Progress report

Serological responses to HBV infection

Twenty years on since the discovery of Australia antigen, the serological complexities of the hepatitis B virus (HBV) are still imperfectly understood. Recent knowledge concerning the structure and biology of HBV\(^1\) together, with the application of molecular biology, has clarified some of the serological events surrounding HBV infection and their relation to chronic hepatitis and cirrhosis. These aspects are considered in this review.

It has been shown that HBsAg, the major component of the outer shell of the HBV virion (Dane particle), is a protein (m wt 24 000 daltons),\(^1\) and exists in both non-glycosylated (PI) and glycosylated (PII) forms. There are at least nine antigenic subtypes as defined by polyclonal antibody specificities. These are grouped into four major subtypes of HBsAg, each containing the common ‘a’ determinant and one of a pair of allelic (mutually exclusive) subdeterminants d/y or w/r.\(^1\) The geographic distribution of subtypes varies; ayw predominates in the Mediterranean, the Middle East, and Africa and is also typically found in high risk subpopulations, namely, patients on maintenance haemodialysis and drug addicts.\(^2\) Infection with one of the four viral subtypes confers protection against reinfection with another subtype via an antibody response to the common ‘a’ determinant and this is utilised in the production of the HBV vaccines.\(^3\) Antibody mounted against epitopes on the subtypes alone, however, is inadequate for cross protection.\(^2,3\)

Production of virus is relatively inefficient. Uncoupling of HBsAg synthesis from the viral assembly situated in the central nucleocapsid core leads to excess production of HBsAg protein alone (up to \(10^{14}\) particles/ml in acute HBsAg positive infection) and spontaneous aggregation into 22 nm spherical and filamentous subviral (sub-unit) particles. These alone are not infectious because they lack viral DNA and other components of the intact virus. Indeed they are used in the production of pure HBsAg immunogen, subunit vaccine.\(^4\)

Unravelling the complexities of the antigenic determinants on HBsAg particles is of more than theoretical interest, for evidence is now accumulating that antigenic determinants in HBsAg particles in HBeAg positive sera may differ from those in anti-HBe positive sera.\(^5,6\) A species-specific receptor for polymerised human serum albumin (RpHSA) has been found on complete virus (Dane) particles and also on the 22nM HBsAg particles.\(^7,8\) The pHSA receptor has been recently located on two polypeptide products (GP31 and GP34) coded for at least in part by the pre-S gene region.\(^9\) Hepatitis B virus replication may be necessary for the synthesis of these polypeptides and for pHSA receptors since they are found in HBeAg but not anti-HBe positive sera.\(^7\) Similar receptors for albumin have been found on the hepatocyte surface and the RpHSA receptor may facilitate penetration of HBV into the liver cell.\(^7-9\)
Serological responses to HBV infection

Production of two widely used plasma derived vaccines (H-B-Vax; MSD and Ny-B-Vax; Biotest NYBC) includes HBeAg positive sera as starting material. Whether the antigenic reactivities in these vaccines differ from those utilising only anti-HBe positive starting material as in that produced by the Institut Pasteur (Hevac B) and, more importantly, have relevance to protective efficacy, is as yet unknown.

The immunogenicity of some of the recombinant hepatitis B vaccines has been disappointing but may be explained by the fact that some of the current recombinants may contain the product of the S gene but not that of the pre S gene which contains the pHSA receptor.

The nucleocapsid contains the genome comprising a double stranded, circular DNA molecule consisting of a short ‘S’ strand of variable length and a long ‘L’ strand of constant length. The DNA polymerase associated with the viral capsid is capable of repairing the gap by elongating the 3’ end of the S strand. The L strand contains the four reading frames S, C, P and X; the S region gene is 678 nucleotides long and codes for HBsAg. Deduction of its amino acid sequence has led to production of synthetic and biosynthetic vaccines.

