis [77]. In addition, association with *HLA antigens* is reported for this autoimmune chronic hepatitis [33] (see Chapter 5 in this issue) but a prevalence of a certain HLA determinant has not been found so far in acute HBV-related hepatitis (see Chapter 3 in this issue).

Immunoregulatory Factors

In patients with early stages of acute viral hepatitis cytotoxicity assays were as in controls but became positive after washing patients' lymphocytes several times before incubation. This was interpreted as supporting evidence for the assumption that in early acute hepatitis, serum factors loosely bound to lymphocyte membranes might inhibit their function [119].

At least six serum factors interfering with in vitro lymphocyte reactivity have been described [16, 18, 24, 36, 84, 103, 118; for a review see 86] but most are poorly characterized and their possible interrelationship remains to be established.

A prime candidate for such an immunoregulatory factor relevant in hepatitis B has been characterized by Chisari et al. [18]. In acute and chronic hepatitis B this group has found a normal number of circulating T-lymphocytes but a marked decrease in in vitro rosetting of erythrocytes by T-cells was observed [20]. Two mechanisms responsible for this disturbance in T-cell function were recognized: (a) an apparently intrinsic defect of T-cells and (b) a virus-induced immunoregulatory plasma lipoprotein, the *rosette-inhibiting factor* (*RIF*) has been identified and purified [18]. The intrinsic defect is found in acute and chronic forms of hepatitis as well as in chronic non-viral liver diseases, e. g., chronic alcoholic liver disease [10].

The presence of RIF, on the other hand, is restricted to viral hepatitis types A, B, and non-A, non-B, and is not found in chronic non-viral diseases nor in hepatitis associated with Epstein-Barr or cytomegalovirus [21]. In all cases of classical acute hepatitis RIF was transiently found and regularly disappeared with recovery [20, 21]. Histologic evidence of ongoing liver cell damage (unresolved hepatitis, chronic aggressive hepatitis) correlated with persistence of RIF in most cases [19].

Pathogenesis of Hepatocellular Injury and of Acute Forms of Hepatitis B

HBV does not appear to be directly cytopathic for infected hepatocytes [31, 32, 34, 47, 86–88]. This is best exemplified by chronic asymptomatic HBsAg carriers (HBcAg-free HBsAg type) [13, 47] and even more so by effectively immunosuppressed patients (e. g. kidney transplant recipients of the generalized HBcAg type) [13, 47]. In both conditions, large amounts of viral antigens are tolerated in liver tissue without substantial biochemical and electron microscopic liver cell damage and little inflammation, if any. By contrast, spotty liver cell necrosis in acute hepatitis of apparently normergic persons illustrates that the hepatitic lesion may result from host defense mechanisms, eliminating hepatocytes altered by the virus.

In *acute spotty necrotic hepatitis*, features of humoral and cellular immunity to HBsAg may be found as early as the onset of illness and liver damage. The widespread presence of HBcAg and HBsAg in liver cells in the preclinical, prenecrotic stage of the infection may thus represent a phase of infection with viral expression and replication *before* an immune response is elicited [14]. The absence of viral antigens from tissue at the height of acute hepatitis and in later stages of the disease may be the result of effective normergic immune responses by which virus-infected cells with full viral antigen expression are eliminated by spotty necrosis, resulting in a self-limited disease. The eliminating hepatitis, as such, may thus be regarded as a beneficial event, and immunosuppression might prevent eradication of virus-producing cells and cause chronicity. Consequently, immunosuppressive therapy should be withheld. *Acute hepatitis with confluent necrosis* may represent a special type of elimination leading to clearance of the virus and limited disease as in spotty necrotic hepatitis. However, persistent infection, usually with expression of the focal HBcAg type in liver tissue, as otherwise seen in chronic aggressive hepatitis [13, 47] may be observed as well. Likewise, persistence of viral antigens in tissue, notably focal HBcAg expression [13, 47] is found in many cases of *acute hepatitis with piecemeal necrosis* and this may predict transition to chronic hepatitis [13, 47].

The pathogenetic mechanisms entailing liver cell necrosis and eradication of the virus are still poorly understood. It is assumed that humoral and cellular immune responses acting in concert hit one or more target antigen(s) exposed on the liver cell surface and trigger necrosis of infected cells. But the main questions remain subject to hypotheses: (a) What is the nature of the target(s) for the immune attack? (b) Which is the potent effector system, humoral or cellular immunity, and notably which effector cell types are involved? (c) Which are the factors responsible for determining type and extent of liver cell necrosis?

Target Antigens

The most likely candidate for a *Virus-Coded Target* is *HBsAg.* Its role in cell elimination is indicated by its association with the cell membrane in very early phases of acute hepatitis B [4]. Several observations point to cellular immunity directed against HBsAg: (1) The time relation between onset of acute illness and demonstration of cell-mediated immunity, i.e., sensitization [31, 32, 62, 70] including T-cell cytotoxicity against HBsAg [3, 119]; (2) The absence of cell damage and the tolerance of viral antigens in effectively immunosuppressed patients [47], in which cellular immunity is far more affected than humoral reactivity, and in carriers who tolerate viral antigens in the absence of demonstrable cell-mediated immunity to HBsAg [39] and T-cell-mediated cytotoxicity in vitro [36].

Anti-HBs-mediated cytotoxicity is unlikely to occur. Passively transferred anti-HBs to HBAg carriers does not appear to cause liver injury [66, 94]. The appearance of anti-HBs usually indicates termination of HBV infection, although the antibody may appear long after the disappearance of HBsAg from blood and liver. Radioimmunoassay does not detect anti-HBs in all forms of active HBV infection. This, however, does not exclude trace amounts of antibody undetectable because of complexing to the target. Indeed, the observed immune complexes during acute viral hepatitis [6] indicate at least involvement of anti-HBs in the elimination of intercellular and circulating HBsAg. Antibody-mediated K-cell cytotoxicity has not been ruled out.

Of particular interest with respect to a possible target are *Dane particle- and tubule-associated antigens*, different from HBsAg and possibly involved in immune complex formation of the Dane particle/tubule type [1, 2, 45, 83]. These antigen/antibody systems are as yet poorly defined. The relation of HBeAg and anti-HBe to such a system has not been clarified but it behaves similarly in that HBeAg is associated with Dane particles and with Dane particle/tubule complexes [46] while anti-HBe is associated with the absence of HBeAg and Dane particles. Cellular immunity against HBeAg and Dane particle-associated antigens in the various forms of hepatitis B have not been studied. Besides the possibility that these antibodies may be involved in K-cell-mediated cytotoxicity for Dane particle-producing cells, such antigen/antibody complexes may have the opposite effect, namely of blocking or suppressing action on cellular immunity [36].

HBcAg has also been considered as a possible target antigen [34]. Indeed, using immunofluorescence HBcAg is found in close connection with the hepatocytic membrane [44, 92]. Electron microscopy, however, demonstrated HBcAg particles only in the intracytoplasmic submembraneous region [44]. Also from a theoretical point of view the core of an enveloped virus is unlikely to be exposed on the cell surface. To our knowledge, HBcAg – unlike HBsAg – has never been demonstrated on the surface of hepatocytes in single cell studies [4].

Anti-HBc appears in virtually all forms of HBV infection, including those without cell damage [37]. This finding speaks against any role of anti-HBc in elimination and in cell damage. Particularly high titers of anti-HBc in chronic active hepatitis [37] might be a secondary phenomenon caused by prolonged immunization by core particles released from cells destroyed by other mechanisms.

Besides viral antigens, *Host Antigens* such as the liver specific membrane protein *LSP* may be considered as a target in acute hepatitis B [75]. The demonstration of cellular immunity to and cytotoxicity against LSP during the acute phase of hepatitis, irrespective of the presence of circulating HBsAg and the loss of sensitization with recovery suggests a loss of tolerance towards LSP in acute hepatitis [75]. The biologic significance of IgG, bound to the liver cell surface, found in rare cases of acute hepatitis B [4] is not established. Autoantibody to LSP or antibody to virus-induced neo-antigens, not yet defined, have been considered. Since RIF is known to be active in very early stages of acute hepatitis B and to be capable of inhibiting active cellular suppressor mechanisms, Chisari et al. [19] have suggested that RIF would facilitate the differentiation of killer T-cells directed against several autoantigens, including LSP.

In acute hepatitis B, the strongest evidence available points to HBsAg and LSP as targets for a *Cellular Immune Attack*. Under normergic conditions, mainly *T-lymphocytes* may be involved in the eradication of infected hepatocytes. In liver biopsies of acute hepatitis B patients T-cells outnumbered B-lymphocytes by a factor of 20 : 1 [79]. Further evidence comes from cases of chronic active hepatitis with substantial liver cell damage in agammaglobulinemia; these patients in fact run an even more severe course [41, 43, 112]. The transiently increased severity of hepatitis after transfer of lymphocytes from convalescent patients, following administration of transfer factor [63, 66] or of agents stimulating cellular immunity, such as levamisole [26], supports T-cell-mediated hepatocytic damage.

Antibodies seem to have mainly protective properties and are involved in inhibiting the spread of virus. It is currently not known which cytolytic cell systems (i.e. K cell, NK cell, T killer cell, armed or activated macrophage) are of prime importance in the pathogenesis of liver cell necrosis.

Immune Regulatory Molecules such as RIF may balance a complex interaction of several T-cell subpopulations and B-cells. The role of HLA genes in acute hepatitis remains to be established [32, 35, 86] (see Chapter 3 in this issue). It should be appreciated that growing evidence from animal studies indicates the need for genetic compatibility between T killer and target cells at major loci of the histocompatibility complex [106]. To be effective, T killer cells require a "dual recognition" [125], e.g., viral antigen on target cell and cell surface HLA marker.

The differences in the pathogenesis of confluent, piecemeal, and spotty *Necrosis* in acute hepatitis are not known. The appearance of neutrophils in confluent necrosis would suggest involvement of complement but this has not been substantiated so far. Endotoxin has been claimed to be operative in confluent necrosis. Chisari et al. [19] have recently offered a fascinating hypothesis to explain the extension of liver cell necrosis in acute viral hepatitis. The presence and differences in local concentration of LEx, an intrahepatocellular immunosuppressive substance from normal human liver [103] may account for the extent of liver cell necrosis. This factor, if released into the extracellular environment as a result of single cell necrosis could modulate lymphocyte effector function on hepatocytes in the neighborhood of the lysed cell and thus limit the further extension of liver cell necrosis. It should be kept in mind that gap junctions allowing molecular cell-to-cell communication disappear with cell injury. The injured cells

are thus sealed-off from their neighbors and the latter are protected from injury [71]. Pathogenesis of piecemeal necrosis, most often associated with chronicity, is far from understood and will be dealt with in the next chapter (Chapter 5).

It is thus premature to design a precise and convincing scenario of the sequence of events in acute hepatitis B. The immunologic and histologic findings observed in acute hepatitis B tempt one to speculate on a concerted action of several virus- and host-dependent steps [19]: (1) Infection of liver cells with HBV; (2) Viral replication; (3) Expression of viral proteins in liver cells, notably HBsAg on liver cell surface, concomittantly with (4) triggering of synthesis of immunoregulatory molecules, e. g., RIF; (5) Loss of tolerance against LSP via RIF; (6) Accumulation of lymphocytes (T-cells) sensitized against HBsAg and LSP (and other, virus-induced neo-antigens?); (7) Production of antibody preventing intercellular spread of viral proteins and involved in antibody-assisted cytotoxicity and/or blocking function; (8) Mainly cell-mediated hepatocytolysis and increasing attraction of macrophages by MIF, involved in eventual phagocytosis. By such a sequence of events, histologic findings of a predominance of lymphocytes in early stages, accentuation of necrosis at the height of the disease, and accumulation of many macrophages in the late phase would be explained.

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The *Immunopathogenesis* of *Chronic HBV* Induced *Liver Disease*

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Introduction

The pathogenesis of hepatitis B virus induced liver disease is not well understood. Because a suitable animal model and methods for in vitro propagation of hepatitis B virus (HBV) have not been available, most current information is derived from clinical studies. Although considerable circumstantial evidence suggests the involvement of immunologic mechanisms [61] it is difficult to determine if the reported immunologic events are primary pathogenetic determinants or merely secondary to the disease.

Potentially important pathogenetic determinants include viral factors such as subtype [9] and dosage [6] mode of transmission [53] and host factors such as age, genetics [8], and sex as well as the immune response [28] to viral or autoantigens. The persistence of viral synthesis in patients without liver injury (the carrier state) suggests that the hepatitis B virus itself is not directly cytopathic for hepatocytes [29]. Nonetheless, viral persistence is frequently associated with the development of chronic hepatitis [61] indicating that the virus is at least a necessary if not a causative factor in the disease process.