The inner nucleocapsid core contains the core antigen system comprising HBCAg (m wt 18 000–19 000 daltons) and HBeAg (m wt 15 500 daltons) which is probably derived from HBCAg by proteolytic cleavage. HBCAg and HBeAg can be easily located in the nuclei of infected hepatocytes using immunofluorescent-labelled anti-HBc and anti-HBe antibodies respectively.

Serum markers of acute infection

In the uncomplicated case, the characteristic serological events during recovery are appearance of anti-core antibodies (IgM and, soon after, IgG) shortly followed by anti-HBe with decline and disappearance of HBsAg and HBeAg antigenaemia within one to three months. The subsequent ‘window phase’ defined by the serological presence of anti-HBc in the absence of HBeAg and anti-HBs persists for two to 16 weeks and ends with appearance of anti-HBs which signals recovery and immunity from future HBV infection.

The results with the newer assays using monoclonal anti-HBs to detect determinants on HBsAg – for example, Auszyme Monoclonal: Abbott Diagnostics, have, however, lead to the window phase being considerably shortened or even eliminated. Subjects hitherto diagnosed as negative for HBV on routine HBsAg screening via polyclonal assay have been found to be positive using monoclonal assays. In 26% of subjects diagnosed as acute HBV infection on anti-HBc positivity alone, HBsAg was detectable by the monoclonal assay only. It has been suggested that the monoclonal antibodies, being of high affinity, allow detection of HBV antigen hidden in immune complexes in the presence of anti-HBs excess. Further support for the enhanced performance of these assays comes from the confirmatory finding of HBV DNA in serum in a substantial number reactive to HBsAg only in the monoclonal RIA assay.

Until recently the detection of HBCAg in serum has been unreliable because of the hidden nature of the antigen in circulating immune
Disruption of HBcAg-IgG anti-HBc complexes in sera of chronic HBsAg positive carriers has now been achieved and should enable studies of the importance of this antigen system to be reassessed in acute infection. The fluctuations in HBeAg/anti-HBe may be artifactual due to the relative insensitivity of the commercial kits.

**Significance of IgM-anti-HBc**

The value of this antibody as an indicator of recent, acute infection is well established. In fulminant hepatic failure, abnormally rapid clearance of viral antigens viz HBsAg and HBeAg may occur, possibly as a result of formation of immune complexes and a diagnosis of a non-B cause considered unless testing includes IgM-anti-HBc. Out of 17 consecutive cases of fulminant hepatitis B admitted to the Liver Unit at King's College Hospital between 1977 and 1980, two were diagnosed on the basis of a positive IgM-anti-HBc alone in the absence of HBeAg and/or HBsAg. Conversely, the absence of this antibody in a patient known to be HBsAg positive suggests superinfection with another agent – for example, hepatitis delta virus, HAV, or non-A, non-B. Three out of 73 patients admitted to the Liver Unit with fulminant viral hepatitis between 1977 and 1980 who were HBsAg positive were subsequently attributed to non-B causes (two delta virus, one non-A, non-B) on the basis of a negative result for IgM-anti-HBc. In two recent reports from Greece and Japan, 62.5% and 56.3% of HBsAg negative cases respectively could be attributed to HBV on the basis of a positive IgM-anti-HBc result.

**Anti-Dane particle (DP) antibody**

An antibody which reacts with antigenic determinants shared by Dane particles and also HBsAg has been found early on during the course of acute infection and disappears with development of anti-HBe and anti-HBs using conventional polyclonal RIA testing. As anti-DP has not been found in patients with evidence of viral replication continuing into the chronic phase, it may play a role in viral neutralisation and clearance based on its ability to interact with the polymerised human serum albumin receptor (RpHSA) expressed on the hepatocyte surface during the HBeAg positive phase.

In the acute phase of HBV infection. Levels of anti-DP antibody activity show an inverse correlation with the expression of RpHSA receptors found on circulating Dane particles. Also, sera containing anti-DP appear free of anti-DP and pHSA binding on Dane particles. One current hypothesis proposes that anti-DP antibody is an anti-pHSA receptor antibody; high levels of which could promote extracellular virus neutralisation, prevent access into the hepatocyte and hence terminate viral replication. Whether cases in which HBV has been found to persist after the acute phase lack anti-DP early on is yet to be established.