Recent work suggests a link between an assortment of human diseases and alleles within the major histocompatibility gene complex (HLA). Conceivably, certain gene products might regulate HBV recognition, processing or replication as well as the qualitative features of the immune response to viral antigens. Nonetheless, despite a weak association between HBV infection and some HLA phenotypes, the most convincing evidence for a genetic role in this disease is its very high prevalence in Down's syndrome and in certain Asian and African populations, although environmental rather than genetic factors could just as easily be responsible for these observations.

A variety of humoral and cellular immune responses to viral and host antigens has been described in hepatitis B virus infection. Antibody to the coat protein,

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hepatitis B surface antigen (HBsAg), is known to neutralize viral infectivity and to contribute to the formation of the immune complexes which produce many of the extrahepatic manifestations of this disease [43, 57]. There is no evidence, however, that antibody to HBsAg is pathogenetically involved in HBV induced hepatocellular injury. Based on observations in other systems, it has been postulated [20] that anti-HBsAg might regulate HBsAg expression at the hepatocyte surface and perhaps viral genome expression as well, but this remains entirely conjectural at present. A second major antibody response is directed to the core antigen of the virus (HBcAg). The persistence of this response is one of the most sensitive serologic markers of continued hepatitis B virus replication, but it has not been implicated in the pathogenesis of hepatocellular injury. An independent group of antibody responses directed to various poorly defined normal hepatocellular antigens [47, 62] has also been identified. However, because the precise nature of the antigen is not known and because similar antibodies are found in patients with an assortment of other liver diseases, the primary pathogenetic involvement of this antigen-antibody system in viral hepatitis B is in doubt. Finally, it is reasonable to question the importance of the humoral arm of the immune response in the pathogenesis of hepatocellular injury since both acute and chronic hepatitis are seen in patients with agammaglobulinemia [44].

Considerable evidence suggests, but does not prove, that the cellular limb of the immune response might be pathogenetically important in hepatitis B virus infection as it is in many other viral diseases [77, 95]. Cellular sensitization to viral and hepatocyte antigens has been observed in the majority of patients with acute and chronic hepatitis who also display cytotoxic effector cell activities specific for target cells bearing viral and hepatocyte antigens [21, 31, 51]. Additionally, chronic active hepatitis has been produced in rabbits chronically immunized with human hepatocyte membrane antigen preparation and the histologic changes correlated well with the cellular immune response to this antigen [62].

Conceivably, therefore, hepatocellular injury in hepatitis B virus infection might be due to a cellular autoimmune response to hepatocyte membrane autoantigens [47]. Since the immune response to self-antigens is strictly controlled by an assortment of mechanisms which collectively constitute the normal state known as immunologic tolerance, one must first demonstrate the failure of one or more of these mechanisms and provide a relevant explanation for their failure before the above hypothesis can be considered tenable. In this regard, it is notable that defects in suppressor cell function putatively involved in maintenance of immunologic tolerance have been identified in patients with viral hepatitis [22, 46]. Furthermore, mechanisms capable of producing suppressor cell dysfunction have been shown to be operative in viral hepatitis [86] and may represent the primary pathogenetic event in this disease.

Based on these observations, we have developed the following hypothesis for the pathogenesis of hepatocellular injury in HBV infection (Fig. 1). HBV may infect the hepatocyte either directly or perhaps via a processing step involving the Kupffer cell which has been shown to play a role in the natural resistance to virus infections [70]. One the HBV genome is integrated into the host chromosomes, hepatocellular metabolism of normal immunoregulatory molecules is disturbed resulting in a complex imbalance, the net effect of which is the partial loss of normal suppressor

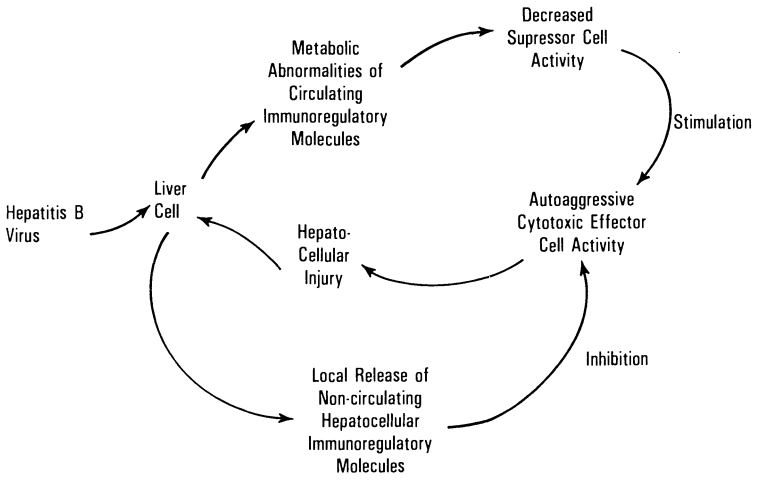

Fig. 1. An immunoregulatory hypothesis for the pathogenesis of hepatocellular injury in viral hepatitis

cell function. This, in turn, leads to the expression of an assortment of auto-immune phenomena including the emergence and expression of autoreactive clones specific for hepatocyte membrane antigens. The ensuing cytolytic process results in the release of normally intracellular proteins that have immunosuppressive properties which down-regulate the cytotoxic effector cells locally within the liver. Normal genetic variation in the hepatocellular content of such immunoregulatory molecules may be responsible for the variable duration and intensity of this virus induced, immunologically mediated disease.

In the remainder of this review, we will discuss the information which forms the basis for the individual tenets of this hypothesis. We will begin with an overview of the cellular constituents involved in this scheme, and discuss the functional characteristics that are germane to the hypothesis. Then we will consider the target antigens to which the immune response may be directed and review the evidence that such a response does indeed occur in this disease. Finally, we will discuss the cellular and molecular immunoregulatory systems which conceivably permit the emergence of this response and consider the possibility that genetic variation in these substances may actually occur. The hypothesis will then be reiterated and new areas of potentially productive future research will be discussed.

Cellular Constituents

The major cellular constituents of the immune system are the thymus-derived (T) lymphocytes, the bone marrow-derived (B) lymphocytes, and the bone marrow-derived cells of the monocyte/macrophage series.

T Cells

T cells possess surface receptors for a glycoprotein ligand [18] on sheep red blood cells (E receptors) and also the Fc [71] portions of IgG (T γ cells) and IgM (T cells) (Table 1). T cells play an important regulatory role in the synthesis of immuno-

Table 1. Properties of the effector cells of the immune system

	T	B	NK	K	Macrophage
slg positive	−	+	−	−	−
E rosette positive	+	−	−	−	−
Esterase positive	−	−	±	−	+
Adherence	±	−	−	−	+
Phagocytosis	−	−	−	−	+
C_3 Receptor	−	+	+	+	+
Fc Receptor	+	−	+	+	+
Genetic restriction	+	−	−	−	−
Antigen specificity	+	−	−	−	−

\+ present
− absent

T = Thymus derived lymphocyte
B = Bone marrow derived lymphocyte
NK = Natural killer cell
K = Killer cell

globulin (lg) by pokeweed mitogen (PWM) activated B cells. No immunoglobulin synthesis occurs in the absence of T μ cells. In the same system, T γ cells function as suppressors of immunoglobulin synthesis [71]. In addition, T cells serve a variety of antigen-specific effector functions which appear to require the positive cooperation between cell surface antigen receptors and certain products of the major histocompatibility complex (the HLA system in man) [27]. Prominent among these are lymphokine production and cellular cytotoxicity [26].

B Cells

The B cell population expresses surface membrane immunoglobulin (slg) [52], receptors for several components of the complement system and for the Fc portion of lgG (Table 1). Its major function is the synthesis and secretion of antibody molecules as a consequence of activation by an antigen for which it expresses highly specific slg receptors.

Null Cells

This class of cell lacks T cell markers (E receptors) and B cell markers (slg) but expresses Fc and C3 receptors [52]. Spontaneous cell mediated cytotoxicity (SCMC) and antibody dependent cellular cytotoxicity (ADCC) are mediated by subsets within this population [73].

Macrophages

Cells of the monocyte/macrophage series originate in the bone marrow from which they migrate either to the vascular compartment where they circulate (monocyte) or to an assortment of organs where they remain as fixed macrophages [70]. They display cytoplasmic esterase and peroxidase activities and surface membrane Fc and C3 receptors and they are adherent to a variety of surfaces [58]. They are functionally heterogeneous, e. g., phagocytosis, antigen processing and presentation, cytolytic activity, secretion of various molecular products (monokines), and they serve immunoregulatory functions as helpers and suppressors of an assortment

of B cell and T cell responses. Approximately half of the body's reserve of fixed macrophages are present in the liver in the form of Kupffer cells [79].

Functional Properties of Lymphocyte Subpopulations in Hepatitis B Virus Infection

Non Specific Parameters

Several laboratories [17, 24, 25, 68] have studied the numbers and distribution of peripheral blood lymphocytes in patients with viral hepatitis (Table 2). Most investigators have observed a decrease in the number of E rosette forming T lymphocytes in the peripheral blood of patients with AVH and CAH [19], with no change in the B lymphocyte population. Since the number of peripheral blood lymphocytes capable of binding neuraminidase treated sheep red blood cells (another specific T cell marker) is normal in hepatitis B [33] reduced E rosette formation is thought to reflect a functional alteration rather than a quantitative depletion of peripheral blood T cells in this disease.

Stimulation of DNA synthesis by polyclonal activators such as phytohemaglutinin (PHA) is another measure of T lymphocyte reactivity. PHA responsiveness is reduced in both acute and chronic viral hepatitis, it returns to normal with recovery [1] and remains normal in the healthy carrier state. These observations have prompted some investigators to suggest that the ultimate outcome of HBV infection may depend on the functional integrity of the T lymphocyte population. Because these systems are modulated by assorted cellular and molecular immunoregulatory events, the functional integrity of regulatory as well as effector cell populations may determine the course of HBV related disease.

Specific Parameters

Using lymphocyte transformation, it has been shown that cellular sensitization to HBsAg occurs during acute and chronic viral hepatitis [30, 48, 55, 56, 88]. Sensitization persists with recovery but it is virtually absent in the carrier state.

Cellular sensitization to liver specific proteins, especially a liver specific hepatocyte surface membrane lipoprotein (LSP), also occurs during the acute and chronic phases of illness but unlike sensitization to HBsAg it disappears with recovery [55, 56, 67]. The latter observation is extremely interesting and suggests that powerful suppressor influences which may normally prevent the expression of this cellular autoimmune response are transiently inoperative during periods of

Table 2. Distribution of peripheral blood lymphocytes in patients with viral hepatitis B

Patients	T	B	Null
AVH-B	Decreased	Normal	Increased
CAH-B	Decreased	Normal	Increased
Carriers	Normal	Normal	Normal

AVH-B = Acute viral hepatitis type B
CAH = Chronic active hepatitis type B

hepatocellular injury but are restored concomitant with recovery. Whether this represents a primary pathogenetic event is conjectural but waarants further consideration.

Cellular Immunoregulatory Systems

Suppressor Cell Function

Suppressor cells within the T cell and monocyte populations play a major role in the regulation of antibody production [40], cell mediated immunity, and some forms of immunologic tolerance [41, 45].

T Suppressor Cells

a) Suppression of B Cell Function. Pokeweed mitogen (PWM) can stimulate human B cells to synthesize and secrete immunoglobulin. A minor subset of T cells which bear surface membrane receptors for the Fc region of lgG (T γ cells) can suppress the synthesis of immunoglobulin by PWM activated B cells [71]. Although this activity occurs without prior mitogen treatment, it is markedly increased when T cells are first incubated with Con A [66]. These cells are exquisitely radiosensitive and comprise 10 to 15% of E rosette positive lymphocytes.

b) Suppression of T Cell Activation. T suppressor cells also inhibit the DNA synthetic response of other T cells to activation by mitogens or allogeneic cells [80]. This suppressor activity requires prior treatment with Con A for expression. Inactivation experiments have demonstrated radiosensitive and radioresistant populations within this subset.

Monocyte/Macrophage Suppressor Cells

There exists a subset within the phagocytic, adherent, esterase positive population in peripheral blood that can suppress the DNA synthetic response of allogeneic cell-stimulated T cells [54]. These suppressors are spontaneously active and radioresistant but depend on active protein synthesis for full expression of their inhibitory activity.

Suppressor Cell Function in Chronic Liver Disease

Numerous studies have demonstrated a mjor defect in suppressor T cell and suppressor monocyte activity in viral hepatitis. T cell suppressor function has been studied by Hodgson et al. [46] and Chisari et al. [22] in patients with acute and chronic active hepatitis (CAH). In CAH, the mean level of suppressor cell activity was reduced (Table 3). There was no correlation, however, between the degree of hepatic inflammation and the altered suppressor response [22, 46]. In acute hepatitis, Tavassolie et al. [86] and Chisari et al. [22] have demonstrated decreased mitogen inducible suppressor cell activity while Hodgson et al. [46] did not. However, in Hodgson's group, three patients with acute hepatitis who were studied before the peak rise in serum glutamic-oxaloacetic transaminase (SGOT) displayed

Table 3. Suppressor cell function in viral hepatitis

Patients	Suppressor cell activity	
Patients	T Cell	Monocyte/Macrophage
AVH-B	Decreased//Normal	Decreased
CAH-B	Normal	Deceased
Convalescent	Normal	Normal

AVH-B = Acute viral hepatitis, type B
CAH-B = Chronic active hepatitis, type B

reduced suppressor cell activity which returned to normal with resolution of the disease [46].

We [15, 22] have demonstrated that monocyte/mediated suppressor cell and mitogen induced (T cell) suppressor cell activity are decreased in acute and chronic hepatitis but return to normal with recovery. As in Hodgson's study, no correlation was observed between suppressor dysfunction and disease activity. Additionally, random association was observed between the two suppressor cell types in terms of functional integrity, suggesting that more than one mechanism was responsible for suppressor cell dysfunction in these patients.

The exact mechanisms responsible for suppressor cell dysfunction are unclear. However, Tavassolie et al. [86] have demonstrated that a factor present in the sera of patients with acute and chronic active liver disease inhibits the function of normal suppressor T cells in vitro. Additionally, we have observed (Fig. 2) that serum from patients with acute viral hepatitis B inhibits monocyte mediated suppressor cell activity. In a study of 48 hepatitis patients and 10 normal controls monocyte suppressor activity was reduced to 60% of reference levels when the assay was performed in the presence of 20% patient's serum. In contrast, normal control serum had no effect on suppressor cell function (F. V. Chisari, -unpublished observations). Thus, it is possible that at least a portion of the defects in suppressor cell activity in viral hepatitis may be due to abnormalities of the network of the immunoregulatory molecules normally present in serum. Since many of these molecules are synthesized in the liver [20], it is conceivable that virus-induced alterations in hepatocellular metabolism may initiate the loss of suppressor cell function by virtue of the induction of circulating inhibitors. Precedent for this hypothesis exists and will be discussed and developed in depth in a later section.

Cellular Cytotoxicity

If cellular immune mechanisms are responsible for hepatocellular injury in viral hepatitis, one would expect that with the variety of cytotoxicity assay systems currently available distinct differences between hepatitis patients and normal controls should be detectable. Several such studies have been performed using peripheral blood lymphocytes from patients with hepatitis B viral infection as effectors against a variety of surrogate hepatocyte target [3, 21, 31, 37, 51, 89–93]. The inability to culture adult human hepatocytes has necessitated the use of other

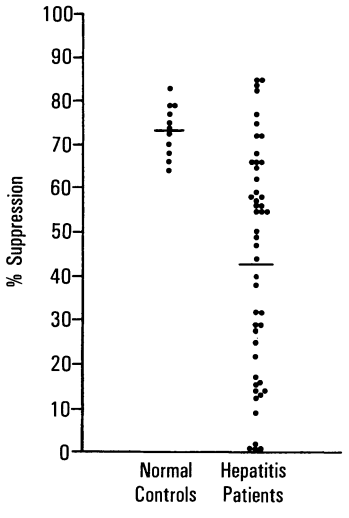

Fig. 2. Effect of serum on monocyte mediated suppressor cell function. The 48 patients studied who had acute hepatitis all had clinical and biochemical evidence of disease for less than 12 weeks. Monocyte suppressor function was fully expressed (70% suppression) in the presence of normal control serum. In contrast, hepatitis sera reduced suppressor cell activity by approximately 40%

target cells [4, 10, 38] in these assay systems: for example, isolated rabbit hepatocytes, autologous human hepatocytes, a human liver derived continuous cell line (Chang), the HBV producing PLC-PRF/5 cell line, the liver specific protein containing SK-HEP 1 hepatoma cell line, hepatitis B surface antigen coated avian red blood cells, and liver specific protein coated pigeon red blood cells. In most of these assay systems, chromium-labeled target cells are employed. However, because unacceptably high levels of spontaneous cell death occur in human autologous hepatocytes, chromium release assays have not been feasible and, therefore, other parameters (adherence to plastic) have been employed to examine target cell viability in these studies [23, 31, 32, 37, 94].

Spontaneous Cell Mediated Cytotoxicity (SCMC)

SCMC is mediated by the natural killer (NK) cell which has the null cell phenotype [89]. NK cell activity is enhanced by interferon and is thought to be important in immune surveillance of cancer and as a pre-immune defense mechanism against an assortment of microbial pathogens. No prior sensitization is required and a wide variety of target cells are susceptible to NK mediated lysis. Genetic restriction does not appear to play a major role in NK cell function.

Antigen Specific Cellular Cytotoxicity (ASCC)

This system is mediated by specifically sensitized T killer cells and thus is a measure of antigen specific cytolysis. The target cells must express cell surface antigen to which the effector cells have been sensitized and for which they have specific receptors. Additionally, T cell mediated cytotoxicity occurs only when the effector cells and target cells share certain histocompatibility antigens [95]. Thus, unlike the other in vitro systems discussed in this section this system requires the generation of two distinct recognition signals before it can be operative: one which is provided by

specific target antigen, and the second which is provided by the shared HLA determined self antigen.

Mitogen Induced Cellular Cytotoxicity (MICC)

This system is mediated by mitogen activated T cells but more closely resembles NK cell function rather than TK cell functions since it is neither antigen specific nor is it genetically restricted [73]. Nonetheless, it provides a functional yardstick for the terminal, effector phase of T cytolytic activity.

Antibody Dependent Cellular Cytotoxicity (ADCC)

Both the humoral and cellular limbs of the immune response are involved in this system. Antigen specificity is determined by the antibody combining site (Fab) of an immunoglobulin molecule, the Fc region of which serves to complete a bridge between a target cell and an Fc receptor-bearing non-T lymphocyte. The effector cell in this system has the null cell phenotype and is designated simply as a killer (K) cell [73].

Target Antigens

If immune mediated cytolysis is an important mechanism in the pathogenesis of viral hepatitis, specific antigens or determinants on the surface of infected hepatocytes must act as targets for one or more of the various effector cells. With the inability to maintain human hepatocytes in vitro it has been impossible to define these entities with any degree of precision. Two major antigens have been implicated as possible candidates. The hepatitis virus or some component of it is a suitable candidate if deposited on the surface of the infected hepatocyte and, therefore, accessible to the immune system. Alternatively, hepatocyte membrane auto-antigens may serve as targets either if they are in some way structurally or conformationally altered by the infection or if tolerance is abrogated as a consequence of decreased suppressor cell function.

Viral Antigens

The coat protein of the hepatitis B virus (HBsAg) is present within the cytoplasm and at the surface membrane of infected hepatocytes. Excess hepatitis B surface protein circulates as 22 nanometer particles with spherical and filamentous morphology. An internal viral core antigen, HBcAg, is found almost exclusively within hepatocyte nuclei. HBcAg is assembled into 27 nanometer particles in the nucleus from whence it moves to the cytoplasm where it is enveloped with surface protein and is transported into the vascular compartment where it circulates as a 44 nanometer Dane particle. The e antigens [59] are a group of poorly delineated molecules which were initially suggested to represent antibodies of restricted heterogeneity that bind Dane particles. Neurath and Strick [74] have proposed that the antigenic determinants of e represent idiotypic determinants of what may be an lgG4 immunoglobulin and thus anti-e would then represent anti-idiotype antibody. Other workers have provided compelling evidence that HBeAg is a structural

component of the viral core [69]. Nonetheless, the e antigen complex has not been identified at the cell surface and it is, therefore, unlikely that e is a viable target antigen in this system. HBsAg is expressed at the cell surface during the acute phase of type B viral hepatitis. In more chronic forms, it appears to be confined primarily to the cytoplasm and is not often expressed at the cell membrane [2]. Unlike HBsAg, which is characteristically located within hepatocyte cytoplasm and at the cell surface where it is accessible as a potential target antigen, the core antigen has never been identified on the hepatocyte surface and is only rarely present in the cytoplasm [75]. Therefore, HBcAg is probably not a potential target antigen in this disease. Since Warnatz et al. [93] and Alberti et al. [3] have demonstrated cellular cytotoxicity to HBsAg coated target cells in patients with CAH, this or some other related, hitherto unknown viral antigen might be a pathogenetically important target in this disease. Of great interest is the reported progression of HBsAg positive acute hepatitis to HBsAg negative chronic hepatitis [78] in which currently identifiable antigens cannot be implicated. Such evidence suggests that host determined antigens may prove to be of paramount importance as pathogenetic determinants regardless of the HBsAg status of the patient.

Liver Specific Antigens

Organ-specific hepatocyte surface membrane antigens [60, 63] might also serve as target antigens under appropriate circumstances. Among these the most likely candidate is liver specific protein (LSP), a hepatocyte membrane lipoprotein [7, 14], which has been demonstrated by immunofluorescence on the surface of rabbit and human hepatocytes. The potential importance of LSP was initially demonstrated by Meyer Zum Buschenfelde [62] and colleagues who chronically immunized rabbits with human LPS. Following an intensive sequence of injections, the rabbits developed histologic evidence of chronic active hepatitis [64] which correlated very well with parameters of cellular immunity to both human and rabbit LSP. More recently, several investigators have detected antibodies to LPS [49] in the serum of patients with HBsAg positive acute and chronic hepatitis. Cellular sensitization to liver specific antigens also occurs in viral hepatitis and has been demonstrated in patients with acute and chronic hepatitis, but most notably it disappears with recovery [55, 65, 84]. Thus, sensitization to LSP appears better correlated with hepatocellular injury than sensitization to HBsAg which persists after recovery. The loss of sensitization also correlates in separate studies with recovery of normal suppressor cell function and suggests that sensitization to LSP may have an auto-immune basis (Table 4). In cytoxicity studies using rabbit or autologous human hepatocytes as targets, nearly all patients displayed LSP specific cytotoxic effector cell activity during the acute and chronic phases of viral hepatitis which disappeared coincident with recovery as above, further supporting the auto-immune hypothesis.

Cytotoxic Effector Function in Viral Hepatitis

The prominent intrahepatic mononuclear inflammatory cell infiltrate characteristic of acute and chronic viral hepatitis B suggests that the associated hepatocytolysis

Table 4. Comparison of suppressor cell function and cellular immune reactivity to liver specific protein in viral hepatitis

	Stage of disease		
	Acute	Convalescent	Chronic
Suppressor cell activity	Decreased	Normal	Decreased
LSP sensitization	Present	Absent	Present
LSP specific cytotoxicity	Present	Absent	Present

may be mediated by cellular immune mechanisms. This impression is supported by reports describing the presence of relevant cytotoxic effector lymphocytes capable of killing autologous [76, 92] and xenogeneic [23, 31, 39, 94] hepatocytes in the peripheral blood mononuclear cell population of patients with hepatitis B virus infection. Since the precise nature of the target in these liver cell mixtures is unknown and because serial observations and examinations of the appropriate normal control groups are not possible, these studies although extremely provocative, are of uncertain significance. Vogten et al. [90] recently described a phagocytic cytotoxic effector cell for pigeon red blood cells coated with liver specific lipoprotein (LPS) in patients with chronic active hepatitis. Alberti and colleagues [3] found cytolytic T lymphocytes specific for hepatitis B surface antigen in the peripheral blood mononuclear cell population in patients with acute and chronic hepatitis. However, the significance of these observations is unclear because, in spite of the apparent antigen specificity in these systems, the expression of antigens chemically coupled to xenogeneic cells from irrelevant organs certainly differs from their natural expression in autologous or allogeneic hepatocytes.

Cytolytically active non-T cells specific for rabbit hepatocytes have been described in patients with chronic active hepatitis [23, 31, 94]. Additionally, rat hepatocyte targets have been shown to be susceptible to injury by peripheral blood mononuclear cells from patients with chronic active hepatitis [39]. Whether these interesting findings using animal targets are pertinent to human hepatocellular injury remains to be seen.

Other groups have examined the functional capabilities of the known cytotoxic effector cells of patients with acute and chronic hepatitis with somewhat conflicting results. Utilizing Chang cells as targets, Wands and Isselbacher [91] and Kakumu et al. [51] demonstrated increased spontaneous cell mediated cytotoxicity in patients with acute and chronic active hepatitis. In contrast, Vierling et al. [89] found no abnormalities in spontaneous cell mediated cytotoxicity, mitogen induced cellular cytotoxicity, or antibody dependent cellular cytotoxicity in systems defined to measure the activity of natural killer (NK), T killer, and K cells, respectively, in patients with chronic liver disease.