**Anti-pre-S antibodies**

Synthetic peptides corresponding to the gene encoding pre-S have been used to detect antibodies in human sera that appear early on during acute
HBV infection and may play an inhibitory role in the attachment of HBV to hepatocyte receptors.\(^{31}\)

IgM-anti-pre S can be detected before HBsAg, using polyclonal kits, and, before the appearance of anti-HBS and anti-HBc. IgG-anti-pre S can be detected early on during the latter phase but thereafter titres of both isotypes decline.\(^{31}\)

The binding of Dane particle-associated (HBV) HBsAg to human liver cells (hepatoma cell lines) was inhibited by anti-pre S antibodies but not using anti-HBs derived from immunisation with pepsin-treated HBsAg subunits as in the hepatitis B vaccine (H B Vax; MSD).\(^{31}\) Furthermore, only human subjects vaccinated with HBsAg envelope protein, but not HBsAg subunits, developed detectable anti-pre S. These results can be explained by the finding of abundant pre-S gene-encoded domains on HBV, but not subunit HBsAg, and the reduction in their reactivity to anti-pre S when treated with pepsin.

Preparation of some of the newer recombinant vaccines has taken these features into account. Subunit HBsAg particles with pHSA receptor activity have been produced in mammalian cells using a plasmid construct that contains the pre-S\(_2\) region and pre-S\(_1\) promoter in addition to the S gene.\(^{32}\) These inclusions improved the immunogenicity of the vaccine and overcame the problem of non-responsiveness that has been found with S gene products alone.\(^{32}\) Whether this approach will enhance the protective efficacy of the subunit vaccines has yet to be explored.

**Impact of molecular biology**

Recent advances in molecular hybridisation techniques and their application to HBV have given further knowledge of cellular events that are occurring in parallel with serological findings during clearance or persistence of the virus following an acute infection. Although up to 90\% of previously healthy heterosexual adults eventually clear the virus, at least 10\% become carriers. In the latter, chronic liver disease, cirrhosis and hepatocellular carcinoma are not uncommon.

Clearance of virus from serum is usually reflected by seroconversion from HBeAg to anti-HBe positivity with loss of both serum HBV-DNA polymerase activity and HBV DNA, the latter denoting loss of HBcAg in liver and free episomal – that is, non-integrated HBV DNA from hepatocytes.\(^{33,34}\) Continued production of HBsAg protein (22 nm spheres) may be maintained by hepatocytes containing integrated, complete viral genome or HBV DNA sequences coding for the pre-S and S regions.\(^{35,36}\) Recent reports have shown that a significant proportion of patients, particularly those from the Mediterranean Littoral or Far East who are either negative for both HBeAg and anti-HBe or become anti-HBe positive, remain potentially infectious with DNA polymerase activity and/or HBV DNA remaining detectable in serum and HBcAg in liver tissue.\(^{37}\) Continuing viral replication may explain why inflammatory activity is maintained in some of these patients. Immunosuppressive therapy can potentiate viral replication with reappearance of HBeAg and/or DNA polymerase in subjects who had become HBeAg negative (without appearance of anti-HBe) before their administration.\(^{38}\) Apparent spontaneous reactivation of liver disease has more recently been described.
for anti-HBe positive subjects.\(^{39}\) Reappearance of HBeAg, DNA polymerase and/or serum HBV DNA has been shown to occur up to 28 months after initial seroconversion in the absence of serological evidence for superinfection with other hepatitis agents including delta virus.\(^{39}\)