We have recently performed a study [21] to determine whether human target cells which naturally express liver specific protein (LSP) and hepatitis B surface antigen (HBsAg) on their surface membrane are preferentially killed by peripheral blood cytotoxic effector cells from patients with acute and chronic hepatitis B. By so doing, we hope to determine whether one or both surface proteins are relevant

antigens in hepatitis B virus induced hepatocellular injury. Chang cells (which lack LSP and HBsAg) were used as specificity controls and also for comparison with the published reports of others. Spontaneous and mitogen induced cytotoxic effector cell activity towards human hepatocyte cell lines which naturally express surface membrane LSP and HBsAg were not observably increased in any patient group in our study. This was surprising in view of published reports that patients with hepatitis B virus associated chronic active liver disease possess phagocytic cytotoxic effector cells active against LSP coated pigeon red blood cells in patients with acute and chronic HBV infections [90] and cytotoxic T cells specific for HBsAg coupled to chicken red cells [3]. This discrepancy is not easily explained except on the basis of the nature of the target cells used in the different studies. Conceivably, prior biochemical purification and conjugation of HBsAg and LSP to non-mammalian cell surface membranes may have exposed antigenic determinants not present on the HBsAg and LSP positive targets used in the present study. Whether a cytotoxic response to such altered antigens is relevant to the putative pathophysiologic response in vivo is not known. Alternatively, although serologically detectable LSP and HBsAg determinants exist on the human hepatoma cell lines we employed, it is conceivable that these molecules express serologically undetectable lymphocyte defined antigens which are either buried in the target cell membrane or genetically deleted from these cell lines.

The data available herein are open to several interpretations. First, contrary to our hypothesis, hepatocyte injury in the course of viral hepatitis may not result from cellular immune effector mechanisms. However, lacking proof of another cause, we consider this interpretation premature. Second, cellular immune mechanisms may be operative but the specific effector cell either does not circulate or is preferentially removed from the peripheral blood compartment by binding to hepatocyte surface antigens. Although this interpretation is testable by examining the cytotoxic effector capability of intrahepatic lymphoid cells, substantial technical difficulties make the execution of these experiments impractical at present. Therefore, this approach is restricted to animal model systems with the assumption that the pathogenetic mechanisms are similar in human and animal species. Third, cellular immune mechanisms may be operative but the target cells employed in the current studies do not express the target antigens recognized in vivo. Alternatively, the relevant antigens may have been present on these target cells but certain biologic requirements for the expression of a cytotoxic response to these antigens may not have been fulfilled in our assay systems including the expression of a latent, antigen-specific cytotoxic response. In this respect, the observations of Zinkernagel and Doherty [95] demonstrating that effector and target cells must share certain histocompatibility antigens as a prerequisite for the expression of a virus specific cytotoxic effector cell response are particularly germane [26].

Molecular Immunoregulatory Systems

A heterogenous assortment of immunoregulatory molecules [11–13, 16, 34–36, 42, 72, 87] has been identified in human plasma (Table 5). A substantial proportion of these molecules is known to be metabolized in the liver. Most of these molecules

Table 5. Plasma immunoregulatory molecules

Lipoproteins
Rosette inhibitory factor (RIF)
Chylomicrons
Very low density lipoproteins
Intermediate density lipoproteins
Low density lipoprotein inhibitor (LDL-In)
Low density lipoprotein
C-reactive protein
Alpha fetoprotein
Fibrinogen degradation products
Immunoregulatory peptide

inhibit lymphocyte activation; some of them inhibit initiation of the immune response and some also suppress the primary induction of cytotoxic effector cells. Others have been shown to inhibit T suppressor cell activity.

Rosette Inhibitory Factor (RIF)

This molecule is found only in patients with viral hepatitis [19] and was the first of the immunoregulatory lipoproteins to be discovered.
It is responsible for the defect in human T lymphocyte E rosette function seen in over half of the patients with acute viral hepatitis but disappears with clinical recovery [17]. RIF is a unique, abnormal serum lipoprotein [16] with distinct biophysical and biologic properties (Table 6). It inhibits E rosette formation in an active metabolic fashion and does not merely interfere with the binding of the T lymphocyte and the sheep red blood cell on a steric hindrance basis. Thus, RIF represents a prototype abnormality in the molecular immunoregulatory system which occurs in patients with viral hepatitis. Other abnormalities are likewise known to occur in this disease: a variety of major structural abnormalities in the plasma lipoproteins have been reported in viral hepatitis and other liver diseases [81] and alpha fetoprotein levels increase in association with hepatocyte regeneration in hepatitis [82]. It is reasonable to postulate, therefore, that certain manifestations of lymphocyte dysfunction observed in patients with viral hepatitis might be related to either hepatic induction of new abnormal regulatory molecules such as RIF or the quantitative and qualitative abnormalities in the hepatic metabolism of other normal molecules. The net effect of altered immunologic reactivity would then depend on the selective induction of specific abnormalities in the biosynthesis of individual immunoregulatory molecules and one would expect these to vary from patient to patient.

Liver Extract (LEx)

Another immunoregulatory molecule has recently been isolated from human livers [13]. This molecule is a protein with a molecular weight of approximately 65 000 daltons and has been shown capable of inhibiting the DNA synthetic response of peripheral blood lymphocytes to mitogens and allogeneic cell stimuli. In addition, it inhibits mitogen activated and basal protein synthesis and reduces immunoglobulin

Table 6. Biologic and biophysical properties of rosette inhibitory factor

Abnormal low density lipoprotein
Restricted to hepatitis A, B, and nonA/nonB virus infections
Apolipopeptides A_{II}, B, C_{III}
Binds to specific T lymphocyte receptors (2900/cell)
Extremely high binding affinity (1×10^{13} L/M)
Molecular weight 2.5×10^6 daltons
Inhibits E rosette function and the mixed lymphocyte response by an active metabolic process
Biologic half-life = 1.5 h

synthesis by pokeweed mitogen activated human peripheral blood lymphocytes. It also inhibits ongoing DNA synthesis indicating that it can modulate biologic events which are already underway.

If one postulates that LEx is released into the extracellular environment as a consequence of hepatocellular injury, it could modulate lymphcyte effector function in the immediate vicinity of adjacent target hepatocytes and thus limit the magnitude and extent of injury according to its concentration in situ. Since the yield of LEx from different livers all obtained and purified under identical conditions has varied over a 20–fold range [13], we suspect that intracellular concentrations of LEx may vary from individual to individual. If this is true, it is possible that the variable severity and focal nature of hepatocellular necrosis in viral hepatitis may be related to local and quantitative differences in the content and the concentration of LEx released during the initial phase of hepatocellular injury.

In recent experiments we have evaluated the effect of LEx on spontaneous and mitogen induced cellular cytotoxicity to test our hypothesis that the local release of LEx within the liver can affect the cytotoxic effector function of resident cytolytically active lymphocytes. LEx was found to inhibit SCMC and MICC at all effector: target cell ratios studied (Table 7). This supports our contention that the effector phase of cell mediated cytotoxicity is susceptible to modulation by hepatic immunoregulatory molecules.

Experimental Viral Hepatitis

The cells of the reticuloendothelial system have largely been neglected as potentially important factors in the pathogenesis of hepatocellular injury in hepatitis B virus infection. It is reasonable to suspect however, that the macrophage and in particular the Kupffer cell, may be involved for two reasons.

a) The macrophage is generally known to be vitally important in natural resistance to virus infections [70, 79].

b) The macrophage is known to determine the outcome of murine hepatitis virus infection in various inbred strains of mice.

The murine hepatitis viruses (MHV) are highly contagious RNA coronaviruses, and produce mild, usually asymptomatic hepatitis, in the wild. However, variant strains produce extensive hepatocellular necrosis in selected inbred mice. Weiser and Bang [5] have demonstrated that hepatocellular injury is linked to the

Table 7. Effect of liver extract on cytotoxic effector cell function

Spontaneous cell mediated cytotoxicity			
Effector/Target	Control (% Cytotoxicity	Liver extract	% inhibition
5	28	18	35.7
20	36	26	27.8
40	48	24	50.0
Mitogen induced cellular cytotoxicity			
Effector/Target	Control (% Cytotoxicity)	Liver extract	% Inhibition
5	40	22	45.0
20	65	54	17.0
40	69	59	14.5

Liver extract added to effector cells at 25 μg/ml for 1 h prior to addition of target cells (Chang cells). In the MICC assay phytohemagglutinin at a final concentration of 4 μg/ml was added to the effector cells simultaneous with the target cells. Results are expressed as percent chromium-51 released after 18 h incubation. Percent inhibition is calculated as follows:

$$1 - \frac{\text{Liver extract cpm}}{\text{control cpm}} \times 100$$

inheritance of a dominant gene that codes for macrophage resistance to viral replication [83]. Although the biochemical basis for variable macrophage susceptibility to infection has not been fully determined, we have identified the production of a serine protease by MHV-infected peripheral blood monocytes in susceptible strains but not in resistant strains of mice (G. Levy, unpublished observation).

The analogy between the variable susceptibility of murine and human populations to MHV- and HBV-induced hepatocellular injury is striking and suggests that similar pathogenic mechanisms may be operative. Because Kupffer cell hyperplasia is a prominent feature of acute viral hepatitis in man, the concept that phagocytic cells within the liver may be involved in the processing of hepatitis B virus cannot be ignored. Nonetheless, considerable differences also exist between MHV and HBV infection and over interpretation of the analogy must be avoided. However, further investigation into the role of the macrophage in human hepatitis B virus infection appears warranted.

Discussion

It should be obvious from the foregoing that the pathogenesis of hepatocellular injury in viral hepatitis is unclear. There is a considerable body of knowledge that indicates that assorted aberrations of the immune response are characteristic accompaniments of hepatitis B virus infection and hepatocellular injury. However, the precise role played by these immune parameters is entirely indeterminant at

present because of our current lack of knowledge about the biology of the virus and the cellular biology of the host-virus relationship.

Despite these handicaps it is possible to construct a hypothesis for hepatitis B virus infection and hepatocellular injury based on the assembly of a large variety of independent observations from separate laboratories over the last few years. This hypothesis is highly conjectural and should merely form the basis for additional experimentation rather than serve as a dogmatic interpretation of available information.

The events leading to a hepatitis B virus induced disease should be considered at two levels: First, the events responsible for parasitization of the hepatocyte by HBV; second, the events which lead to hepatocellular injury.

Following entry into the body the virus must gain access to the hepatocyte before replication and hepatocellular injury can occur. The reticuloendothelial system, and in particular the intrahepatic fixed macrophages (Kupfer cells), may conceivably play a pivotal role in removal of infectious *virions* from the circulation before they gain access to hepatocytes. Indeed, it is likely that in most instances of HBV infection the reticuloendothelial system adequately destroys the virus and protects the hepatocyte from viral parasitization and subsequent disease. This suggestion is compatible with the relatively high frequency of subclinical or inapparent hepatitis B virus infection in most human populations. If the viral inoculum is sufficiently large to overwhelm the reticuloendothelial system or if the macrophage is unable to adequately neutralize the virus, then hepatocyte infection and disease may follow.

Alternatively, macrophage functions other than phagocytosis may also influence the infection process. Based on the genetically-determined macrophage-mediated variation in susceptibility to murine hepatitis virus infection and the identification of a biochemical correlate of this genetically determined macrophage heterogeneity, it is conceivable that macrophage activation products, such as serine proteases, might alter the hepatocyte surface membrane so as to render it either more or less capable of binding and internalizing infectious viral particles. Additionally, other macrophage secretory products might conceivably be directly toxic to hepatocytes such that viral induced macrophage activation might result in hepatocellular injury and necrosis irrespective of the coexistent presence of the viral genome and gene products within neighboring hepatocytes.

If the mechanisms which produce hepatocellular injury are initiated because of viral genome incorporation within hepatocytes rather than by non-specific activation of neighboring macrophages, then a complex sequence of events may be responsible for the ultimate production of hepatocytolysis. Hepatocellular injury may result from cellular attack systems which are specific for surface membrane autoantigens such as LSP or for viral antigens if and when they are expressed at the hepatocyte surface. The specific cytolytic effector systems which may be responsible for hepatocytolysis cannot currently be identified. Indeed, the specific target antigen is unknown and the requirements for effective cytolytic activity in vivo are unclear. If T cell mediated cytotoxicity is important in this process, the tenets of the genetic restriction hypothesis would have to be fulfilled. Identification of this pathogenetically important phenomenon with in vitro assay systems must therefore await the

development of a target cell which expresses both the relevant target antigens and shares the necessary histocompatibility loci with the effector cells.

The emergence of anti-hepatocyte, cell-mediate immune reactivity appears to be normally precluded by powerful suppressor influences which serve to maintain the state of tolerance towards hepatocyte antigens. It appears as if activation of normally suppressed cytolytic effector functions may be a consequence of a dysfunctional state of immunologic homeostasis due to an imbalance in immunoregulatory molecules normally synthesized by the liver. Since the biosynthesis of many of these molecules depends upon normal hepatocellular function and since qualitative and quantitative abnormalities of the molecular immunoregulatory system are known to occur during viral hepatitis (e. g. RIF), it is reasonable to postulate that viral genome incorporation into hepatocyte chromosomes results in dysfunctional immunoregulatory metabolism and secondary inhibition of normal suppressor cell function. Indeed we have shown (Fig. 2) that molecules which inhibit suppressor monocyte activity are detectable in the circulation of patients with acute viral hepatitis. Conceivably, they might allow the emergence of a normally suppressed cellular immune response to host or viral antigens on the hepatocyte surface resulting in hepatocellular injury.

Two mechanisms might be responsible for either the resolution of the disease process or its continuation in a chronic form. First, hitherto unrecognized events may suppress viral genome expression permitting the resolution of dysfunctional hepatocellular biosynthetic activity restoring hepatic metabolism of immunoregulatory molecules to normal. This would then the reimposition of normal immunologic homeostasis with the reconstitution of the suppressor cell population. Precedent for viral genome suppression is found in the model systems of measles and herpes virus infection developed by Joseph and Oldstone [50] and Stevens and Cook [85].

The second mechanism is based on our observation that hepatic LEx concentrations appear to vary from person to person [13]. If LEx is released into the extracellular intrahepatic microenvironment, as a consequence of cell mediated hepatocellular injury, it could inhibit local cytotoxic effector cell activity. Depending on the original hepatocellular concentration of LEx, cytotoxic effector cells at the site of injury would be inhibited to varying degrees. This might result in the range of well-known clinical syndromes characteristic of hepatitis B virus infection. Additionally, inhibition of cytotoxic effector cell activity within the liver might result in the focal distribution of injury also characteristic of viral hepatitis.

Conclusion

In conclusion, until the cellular biology of hepatitis B virus infection is understood and until the technology for the development of suitable virus infected autologous target cells is available, it will not be possible to definitively establish the mechanism involved in the pathogenesis of hepatitis B virus induced hepatocellular injury. Clearly, considerable effort must now be focused on the basic biology of virus infection, replication, and effects on hepatocellular metabolism before it will be possible to perform the definitive experiments to elucidate the role played by the immune system in the pathogenesis of this disease.

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Immunopathogenesis of the Extrahepatic Manifestations of Hepatitis B Virus Infection

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Introduction

Our understanding of the pathogenesis of liver disease associated with acute and chronic hepatitis B virus (HBV) infection remains limited; hypotheses which implicate host immune responses to the virus or virus-altered hepatocyte membranes are speculative and insufficiently supported by experimental observations. In contrast, participation of the host's immune response in the pathogenesis of several extrahepatic features of HBV infection appears to be established more firmly; these syndromes are reminiscent of experimental and naturally occurring "immune complex" diseases. From the study of these syndromes and their comparison with animal models of immune complex injury, we have made progress in understanding the pathogenesis of these extrahepatic features; however, many important questions about the association among HBV, immune complexes, and extrahepatic tissue injury remain unanswered.

Extrahepatic Syndromes Associated with HBV

The most frequently encountered extrahepatic disorder associated with HBV infection is the *prodromal arthritis-dermatitis syndrome* [2, 4]. In 10–20% of patients with acute hepatitis type B, transient, prodromal arthritis or arthralgias, rash, fever, and occasionally angioedema develop. Arthritis and/or arthralgias occur several days (rarely weeks) before the onset of jaundice and sometimes are unaccompanied by jaundice or other obvious signs of liver disease [79, 80]. The articular manifestations are almost indistinguishable from those of acute rheumatoid arthritis and are characterized by nonmigratory, symmetrical polyarthritis/arthralgias affecting primarily distal joints of the extremities, occasionally larger appendicular joints, and rarely the axial skeleton. Morning stiffness is also common. The

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joint manifestations are frequently accompanied by a similarly transient skin rash, most commonly urticarial or maculopapular, but occasionally purpuric and petechial lesions and, even less frequently, target-like lesions resembling erythema multiforme [15, 43, 44, 79, 85, 91, 92]. Not uncommonly, rash may be the sole extrahepatic feature of HBV infection [79]. As the acute episode of hepatitis evolves, the prodromal arthritis and rash resolve without sequelae. On the other hand, both articular and dermatologic features have been observed in patients with chronic HBV infection [89]. In these cases the extrahepatic features may appear intermittently or persist indefinitely. Still, joint destruction, if it occurs at all, is very rare.

In patients with prodromal arthritis, immunologic investigations have shown depressed serum complement levels associated with high titers of hepatitis B surface antigen (HBsAg) [4]. As arthritis resolves, complement levels return to normal and HBsAg titers fall [4, 5]. In addition, these patients may have depressed synovial fluid complement levels, and HBsAg can be detected in their synovial fluid [63]. This prodromal syndrome associated with acute hepatitis type B has been characterized further by the demonstration of circulating immune complexes (CIC) containing HBsAg and anti-HBs [15, 50, 90]; deposition in synovium [76] and cutaneous vessel walls [15, 91] of immunoglobulins, complement, and HBsAg; and the presence of cutaneous vasculitis [15, 70, 91]. Similar immunologic features have been described in patients with articular or dermatologic manifestations of chronic HBV infection [89]. Very recently a dichotomy has been reported between the dermatologic features of acute and chronic hepatitis. In patients with acute HBV infection, urticarial and maculopapular lesions are the rule and morphologically consist of a primarily lymphocytic venulitis with focal necrosis. In contrast, patients with rash associated with chronic hepatitis are more likely to have palpable pupura, which histologically is a primarily neutrophilic necrotizing vasculitis involving small vessels [30, 70].

In contrast to the relatively benign arthritis-dermatitis syndrome which is associated predominantly with acute HBV infection, other extrahepatic syndromes occur characteristically in persons with chronic HBV infection and tend to be more serious, even life-threatening. The first of these to be recognized was *systemic necrotizing vasculitis* or, as it is more frequently labeled, polyarteritis nodosa (PAN) [28, 29]. Approximately 30–40% of all patients with this disorder are infected with HBV, as documented by the presence of HBsAg in their circulation. The vasculitis in HBsAg-positive persons, indistinguishable from that in HBsAg-negative persons, has a variable relationship to liver disease and may occur either prior to, coincident with, or following the onset of liver disease. Although all varieties of liver involvement have been recorded, chronic active hepatitis and chronic persistent hepatitis are the most frequently encountered [20, 28, 29, 77]. Typical of any patient with PAN, these patients with HBsAg-positive PAN present initially with the acute onset of fever, rash, polyarthritis, and polyarthralgias, but eventually progression to a multisystem diffuse necrotizing vasculitis ensues, with involvement of the gastrointestinal, renal, and peripheral and central nervous systems. As in classical PAN, the pathologic lesions of affected organs are characterized by fibrinoid necrosis and perivascular infiltration of medium and small arteries and arterioles; in some patients the vascular lesion more closely resembles a hypersensitivity angiitis.

Response to immunosuppressive/anti-inflammatory therapy in HBsAg-positive patients with PAN is as unpredictable as in HBsAg-negative patients.

During active vasculitis, levels of serum and synovial complement in these patients are low; HBsAg can be detected in synovial fluid; vascular deposits of HBsAg, immunoglobulins, and complement have been demonstrated along internal elastic laminae of affected arteries and in lymphoid organs [28, 29, 53]; and circulating immune complexes, at levels correlating with disease activity, appear [26]. In inactive, scarred lesions, viral and host humoral components are not detectable, and in some patients the disease becomes quiescent with clearance of HBsAg [53, 87]. In short, this syndrome appears to be the first example in man of a chronic rheumatic disease caused by a viral infection.

Another extrahepatic feature that has been associated with chronic HBV infection and that has received growing attention is *glomerulonephritis* (GN), which occurs in the setting of either clinically recognized or covert chronic liver disease and occurs as an isolated manifestation or as a component of a more generalized vasculitis. Although membranous GN is the lesion most frequently encountered, membranoproliferative and diffuse proliferative lesions have been described as well. As is true for the extrahepatic disorders described above, there is evidence that GN associated with HBV infection is mediated by glomerular deposition of HBV antigen-antibody complexes. This view is supported by the following observations: Demonstration of circulating HBsAg-anti-HBs immune complexes; deposition of HBsAg, immunoglobulins, and complement components in glomeruli; the presence of elutable anti-HBs, at a titer greater than that in serum, in glomeruli; visualization of dense deposits, similar to those seen in other immune complex diseases, in a thickened glomerular basement membrane; and demonstration of virus-like particles, some of which bear HBsAg immune reactivity, within glomeruli [8, 14, 35, 36, 64]. Whereas these investigators have detected HBsAg within glomeruli, more recently others have demonstrated HBeAg in glomeruli [81], while still others have detected HBcAg in glomeruli of HBsAg-positive children with GN [59].

Although the association between HBV and GN has been accepted, the frequency of the association has been a matter of controversy. The presence of stainable HBsAg was detected in 35% of unselected renal biopsies from Polish children with GN or the nephrotic syndrome. Moreover, the frequency of glomerular deposition of HBsAg was even higher, 56%, when biopsy specimens were preselected to include only those with glomerular deposits of immunoglobulins and complement [8]. Frequencies of this magnitude, however, have not been observed in other European countries [39, 57] or in Japan [82], and in the United States the estimated frequency of HBV infection in patients with GN is approximately 1% [36]. In a recent report from France, 42% of 33 children with membranous nephropathy had HBsAg detectable in serum but *not* in renal tissue [34]. Unfortunately, the differences among these reported frequencies and these conflicting findings have never been explained satisfactorily. Moreover, the validity of the association between HBV and GN has been questioned by a group of investigators who could not detect HBsAg in kidney tissue of HBsAg-positive patients with GN but who did detect HBsAg in glomeruli of HBsAg-negative patients [38]. False-positive staining for HBsAg was found to be secondary to binding of the Fc fragment of the fluoresceinated anti-HBs IgG to IgM rheumatoid factor deposited

in the glomeruli. It is also difficult to understand how an immune complex large enough to contain HBV particles or even HBV polypeptides could migrate to the subepithelial surface of the glomerular basement membrane. Furthermore, the lack of unanimity about the HBV antigen involved in GN, HBsAg [8, 14, 35, 36, 64], HBcAg [59], or HBeAg [81] adds to the confusion about this extrahepatic disease manifestation.

There are other extrahepatic manifestations of HBV infection as well. A relatively well-documented and exhaustively studied patient was described who had chronic active hepatitis type B and disabling *peripheral neuropathy* [22]. Beyond this one case, however, there have been no additional reports of isolated peripheral neuropathy associated with HBV infection; perhaps this is a forme fruste of systemic necrotizing vasculitis (PAN). In addition, several investigators have reported an association between HBV infection and *polymyalgia rheumatica* [6] as well as *essential mixed cryoglobulinemia* [41]. These associations, however, have not been accepted universally and remain controversial, as detailed below. Finally, isolated reports have appeared in which HBV infection was associated with a variety of myopathies [7, 54, 58, 65, 67] and cardiopulmonary disorders [1, 9, 24, 55] (see below), but studies of pathogenesis were not attempted.

Pathogenesis of Extrahepatic Features of HBV Infection

The clinical syndrome, sequence of immunologic events, tissue deposits, and tissue injury associated with the prodromal arthritis-dermatitis syndrome of acute HBV infection resemble those seen in acute "one-shot" experimental serum sickness in rabbits [11, 18]. Indeed, this constellation of prodromal features is referred to commonly as a "serum sickness-like" syndrome. In the animal model, one or several closely spaced large injections of a heterologous serum protein is administered to a rabbit. If the antigen persists sufficiently long in the rabbit's circulation for antibody to appear, if there is protracted interaction between antigen and antibody in the circulation, and if other conditions as detailed below are appropriate, antigen and antibody form complexes which circulate and deposit in tissues, which are injured as innocent bystanders. In experimental acute serum sickness, injected antigen is cleared from the circulation in three discrete phases. First, equilibration of antigen concentration occurs between the intravascular and extravascular compartments. During this period, which lasts approximately two days, there is a rapid but limited fall in the intravascular antigen concentration. Second, slow non-immune elimination occurs, lasting on the order of one week, as a result of normal catabolism of the protein by unknown mechanisms. Third, once sufficient antibody has appeared to allow formation of antigen-antibody complexes of the appropriate composition, rapid and precipitous "immune elimination" occurs. This phase is brief, lasting approximately two days, and is accompanied by a depression in serum complement levels and the development of lesions in the kidneys, arteries, joints, and endocardium of the injected animal. Within these lesions, concentrated antigen (relative to serum), host antibody, and complement can be identified by immunohistochemical techniques. Once sufficient antibody is present to achieve a state of antibody excess, free antibody alone is detectable in the circulation, complement

levels return to normal, and tissue lesions resolve. In many details, therefore, the prodromal serum sickness-like syndrome of acute HBV infection is analogous to experimental one-shot serum sickness.

In one-shot acute serum sickness, the host experiences exposure of brief duration to a large concentration of antigen, and the tissue lesion, which involves vessels in a variety of organs, is characterized by polymorphonuclear leukocytic infiltration and proliferative endothelial lesions with necrosis. If, on the other hand, experimental conditions are changed, a different syndrome develops. When small doses of antigen are administered daily over an extended period, if the host's immune response is appropriate, *chronic* serum sickness may ensue [17]. In this setting, generalized arteritis does not occur; instead the lesions are confined to glomeruli, and distinct from those of acute serum sickness are characterized by a mononuclear infiltrate associated with hyaline degeneration of vessel walls. What begins as a membranous glomerulonephritis progresses to a proliferative lesion with irreversible scarring.