Hepatitis B virus is not directly cytopathic. Persistence of virus depends on several factors including its ability to integrate into the host (hepatocyte) genome and whether it can evade the host's immune attack via T-cytotoxic cells directed against HBcAg displayed on the hepatocyte membrane of cells containing actively replicating virus.\(^{40}\) During the initial months or years after acute infection, when viral replication is active, free HBV DNA has been shown to predominate in both liver cells and serum, although integrated sequences in the former have been reported.\(^{51}\) Later, for reasons which remain unclear, viral replication ceases. Liver cells may contain HBV DNA, now in integrated form. These no longer display viral antigenic determinants and consequently can evade the host's cytotoxic attack.\(^{40}\) In patients who remain HBsAg, HBeAg positive, HBcAg can be detected by immunofluorescence in the nuclei of infected hepatocytes. IgM anti-HBc antibody may persist and levels reflect on-going inflammatory activity. The titres, however, are lower than in the acute phase and hence differentiation from the latter can usually be made.\(^{21-24}\)

HBV DNA hybridisation techniques, together with monoclonal antibody assays for HBsAg determinants, have also provided new knowledge in liver diseases previously thought to be of non-B aetiology. In a study involving 134 patients with HBsAg-negative chronic liver disease (defined on conventional polyclonal assay), out of 105 tested for serum HBV DNA, 10 were positive and in six this was the only marker, being negative for anti-HBc and/or anti-HBs.\(^{42}\) Monoclonal anti-HBs radioimmunoassay was performed on 31 of the 134 samples. Serum HBsAg – associated determinants were found in five of 17 HBV DNA positive; two of which were negative for anti-HBs and/or anti-HBc. None of 14 HBV DNA negative sera were positive by monoclonal assay. Interestingly, of the five HBV DNA positive samples, two came from renal transplant recipients with chronic persistent hepatitis and three had chronic active hepatitis.\(^{42}\) Thus serum HBV DNA appears to be even more sensitive than the monoclonal assay. That HBV DNA may be present in sera where HBsAg is not detectable suggests that either the serological testing is still not sufficiently sensitive or, more likely, that viral multiplication can occur in the absence of detectable HBsAg.\(^{42}\) The same study included 20 patients with hepatocellular carcinoma (HCC) and, in 17 of these, viral sequences were found on nucleic acid hybridisation of liver specimens.\(^{42}\) By contrast, serum HBV DNA was not found in any of the nine samples tested including six with anti-HBc and anti-HBs.\(^{42}\) In HCC, serum HBV DNA is a much less reliable marker of viral infection than in chronic hepatitis. One possible explanation is that by the time the tumour is clinically evident the virus may have cleared from the serum.

**Hepatitis delta virus co-infection and superinfection**

Hepatitis delta virus (HDV), as it is now known, was discovered by Rizzetto in 1977 when he detected a new antigen/antibody system
Serological responses to HBV infection

Serological responses transcribed and obtained from nucleic RNA as simultaneously (bAg/bAb), with particularly common in I.V. drug or by serous delta antigen using Northern Italy, Scandinavia,50 by the from that clinical delta antigen and will show delta sera from Italy, France and the United Kingdom, 43 (39%) had delta markers compared with 19% of cases of acute hepatitis from Italy.53 Among the 43 delta positive cases, primary hepatitis B coinfection was diagnosed in 25 and superinfection with delta virus in 18 on the basis of positive or negative tests respectively for IgM-anti-HBc.53 Furthermore,
HDV interferes with synthesis of HBV leading to a fall in titre of HBsAg, levels of DNA polymerase activity and serum HBV DNA.\textsuperscript{44, 45} Diagnosis of HDV superinfection in a chronic HBsAg positive carrier may include detection of delta antigen (\(\delta\)Ag) in the acute phase\textsuperscript{47} but assays are not routinely available. IgM and, later, IgG-anti-\(\delta\) antibodies are more likely to be present. IgM-anti-\(\delta\) may persist in high titre and presumably reflects ongoing HDV replication analogous to the persistence of IgM-anti-HBC in chronic HBV infection. This carries an adverse prognosis since it is invariably found in those HBV carriers with evidence of liver disease (chronic active hepatitis/and cirrhosis).\textsuperscript{48, 54}

Reliable commercial assays are now available for the detection of total anti-\(\delta\) (IgM and IgG) antibodies and some centres have developed a solid-phase radio-immunoassay for the specific detection of the IgM isotype.\textsuperscript{54} These should find particular use where the serum IgM anti-HBc is negative in a HBsAg positive subject with an acute exacerbation of hepatitis.