In addition to experimental chronic serum sickness, there are models in nature of chronic immune complex disease, and in these, lesions are not limited to glomerulonephritis. Chronic immune complex diseases have been described in such viral infections of animals as lactic dehydrogenease virus; lymphocytic choriomeningitis virus; Maloney sarcoma virus; Gross, Friend, and Rauscher leukemia viruses of mice; Aleutian mink disease; polyoma equine anemia; hog cholera; and the immune complex disease of NZB mice. In these diseases, persistent infection with viruses of low pathogenicity occurs, generally in the setting of low levels of host antibody. Immune complexes consisting of virus antigens and host immunoglobulins circulate throughout life and deposit not only in glomeruli, where lesions are most pronounced, but also in medium-sized arteries, and the choroid plexus [13, 62]. In most of these diseases, free antibodies to viral antigens do not circulate but can be eluted from tissue deposits of immune complexes, which also contain viral antigens and complement components. In many respects, the extrahepatic syndromes associated with *chronic* HBV infection are reminiscent of experimental chronic serum sickness in rabbits and the naturally occurring viral immune complex diseases of animals. Unlike most human diseases with features suggestive of an immune complex pathogenesis in which the nature of the antigen is unknown, the viral immune complex diseases of animals and the extrahepatic features of HBV associated with chronic immune complex injury are attributable to CIC of defined antigenic composition. In these diseases, viral antigens complexed with antibody deposit in blood vessels and glomeruli, where the specificity of virus antigens and antibodies can be demonstrated. Moreover, like some of the viruses of naturally occuring immune complex disease in animals, HBV is of limited pathogenicity [21] and has a tendency to persist chronically in its host.

On the other hand, our understanding of why or how immune complex-mediated injury occurs in HBV infection is limited. In addition to studies cited above which demonstrate vascular deposition of HBsAg-containing immune complexes in affected organs, there is a wealth of data supporting the frequent occurrence of CIC in acute and chronic HBV infection. Employing techniques to identify non-antigen-specific CIC [61, 83] as well as techniques to identify HBsAg-containing CIC [3, 37, 46, 47, 50, 66, 73, 78, 83, 88–90], investigators have demonstrated such complexes in patients spanning the entire clinical spectrum of

HBV infection, usually in the absence of extrahepatic features [72]. Although some investigators reported qualitative differences in cryoprecipitates between patients with and without extrahepatic features [89, 90], others detected cryoprecipitates with features of immune complexes in patients without extrahepatic manifestations [50, 86]. Similarly, although transient complement activation has been described in patients with extrahepatic features of HBV infection [4, 15, 89, 90], it has been found also in the absence of extrahepatic features [71, 84]. Thus, as in so many other infectious and inflammatory diseases, HBV infection is frequently accompanied by detectable CIC. Why these complexes are of no consequence in most patients remains obscure. The biologic activity of HBsAg-anti-HBs complexes has been demonstrated by the production of cutaneous injury in experimental animals injected with these complexes [49, 60]. Moreover, circumstantial evidence that deposition of HBsAg-anti-HBs complexes can induce tissue injury comes from one report of progressive focal glomerulonephritis in two baboons which had received injections of HBsAg-rich plasma [31] and another report of lowered HBsAg titer and hematuria in patients with HBsAg-positive chronic active hepatitis who had been infused with high-titer anti-HBs [75].

Some insight into the factors which favor immune complex injury in HBV infection may be derived indirectly from studies of experimental serum sickness. Among these are size and composition (intermediate size in moderate antigen excess), antigen or antibody valence (lattice structure), antibody affinity, duration of antigen exposure, antibody subclass (complement fixing immunoglobulin subclasses), the presence of rheumatoid factors (which increase the size of complexes), the functional status of the mononuclear phagocyte (reticuloendothelial) system (if impaired, large complexes which would ordinarily be cleared could deposit in vessel walls), and the capacity for local increases in vascular permeability by vasoactive amines (required for trapping of immune complexes in vessel walls to occur) [11, 12, 17, 48]. If we extrapolate to the case of acute and chronic HBV infection, the intricacy of the interaction among virus, host antibody, and the mononuclear phagocyte system are such that kinetics of antigen and antibody development would vary considerably from person to person. Therefore, differences in duration of antigenemia, in antigen-antibody ratios, in impairment of mononuclear phagocyte function, etc., could distinguish between those patients with CIC who do and those who do not experience clinically apparent immune complex injury. Unfortunately, quantitative studies to substantiate this speculation have not been done. Similarly, there may be differences among individuals in immune responsiveness to HBV, but none have been identified.

Controversial Associations with HBV Infection

In contrast to the generally accepted associations between HBV on the one hand and the prodromal arthritis-dermatitis syndrome, systemic necrotizing vasculitis, and glomerulonephritis on the other, associations between HBV and two other syndromes with clinical features of an immune complex disease, polymyalgia rheumatica and essential mixed cryoglobulinemia, have been challenged.

In the first report associating HBV and polymyalgia rheumatica, Bacon et al. [6] detected anti-HBs in the absence of HBsAg in nine of twelve patients, compared to in only one of 12 controls. This observation in Great Britain was followed very shortly by conflicting data from the United States, where 15 patients were evaluated, and the prevalence of HBV antibodies was found to be no greater than excepted prevalences for a population of comparable age [42]. More recently another report, originating from Switzerland, claimed an increased prevalence of anti-HBs in patients with polymyalgia rheumatica and temporal arteritis [56], and a report from France described a woman with polymyalgia rheumatica and HBsAg in serum. This patient had deranged indices of liver function and a superficial temporal artery biopsy which, though demonstrating a histologically normal vessel, stained for IgM, C3, and HBsAg [68]. The hypothesis that polymyalgia rheumatica can be an HBV immune complex-mediated disorder has not been accepted universally, however. Unlike other diseases in which immune complexes are presumed to be pathogenic, polymyalgia rheumatica is *not* associated with depressed serum complement levels. Specificity of HBsAg staining was not described in the report from France [68], and the significance of immune deposits in a histologically normal vessel is also unclear. Moreover, in the original report by Bacon et al. [6] the disappearance of serum anti-HBs in more than half the patients within six months of acute illness defies conventional explanation. The controversy is far from resolved. Do the tests for anti-HBs and HBsAg represent HBV as a primary agent in the pathogenesis of the disease, or is HBV contracted coincidentally in this population as a result of enhanced exposure?

Similarily, for essential mixed cryoglobulinemia (EMC), an association with HBV has been suggested [40, 41, 74] and contested [16, 69]. Essential mixed cryoglobulinemia is a disorder of unknown etiology characterized clinically by arthralgias and/or arthritis, purpura, weakness, glomerulonephritis, and generalized vasculitis [45, 51, 52] and immunologically by mixed cryoglobulinemia (a mixture of cryoglobulins of two or more immunoglobulin classes), deposition of immunoglobulins and complement in blood vessel walls and glomeruli, and frequently depressed serum complement levels. The disorder is suspected in patients with these clinical and immunologic features in the absence of infection, neoplasm, or a well-defined connective tissue dirsorder. Realdi et al. [74] suggested that there might be an association between EMC and HBV when they detected HBsAg in serum and cryoprecipitates obtained from six of 12 patients with EMC. Five of the six also had anti-HBs in their serum and cryoprecipitates, and these investigators provided evidence to suggest that HBsAg-anti-HBs immune complexes in EMC were in a state of relative antibody excess.

These findings were extended by Levo and colleagues [40, 41] who described liver involvement in 26 of 30 patients and reported the presence of HBsAg in three of 25 serum samples and in six of 19 cryoprecipitates, and anti-HBs in 12 of 25 serum samples and 11 of 19 cryoprecipitates. Seventy-four percent of cryoprecipitates contained HBsAg, anti-HBs, or both, and four cryoprecipitates examined by electron microscopy contained particles said to resumble HBV. In contrast, among 49 patients with rheumatoid arthritis or systemic lupus erythematosus, none had HBsAg and only 12 (24%) had anti-HBs in serum. Both Realdi et al. [74] and Levo et al. [40, 41] postulated that the occurrence of immune complexes primarily in

antibody excess was more likely to initiate EMC than complexes in antigen excess, which had been associated with other extrahepatic syndromes of HBV infection.

Prompted by these findings associating HBV with yet another chronic immune complex disease, Popp et al. [69]. evaluated a group of EMC patients and compared them to a comparably-aged control group with mixed cryoglobulinemia secondary to well-defined disease processes, "secondary mixed cryoglobulinemia". Although these EMC patients were similar in non-hepatic clinical features to those described by Levo et al., Popp et al. [69] found little evidence for liver disease or HBV infection in their study population. Two of the 12 patients had abnormalities of liver function or histology, but so did a comparable proportion of controls with secondary mixed cryoglobulinemia. None of the 12 serum samples from EMC patients contained HBsAg or anti-HBs, while five of 22 patients with secondary cryoglobulinemia had HBV markers. Even in cryoprecipitates, Popp et al. [69] did not detect HBsAg by radioimmunoassay or HBV particles by electron microscopy but did detect anti-HBs in a sample from one of the two patients with liver disease. In this patient, however, the onset of liver disease followed the onset of EMC by two years, suggesting that liver disease, perhaps related to HBV, occured as a coincidental event and was not involved in the pathogenesis of EMC. Moreover, there was more evidence of HBV infection in cryoprecipitates from patients with secondary mixed cryoglobulinema, a group which included patients with a variety of neoplastic, inflammatory, and infectious diseases unlikely to have been caused by HBV. In these patients, HBV infection occurred more likely as a consequence of exposure to blood products, hospital environments, and the like. In fact, even the control group described by Levo et al. [41] had a higher prevalence of anti-HBs than a comparable normal population would have been expected to have. Conceivably, their control group of patients with systemic lupus erythematosus and rheumatoid arthritis would have had an even higher prevalence of HBV markers had their cryoprecipitates been examined. Because these two diseases are not caused by HBV, the high anti-HBs prevalence in these patients probably reflects increased exposure to HBV in this "control" population. Therefore, the importance of the high prevalence of HBV serologic markers in the EMC patients from the same institution is open to question.

A plausible explanation for the difference between the results of Levo et al. [40, 41] and Popp et al. [69] may be a semantic one. Many patients with chronic liver disease have mixed cryoglobulinemia [16, 19, 22, 23, 25, 32, 33, 50, 89, 93]; in many such patients, underlying liver disease is not overt, and many have extrahepatic features such as purpura, arthralgias, Raynaud's phenomenon, and even glomerulonephritis. In short, many of these patients with chronic hepatitis have extrahepatic features which resemble the clinical features of EMC. Conceivably, then, some of the patients with EMC described by Levo et al. [40, 41] could have had primary liver disease and secondary cryoglobulinemia and would have been classified as patients with secondary mixed cryoglobulinemia, not EMC, by Popp et al. [69], whose patients with EMC rarely had liver disease.

In addition to questions about the importance of CIC in patients with HBV infection in the absence of extrahepatic disease and to the controversy about the association between HBV infection and both polymyalgia rheumatica and EMC, there are other extrahepatic disorders associated with HBV infection the patho-

genesis of which remains unexplained. These include such hematologic complications as aplastic anemia [10]; such neuromuscular disorders as Guillain-Barré ascending paralysis [58], seizures [7], dermatomyositis [67], polymyositis [54], and lipid storage myopathy [65]; such cardiopulmonary complications as myocarditis, pericarditis, pleuritis, and fibrosing alveolitis [1, 9, 24, 55]; and the nonvasculitic cutaneous lesion, papular acrodermatitis of childhood [27].

Conclusion

The association between HBV, immune complexes, and extrahepatic tissue damage has been studied intensively. Still, many links are missing in our understanding of this complex set of interactions. Although the conclusion is inescapable that several extrahepatic features of HBV are mediated by immune complex injury, several of the associations are debatable, and we know very little about how and why such injury occurs. In most instances, the presence of circulating immune complexes in HBV infection is probably of no pathogenic importance but may be related to normal mechanisms of immune clearance of virus antigens.

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Primary Liver Cancer and Its Relationship to Chronic Infection with the Hepatitis B Virus

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Introduction

Evidence from numerous studies published since 1970 [12, 33, 36, 43] indicates a clear-cut association between primary liver cancer (PLC) and chronic infection with the hepatitis B virus (HBV). This association is currently believed to represent a causal relationship and is specifically restricted to hepatocellular or liver-cell carcinoma (HCC), which accounts for more than 80% of all primary liver tumors [9]. Cholangiocarcinoma or bile-duct carcinoma, the second malignant tumor grouped under the broader term of PLC, is not associated with chronic HBV infection and will not, therefore, be considered in this article. It must, however, be noted that in many studies, particularly of epidemiologic character, no distinction between HCC and cholangiocarcinoma is made and the non-discriminating term of PLC is used. Even so, data from such studies have not concealed the fact that the association with HBV specifically concerns hepatocellular cancer and not all PLCs [49].

Primary liver cancer is one of the commonest forms of cancer affecting man. Though rare in most European countries, in the USA, and the rest of the Western world, it is a major cause of death in some of the most populous areas of the earth, i.e., China, South-East Asia, and Sub-Saharan Africa [51]. In the same areas the prevelance of chronic HBV infection in PLC is very high indeed ranging from 40 to 95% [4, 9, 43]. The role, therefore, of the HBV in HCC should not be viewed as a minor problem, limited to a rare form of cancer, but as a major issue and challenge in cancer etiology and prevention. In this context significant emphasis is given in this article to the analysis of data supporting the notion of a causal link between HBV and HCC and to the discussion of possible pathogenetic mechanisms in HBV-related hepatocarcinogenesis.

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Data reviewed in this article cover several aspects of the above mentioned association of HBV with HCC such as clinical, laboratory, epidemiologic, immunochemical, virological, and biomolecular. These have been grouped into the following five categories: (1) chronic HBV infection in PLC, (2) clinical and laboratory aspects of "HBV-related HCC", (3) the causal role of HBV in HCC, (4) possible pathogenetic mechanisms in hepatocarcinogenesis related to HBV, and (5) possibilities in prevention of HBV-related HCC.

Chronic HBV Infection in PLC

The association between PLC and HBV, first reported in 1970 among Mediterranean patients with hepatocellular cancer [12, 36], is now well established and has been confirmed in studies from most parts of the world. Several authors have reviewed the available information, which clearly indicates that in HCC but not in cholangiocarcinoma markers of HBV infection are much more frequently present than in suitable controls [9, 30, 33, 42, 43, 48]. However, there is a certain amount of confusion as to whether this association refers to active HBV infection or if it covers past HBV infection as well, and as to whether it concerns only HCC developing as a result of cirrhosis or if it also extends to HCC unassociated with cirrhosis. Moreover, there are differing views on the significance of the presence of antibodies to the hepatitis B core antigen (anti-HBc) alone in the serum and accordingly to its interpretation in relation to activity of HBV infection.

Current views on the interpretation of several markers, widely used in the study of HBV infection, can be summarized as follows:

Current (active) HBV infection is marked by the presence of the hepatitis B surface antigen (HBsAg) in the serum and liver, by the presence of hepatitis B core antigen (HBcAg) in the liver, and by high titers on anti-HBc in the serum. In practice the serum HBsAg appears to be the most sensitive and easily detectable HBV marker. It must, however, be emphasized that since the amount of circulating HBsAg in HCC is frequently low, highly sensitive techniques such as solid phase radioimmunoassay (RIA) have to be used for its detection.

Previous HBV infection is marked by the presence of circulating antibodies to HBsAg (anti-HBs), usually in association with anti-HBc. Low titers of anti-HBc alone indicate previous rather than active HBV infection [9, 16, 19].

Interpretation of available data on the relationship of HBV infection to PLC permits the following general remarks:

1. There is a strong association between HCC and current HBV infection all over the world [30, 43, 45, 48]. In the vast majority of serologic studies the prevelance of HBsAg in PLC has been found to be much higher than in controls and in some countries figures up to 80 and even 90% have been reported (Table 1). Since the association is restricted to HCC, inclusion of cases of cholangiocarcinoma may have resulted in the underestimation of the association and this may be particularly true for some areas of South-East Asia where cholangiocarcinoma, included under PLC, is much more frequent than in the rest of the world. The association between HBV and HCC concerns not only cases developing on the grounds of cirrhosis but also of HCC unassociated with chronic liver disease (CLD). It appears, however,

Table 1. Prevalence of HBsAg in HCC patients and controls and relative risks in selected countries from four continents. From data reviewed by Hadziyannis [9]

Country	Prevalence of HBsAg%		Crude relative risks
	In HCC	In controls	
A. *Africa*			
Senegal	61.2	11.3	12
Mozambique	66.0	14.0	12
S. Africa	59.5	9.0	15
Uganda	47.0	6.0	14
B. *Asia*			
India	55.0	2.5	42
Vietnam	80.3	24.5	15
Japan	45.9	4.0	20
Taiwan	90.0	20.0	36
C. *Europe and USA*			
Greece	55.0	5.0	23
Great Britain	16.0	0.2	95
USA	26.0	0.25	140

that in the latter group the prevelance of HBV infection is comparatively lower than in the former [18, 25, 34, 48].

2. The prevelance of anti-HBc alone in HCC has been found to range from 0–26.5% [17, 20, 48] but neither its prevelance in matched controls nor its titers have been always properly investigated. Therefore, its contribution to the study of the association between HCC and current HBV infection is as yet limited [9].

3. There is no association between HCC and previous HBV infection at least in countries with high or intermediate PLC incidence, where the described relationship with active HBV infection is striking [9, 48].

4. There is mounting evidence that the association of chronic HBV infection with HCC preceeds the development of HCC and this is further supported by vertical studies of patients with HBsAg positive CLD without HCC, who after years of observation, have been found to develop HCC [15, 21].

The above observations indicate the existence of a clearcut relationship between ongoing (active) HBV infection and the development of HCC which appears to be particularly strong in countries with high PLC incidence. Although the meaning of this relationship in terms of pathogenesis of HCC is still unknown, chronic HBV infection can now be definitely accepted as a significant risk factor of HCC, and HBsAg positive patients with HCC can be reasonably grouped under the proposed term of "HBV-related HCC".

Clinical and Laboratory Aspects of "HBV-Related HCC"

Before discussing the evidence suggesting some etiologic role played by HBV in HCC, I will review some data on the clinical and laboratory aspects of the disease and on "early" diagnosis. It is of course possible that "HBV-related" HCC may not

differ at all from HBV-unrelated cases. However, since the "HBV-related" HCC may indeed represent an etiologically homogeneous and distinct form of the disease it is worth trying to attempt its clinical description. This may well serve as a basis for future comparisons with other etiologic forms of HCC.

Age and Sex

There is a striking geographical variation in the age distribution of "HBV-related" HCC. In Greece the mean age is 61.3 years, in Japan 57.1, in South Africa (Bantu) 36.1, and in Uganda 33.9 years [9]. These age differences may be related to acquisition of HBV infection in the tropics at a younger age but also to geographical differences in the natural course of HBsAg positive chronic liver disease (CLD), with an earlier termination into HCC in the tropics. It is also noteworthy that in a given area patients with HBsAg positive HCC are somewhat younger compared to those with HBsAg negative tumors. In Greece the difference in mean age between HBsAg positive and negative cases is four years and in Uganda nine years [25, 50]. This again fits with a relative earlier development of HCC in the course of HBsAg positive than negative CLD and with a decreasing HBsAg prevalence in HCC with age, observed by several authors [26, 34].

There is a striking male predominance, the male/female ratio of HBsAg positive cases ranging from 5/1 to 17/1 [9]. It also appears that in a given area the male/female ratio is higher in the HBV-related than in the unrelated cases.

History of Hepatitis and of Chronic Liver Disease (CLD)

Data from the history of the patients are not very contributory. A history of hepatitis is present in less than 20% of the cases [5, 9, 34, 48]. Acquisition therefore of HBV infection and transition to chronicity apparently occurs in these patients in a subclinical way. It is nevertheless true that in the majority of "HBV-related" HCCs there is an underlying well-developed CLD, most frequently macronodular cirrhosis. This is true for about three quarters of the cases all over the world [28, 37]. In some cases the pre-existing CLD may be clinically overt but in the majority of the cases it represents a rather mild and clinical latent type of disease. Thus, more than three quarters of HBsAg positive HCC patients in Greece first present with features related to the tumor itself rather than to underlying cirrhosis [12]. It must, however, be remembered that the proportion of these patients appears to differ from series to series and from country to country [9, 29, 43].

"HBV-related" HCC appears to be associated with CLD, particularly cirrhosis, rather more frequently than the HBsAg negative group of HCC. For example in South Africa the prevalence of cirrhosis in HBsAg positive HCC was 70% compared to 50% in the HBsAg negative HCC [18]; and in Greece 72% and 27.5% respectively [26]. HBsAg positivity has also been detected more frequently in HCC with than without cirrhosis [30, 48]; however, the absence of cirrhosis has not always been properly documented by detailed autopsy studies.

The existence and severity of CLD underlying the tumor appears to determine largely the clinical and laboratory features of HCC. In this respect it is noteworthy that underlying CLD in "HBV-related" HCC can be roughly divided into two types: (a) severe, usually postnecrotic cirrhosis with clinical features of liver failure

and/or of portal hypertension [29] and (b) inactive, usually incomplete septal cirrhosis, which is frequently clinically latent [37]. In a number of patients the underlying CLD may have not even reached the cirrhotic stage. In the second group the tumor can increase to a considerable size and this may occur even at the presymptomatic stage [21, 28].

Clinical Presentation

The clinical presentation of "HBV-related" HCC can be roughly grouped into three main categories [9]:
a) Presentation with symptoms related to the tumor itself rather than to an underlying CLD. Such patients may present with right hypochondrial pain, a palpable hepatic mass, weakness, weight loss, fever, etc. In this category the icteric and metastatic-type of HCC can also be included.
b) Presentation with symptoms of CLD particularly of cirrhosis. Patients in this category are either known cirrhotics with an otherwise unexplained rapid deterioration or have subclinical CLD which suddenly becomes apparent due to major manifestations such as development of ascites or esophageal bleeding.
c) Lack of relevant symptoms (occult type). In such cases HCC is diagnosed either at autopsy of an end-stage cirrhosis or it is incidently discovered at routine laboratory studies of patients with CLD as for example by serum a_1-fetoprotein (AFP) or by liver scan. In the last cases the detection of HCC is a preclinical one and clinical manifestations are expected to follow soon, though the clinically occult period may last as long as two and even three years [15].

The percentage of patients with "HBV-related" HCC that fits the above three categories of clinical presentation appears to vary significantly geographically [4, 26].

Laboratory Findings

Except for the presence of markers of current HBV infection no other laboratory findings specific for the "HBV-related" HCC have been hitherto described and no major differences from HBsAg negative cases have been observed. Serum AFP appears to be positive in 50 to 85% of the cases, being rather more frequently increased than in the HBsAg negative group [34, 35].

"Early" Diagnosis

Reports from Japan, Germany, and Greece [15, 21, 23] indicate that follow-up of patients with CLD by serial testing for serum AFP permits the diagnosis of developing HCC at a preclinical stage. In the Greek series 5.5% of the HBsAg positive patients developed a positive test annually. Their mean survival, without any treatment at all, was 12.5 months. A few cases were diagnosed more than two and even three years prior to clinical evidence of the developing tumor. It is, however, questionable if such patients can be helped by a radical therapeutic approach because in most cases the tumor appears to be ab initio multicentric [15]. Whether or not chemotherapy would be more effective at this earlier stage remains to be evaluated.

The Causal Role of HBV in HCC

Existing evidence, supporting the view that the link between HBV and HCC is a causal one is summarized in Table 2. In several recent articles this evidence, particularly the epidemiologic evidence, has been reviewed in detail [9, 43, 49]. It has been stressed that there is an impressive international correlation between the mortality from PLC and the prevalence of the HBsAg carrier state. In general the higher the incidence of PLC the higher the prevalence of the HBsAg carrier state. This is shown in Table 3 in which PLC mortality has been classified into three categories, i. e., high, intermediate, and low and has been compared to the respective prevalence of the HBsAg carrier state. It must, however, be noted that in certain countries like Egypt and Greenland there is a high rate of HBV infection but HCC is rather uncommon [9, 39].

The validity of this international correlation has been questioned [11] because of several reasons such as poor quality of existing epidemiologic data, lack of distinction between HCC and other primary liver malignancies particularly cholangiocarcinoma, differences in the populations compared and their ecologic conditions, differences in methology of HBsAg detection, etc. However, the same correlation was also observed in populations living under similar ecologic conditions studied by comparable methods. This has been clearly shown in two independant intracountry studies in Greece [25, 37] which have additionally shown [11] that the correlation between prevalence of HBsAg and PLC mortality is specifically restricted to mortality from HBsAg positive HCC; a finding supporting the notion that the association is probably of a causal nature.