Particular questions that remain unanswered relate to the diverse frequency of HDV superinfection in communities with high HBV carrier rates. Why has HDV so far escaped the European homosexual but not drug addict population, and also why is it uncommon in certain Oriental communities?\textsuperscript{55} Most HBsAg positive adults with delta superinfection are anti-HBe, rather than HBeAg positive. It is possible that low levels of HBV replication may support, and high levels inhibit replication of HDV.\textsuperscript{44, 47}

A striking feature of superinfection with HDV is the tendency of the resulting hepatitis to remain active. Chronic persistent (CPH) or chronic lobular (CLH) hepatitis are uncommon and greater than 50% have histological evidence of CAH, with progression to cirrhosis in up to 25 per cent.\textsuperscript{45, 48} The rapid evolution of chronic delta hepatitis may explain the infrequent occurrence of HDV positive HCC.\textsuperscript{45, 48}

At present there is no specific therapy for HDV infection and current aims are directed towards prevention or irradication of HBV infection. Certain antiviral agents namely the interferons have shown promise in the treatment of chronic HBsAg positive liver diseases.\textsuperscript{56} Their use in patients superinfected with HDV would appear rational since interferons can inhibit RNA as well as DNA virus replication.

**Superinfection with other viral agents**

In addition to HDV, HBsAg positive carriers are at risk of superinfection with other viruses. This is well known for the drug-addict population where multiple attacks of hepatitis occurring in HBsAg positive individuals have been shown to be due to hepatitis A, non-A, non-B and Epstein-Barr viruses amongst others. Evidence is also accumulating that HBsAg positive carriers are at high risk from developing fulminant non-A, non-B hepatitis.\textsuperscript{27, 28}

In Greece, where the prevalence of HBsAg positivity is high, among 65 cases of fulminant viral hepatitis, 48 were HBsAg positive.\textsuperscript{57} Superinfection with non-A, non-B agents was considered responsible, however, for 10 (20.8%) who were HBsAg positive cases but IgM-anti-HBc negative on subsequent testing.\textsuperscript{27} In addition, among 38 HBsAg positive cases diagnosed as acute fulminant B hepatitis on the basis of IgM-anti-HBc, in
addition, one had δ antigen and another IgM-anti-δ, i.e. HDV co-infection. Synergistic viral infections may play a more important role in the development of severe or even fulminant hepatitis than hitherto realized. Acute hepatitis A virus (HAV) infection is common in subjects known to be prone to multiple viral infections – namely, homosexuals, drug addicts, etc. Simultaneous acute infection with HAV and HBV has been described in a chimpanzee57 and also a female laboratory technician58 resulting in a clinically more severe course than that anticipated with either virus alone. That fulminant hepatitis may be related, at least in part, to multiple viral aetiologies, as well as being determined by host/genetic factors, is an attractive concept.

ELIZABETH ANN FAGAN AND ROGER WILLIAMS

Liver Unit,
King's College Hospital,
Denmark Hill,
London

Received for publication 17 October 1985

References


Trepo CG, Robert D, Motin J, Trepo D, Sepetjian M, Prince AM. Hepatitis B antigen (HBsAg) and/or antibodies (anti-HBs, anti-HBc) in fulminant hepatitis: pathogenic significance and prognostic significance. Gut 1976; 17: 10–18.


Eddleston ALWF, Mondelli M, Mieli-Vergani G, Williams R. Lymphocyte cytotoxicity to
Serological responses to HBV infection

autologous hepatocytes in chronic hepatitis B virus infection. *Hepatology* 1982; suppl. 2: 122S–127S.


Serological responses to HBV infection.

E A Fagan and R Williams

Gut 1986 27: 858-867
doi: 10.1136/gut.27.7.858