The strength of the association between HBV and HCC has been further measured by estimation of the magnitude of the relative risk of HCC associated with chronic HBV infection [9, 43, 49]. In all studies high relative risks ranging from 12 to 95 and even more were reported (Table 1). Such high relative risks represent per se good evidence for a causal rather than a non-causal relationship. In addition to the high relative risk associated with active HBV infection, measurement of the actual risk (incidence) of HCC in the HBsAg carrier population has been attempted by Hadziyannis [9] on the basis of existing data on HCC incidence and of prevalence of

Table 2. Evidence for a causal link between hepatocellular carcinoma and the HBV

1. International correlation between mortality from PLC and prevalence of the HBsAg carrier state [9, 43].
2. Intracountry correlation between regional PLC-mortality and HBsAg carrier state [11, 25, 47].
3. Impressively high crude relative risks for development of HCC of persons with chronic HBV infection. See Table 1 [9, 43, 48].
4. Data on immigrant populations [25, 44].
5. Other evidence:
   a) More frequent and earlier development of HCC in HBsAg positive than negative chronic liver disease [15, 21, 23].
   b) Possible precancerous liver changes associated with chronic HBV infection.
   c) Detection of HBsAg in some well-differentiated HCCs, partial integration of the viral genome and HBsAg production by some cancer lines [1, 6, 27, 38, 40].
   d) Experimental liver tumors caused by viruses and association of hepatoma in woodchucks with a virus similar to HBV [22, 41].

Table 3. Mortality from primary liver cancer (PLC) compared to prevalence of HBsAg carrier state. From data reviewed by Hadziyannis [9]

Area	PLC mortality per 100 000	Prevalence of HBsAg carrier state (%)
A. "High" PLC incidence	20–100	6.0–14.0
B. "Intermediate" PLC incidence	12–15	2.5–5.0
C. "Low" PLC incidence	1–5	0.1–0.25

HBsAg among HCC and in controls. This has been found to be higher than 120/100 000 of the carrier population per year not only in the high but also in the intermediate and low HCC incidence areas of the world [9, 49].

Additional epidemiologic evidence for a causal link between HBV and HCC has been obtained from studies on immigrant populations showing that such populations exhibit prevalence of HBV markers and of PLC mortality similar to those of the place of their origin rather than to those of their residency [25, 44]. These observations suggest that HBsAg positive HCC is unrelated to other enviromental factors, like aflatoxine, which may also prevail in the high incidence areas of HCC.

The etiologic character of the association between chronic HBV infection and HCC is further supported by the following information:

a) In a given country or area HCC seems to develop more frequently and earlier in HBsAg positive than HBsAg negative cirrhosis. This has been shown in studies from Japan and Greece [15, 21]. In the Greek series the annual rate of development of HCC was calculated from 345 patients with various forms of CLD, followed-up over a period of eight years. Development of HCC was more frequent in HBsAg positive cirrhosis than in any other form of CLD. Thus the annual rate of HCC development was zero in primary biliary cirrhosis, 1.83% in cryptogenic cirrhosis (HBsAg negative), 2.92% in HBsAg positive chronic hepatitis, and 9.20% in HBsAg positive cirrhosis. These data clearly indicate that though cirrhosis per se regardless of its etiology, may well represent a risk factor for HCC, this risk becomes extremely high when cirrhosis is etiologically related to HBV and that development of HCC may even occur long before HBsAg positive chronic liver disease reaches the cirrhotic stage. On the other hand such information should not necessarily be interpreted as evidence that HBV itself is the cause of malignant transformation of hepatocytes. This will be further discussed in the chapter on pathogenesis of HCC.

b) Liver cell dysplasia, a probable precancerous change in cirrhosis, has been shown by Anthony et al. [3] to be linked with the presence of chronic HBV infection. Moreover, hepatocytes loaded with cytoplasmic HBsAg, known as "ground-glass" hepatocytes [13], have an impressive morphologic similarity to liver cells in putative preneoplastic hyperplastic nodules in rats exposed to chemical hepatocarcinogens [31]. Ground-glass hepatocytes are extremely abundant in HBsAg carriers [13] but are also frequently seen in the non-cancerous areas of HBV related HCC [10, 14, 37]. Such cells have been occasionally found [10] to produce a_1-fetoprotein and they may represent a dysplastic cell population, i.e., a probable precancerous change. However, the evidence for this is still very limited.

c) Early studies of HBsAg and HBcAg in the liver [14] have clearly shown that these

two antigens of the HBV can be clearly demonstrated in the non-cancerous areas of HBsAg positive HCCs but are absent from the malignant liver tissue. Further investigations [27] have largely confirmed these findings but have additionally shown that in well-differentiated hepatocellular carcinomas several tumor cells may also be positive for HBsAg but not for HBcAg. It can, therefore, be assumed that malignant liver cells are, in general, resistant to the ordinary replication of HBV and that when expression of HBV occurs in such cells it is a rather defective one, restricted only to the production of the surface antigen. This is further supported by studies of two cell lines (PLC/PRF/5 and Hep 3B) derived from biopsies of well differentiated human hepatomas [1, 24, 38]. These cells lines produce HBsAg but no other viral proteins and have part of the viral DNA integrated into their genome [6, 40]. The presence of integrated HBV genes with limited expression of HBV proteins does not, of course, prove viral oncogenicity. However, these observations are clearly compatible with such a view because malignant transformation from viruses appears to result from altered cellular gene expression rather than from the function of viral genes in permissive host cells.

d) PLC has been produced in experimental animals by several viruses like the MC-29 avian leukosis virus and the CELO avian adenovirus [8, 22]. An HBsAg positive hepatocellular carcinoma has also been produced in athymic mice by the PLC/PRF/5 (Alexander) cancer line [6]. Finally woodchucks infected with a virus similar to HBV exhibit chronic liver disease complicated by the development of primary liver cancer [41]. This observation gives further unexpected support to the etiopathogenic role of HBV in HCC and offers a natural model, suitable for research on viral hepatocarcinogenesis.

The above data, particularly the epidemiologic material, on the association between HCC and chronic HBV infection, though clearly indicative of a causal link, have been interpreted by some authors in other ways which have been much discussed in the past [32, 34, 42]. The mere fact that more than two thirds of all HCCs in the world arise as a result of CLD caused by the hepatitis B virus indicates that, regardless of mechanisms and of factors involved in hepatocarcinogenesis, chronic HBV infection itself represents the major cause of primary liver cancer in man. Criticism, therefore, should now be restricted on possible mechanisms of hepatocarcinogenesis related to HBV and on the major question of whether or not the HBV is a directly oncogenic virus.

Possible Pathogenetic Mechanisms

Before discussing several pathogenetic possibilities in hepatocarcinogenesis related to HBV it is first necessary to stress that such a discussion is completely meaningless without understanding the following three points [9]:

1) Chronic HBV infection may well represent a universal oncogenic factor but is not the only factor of HCC, since several cases of HCC, all over the world, are clearly unrelated to any evidence of HBV infection.

2) Chronic HBV infection per se is not sufficient to cause HCC, for only a fraction of infected subjects developes HCC. Therefore, additional factors must be cooperating (multifactorial disease).

3) Individual susceptibility, duration of infection, age and mode of acquisition of the infection, immunologic surveillance, concurrent infections, aflatoxins, and other factors may play a significant adjuvant role in the genesis of HBV-related HCC.

Several mechanisms could be implicated in the pathogenesis of HCC-related to the HBV. Some of them have already been discussed in the past and others have been considered only recently [7–9, 14, 31]. These can be summarized as follows:
a) Long-standing liver-cell damage caused by the HBV (or by immunologic mechanisms against HBV) may represent an intense stimulus for regeneration and growth, in other words an appropriate selection pressure [7, 8], which is the required prerequisite, leading some initiated dysplastic hepatocytes to malignant transformation. Liver-cell dysplasia could be induced either by HBV itself or by some others factors or it could even arise spontaneously. Such a mechanism of hepatocarcinogenesis could be responsible for the development of any HCC arising as a result of chronic liver disease – regardless of its etiology – provided that there is a persisting high rate of cell loss and consequently of liver-cell replacement by regeneration. This condition appears to exist in all forms of cirrhosis but seems to be particularly pronounced when the underlying cause of liver damage can not be eliminated by the host, e. g., chronic viral liver disease. Increased susceptibility of cells in mitosis to malignant transformation appears to be related to enhanced replication of their DNA which may occur before restoration by the host of damage caused to it by any potential carcinogen [7].
b) Patients with chronic HBV infection have been found to have characteristic hepatocytes known as "ground-glass" hepatocytes [13]. These cells have abundant centrocellular HBsAg, little if any HBcAg, and an increased smooth endoplasmic reticulum [10]. The biotransformation system of the ground-glass hepatocyte appears, therefore, to be altered and this may well be implicated in HBV-related hepatocarcinogenesis either by increased activation of some potential carcinogen to "ultimate" carcinogen or by decreased degradation of an "ultimate" carcinogen, e. g., chemical, mycotoxin, etc. [9, 31]. It is also possible that ground-glass hepatocytes represent a cell population, dysplastic or not, which is partially resistant to the growth of HBV. As already mentioned expression of HBV in these cells appears to be largely confined to HBsAg. They also appear to differ from ordinary hepatocytes regarding their capacity to produce and particularly to excrete several host proteins [10, 46]. In this regard they appear to have certain similarities with some lines of well differentiated hepatocellular carcinomas, already mentioned in the previous pages [1, 38]. Such an altered liver-cell population, partially resistant to the growth of HBV, may represent a protective host mechanism analogous to the one described by Farber [7] in the induction of hyperplasia and liver-cell cancer by chemical carcinogens in experimental animals. Further development of complete resistance to the ordinary growth of HBV may represent the next step to hepatocarcinogenesis [9]. In keeping with this possibility is the already mentioned lack of demonstration of HBsAg and HBCAg in histochemical studies of most HCCs except for some well differentiated cells [14, 27].
c) Development of HCC in chronic HBV infection may be due to malignant transformation of hepatocytes by integration of the viral genome within the genetic apparatus of the cells. Tumors induced in experimental animals by DNA oncogenic

viruses are known to arise from cells non-permissive to an ordinary growth of the infecting virus [2]. Therefore, they do not contain the ordinary capsid and coat antigens of the virus particles – usually detectable in cells suffering cytopathic infection – but appear to produce other DNA virus-specific antigens [2, 14]. If this is indeed the case with HCC it must then be assumed that, for one or other reason, certain hepatocytes first become resistant to HBV infection and are subsequently transformed by the virus. The mentioned hybridization studies showing the presence in some well differentiated HCCs of integrated HBV genes with limited expression of HBV proteins [6] and the early histochemical studies showing lack of HBsAg and HBcAg in the majority of HCCs [14], are compatible with this hypothesis. It is, however, important to remember that if this is indeed the case then transformed hepatocytes may not synthesize much of the known antigens of HBV but should produce new virus specific proteins like tumor antigens and transplantation-tumor specific antigens [2, 14]. Such a finding has not yet been reported in the literature though in a recent report three viral specific RNAs were detected in the PLC/PRF/5 which produces only one viral protein, i. e., HBsAg [6].

Possibilities in Prevention

Whatever the mechanisms in the pathogenesis of HCC, the possibility that the hepatitis B virus either directly or, most probably, in conjunction with other factors is causally related to HCC, raises some hope for future prevention. An effective hepatitis B vaccine appears to be now available [52]. Application of this vaccine in early life may lead in the future to a considerable reduction of HCC-mortality. This could prove true even if HBV represents nothing more than the cause of underlying chronic liver disease. There are about 200 million HBsAg carriers in the world with an impressively high relative risk for HCC. In fact three quarters of the future HCC cases are expected to arise from this population of HBsAg carriers. This is indeed a challenge to preventive medicine. Whether or not early vaccination with the emerging HBsAg vaccines will prove the right preventive strategy for HCC, the future will show.

Conclusions

Chronic infection with the hepatitis B virus is etiologically related to the development of hepatocellular carcinoma, one of the most frequent cancers in several heavily populated areas of the world. Chronic infection with the HBV is extremely frequent among HCC patients and it may be true that more than three quarters of the world cases of HCC are positive for HBsAg. The clinical and laboratory features of HCC associated with chronic HBV infection appear to vary geographically. An attempt was made, however, to outline its main clinical aspects including possibilities in "early" diagnosis. Although cirrhosis is present in more than two thirds of HBsAg positive HCCs, it is usually compensated and even clinically latent, particularly in the high-incidence areas of HCC. HBsAg positive HCC can also arise as a result of chronic hepatitis, in only slightly fibrotic or even

normal livers. The causal role of HBV in HCC has been discussed on the basis of existing evidence. Chronic HBV infection in conjunction with other factors may lead to the development of HCC by creating a continuous stimulus for regeneration and growth, by modifying the biotransformation system of hepatocytes, by initiating and promoting resistant dysplastic hepatocytes, and by transforming directly liver-cells to malignant ones after integration of the viral DNA into the host genome. Regardless of these speculations it is hoped that prevention of chronic HBV infection may significantly reduce, in the future, mortality from HCC.

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Subject Index

Immuno-stimulation

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The history of pharmacological research can be viewed as a long avenue leading from ill-defined plant and animal extracts to the well-defined molecules of today. Until recently, immunostimulation research was itself still in an archaic phase, emerging only in the last few years to the use of well-defined molecules for stimulating immune responses in animals and man.
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