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Leading article – Hepatology series

Host immune response and variations in the virus genome: pathogenesis of liver damage caused by hepatitis B virus

Recent understanding of the mechanisms responsible for liver damage during an infection with hepatitis B virus has changed significantly with the application of molecular biological techniques, the formulation of new concepts for T cell recognition of viral antigens, and experience with orthotopic liver transplantation.

Infection with hepatitis B virus (HBV) is associated with a wide spectrum of liver damage ranging from fulminant acute hepatitis to chronic HBV carriers with minimal hepatic changes or liver cirrhosis. This variation in liver damage was thought to be due to variations in the intensity of the host immune response to the virus.[1] From a virological point of view two major subgroups of chronic HBV carriers were identified – HBeAg seropositive with active HBV replication, and patients seropositive for anti-HBe, characterised by the absence of virus replication. In the early phase of chronic infection HBV carriers are HBeAg seropositive with a high level of virus replication, but with minimal liver damage.[2] This immunotolerant phase is particularly long in cases with vertical transmission of HBV. With time there is a gradual loss of the immune tolerance to the virus, manifested by the onset of the active phase of chronic HBV infection. Histological examination of the liver shows chronic lobular, chronic persistent or chronic active hepatitis and an increase in serum aminotransferase activity. HBeAg and HBV-DNA are still present in the serum as markers of ongoing virus replication. The duration of this active phase has an important impact in determining whether a transformation of the normal liver architecture into a liver cirrhosis will follow. The immune pressure is concentrated on hepatocytes supporting HBV replication and eventually may eliminate these cells, leading to seroconversion to anti-HBe, undetectable HBV replication, and no hepatic inflammation.

Recent knowledge has challenged this conventional concept of the immunopathogenesis of HBV related liver diseases. Immunology has made huge conceptual advances. It has been shown that T lymphocytes do not recognise large protein antigens but small peptide fragments of 8–16 amino acid residues, presented within the groove of an HLA molecule.[3] The use of the polymerase chain reaction technique, DNA sequencing, and cloning has permitted detailed examination of the virus genome and has shown the existence of a number of genomic variants of HBV,[4] often existing as a mixture of different strains in a patient with chronic infection.[5 6] Direct detection of the nucleotide sequence of the virus genome has also shown the persistence of HBV replication in chronic carriers, seropositive for anti-HBe, previously believed to be in the 'non-replicative' phase of chronic HBV infection, which made the serological testing for HBeAg/anti-HBe irrelevant as a marker of ongoing virus replication. Mutations in the viral genome during chronic infection will result in amino acid alterations in translated proteins and consequently in the viral peptides presented to the immune system. Can a new hypothesis for the mechanisms of HBV and host interactions embrace these new findings?

Immune recognition of viral antigens

Our early attempts to analyse the interactions of cytotoxic T cells with virus infected hepatocytes were based on the autologous microcytotoxicity assay.[7 8] Despite some obvious limitations in this system, it had the great advantage of using fully HLA compatible effector and target cells with naturally processed viral peptides. The results showed that in chronic HBV infection T cell cytotoxicity is directed to viral nucleocapsid protein[7] and that T lymphocytes selectively lyse hepatocytes expressing HBcAg, but not hepatocytes with HBsAg only.[8] In addition, the results of these in vitro experiments suggested that IgG anti-HBc, bound to hepatocytes expressing HBcAg can modulate the T cell attack.[8] Important evidence for the modulatory role of anti-HBc has also been obtained in vivo in chimpanzees, infected with HBV.[9]

More recently, a transgenic mouse model for hepatitis B surface antigen has been used to study the cytolytic effector T cells against hepatocytes expressing HBsAg.[10] The transgenic mouse lineage expressed non-cytotoxic amounts of the large and major virus envelope proteins in hepatocytes. Spleen cells from a non-transgenic mouse were primed by immunisation with a recombinant vaccinia virus containing HBV envelope polypeptide. When injected in the HBsAg transgenic mouse, these primed spleen cells caused liver cell necrosis. The hepatocellular injury in this model was transient and this system does not reproduce adequately the wide range of viral protein expression in humans. Pre-S2 viral protein has also been shown to be recognised by cytotoxic T lymphocytes, isolated from a patient with chronic hepatitis B.[11] The strong correlation, however, between ongoing HBV replication with HBcAg in hepatocytes and the persistent inflammatory activity in the liver has established the nucleocapsid protein of HBV – HBcAg as the main focus in HBV/host interactions both with respect to the pathogenesis of liver cell necrosis and the clearance of infected hepatocytes. Nucleocapsid proteins have also been shown to be important targets for the cytotoxic T lymphocyte response against several other viruses.[12–14]

HBV nucleocapsid (HBcAg) and its secretable, non-particulate form (HBeAg) are highly cross reactive at the T helper cell level.[15 16] HBeAg is a non-structural protein encoded by the precore/core gene of the HBV genome, which is present in the serum of patients, infected with the wild type of HBV. Recently, HBeAg has also been

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shown to be expressed on the cell membrane,[17] leading to speculation as to whether HBeAg or HBcAg is the target for the cytotoxic T cell response at different phases of chronic HBV infection. The fundamental concept of T cell recognition is that T lymphocytes, whether helper or cytotoxic, recognise small peptide fragments of about 8–16 amino acids in length, which are presented to the antigen receptor of HLA compatible T cells in a special groove on the top of an HLA antigen.[3] Cytotoxic T cells use their surface CD8 molecule to probe for a corresponding epitope on the side of the class I HLA molecule and their antigen receptor to probe for a complementary antigenic peptide in the groove and this complex will then trigger the cytolytic process. Similarly, helper T cells probably use CD4 molecules to search for HLA class II molecules on the antigen presenting cell. The presence of an antigenic peptide in the groove, complementary to the helper T cell antigen receptor will then trigger the release of cytokines. Thus, although serologically distinct, HBcAg and HBeAg share a substantial aminoacid homology and are not different in respect to T cells, to which only small peptide fragments are presented. In this context, viral peptides expressed within the groove of an HLA molecule on the surface of infected hepatocytes will not be detectable by antibodies. Thus, viral antigens – HBcAg, HBeAg, and HBsAg-detectable on the hepatocyte membrane using monoclonal or polyclonal antibodies are probably not being displayed in HLA molecules and therefore not recognisable by the host T lymphocytes.

Both CD4 and CD8 positive T cells, specific for HBcAg, have been isolated from liver specimens from patients with chronic hepatitis B. These T cells produced and secreted several cytokines including interleukin 2, interferon gamma, and tumour necrosis factor.[18] CD4 cells in the peripheral blood show a strong proliferative response to HBcAg or to HBeAg in patients with acute hepatitis B.[19] During the exacerbations of hepatitis in chronic HBV carriers peripheral blood T cells show an increased proliferative response to HBcAg as well as increased production of interferon gamma.[20] These data suggest that HBcAg specific CD4 positive, T helper cells may also participate in the pathogenesis of liver damage in HBV infection. The correlation between the helper T cell response to HBcAg and HBV clearance in patients with acute hepatitis B may be explained by the requirement of a helper T cell function for the induction of HBcAg specific cytotoxic T lymphocyte response or the effect of cytokines released from CD4 cells. The potential importance of the HBV nucleocapsid protein as a target for the host immune response in HBV infection is emphasised by the fact that HBcAg is about 100-fold more immunogenic in comparison with HBsAg at both the T cell and the B cell level.[21] In addition, HBcAg specific T helper cells can elicit the production of anti-HBs antibody by B cells, specific for the HBV envelope antigen when stimulated by virions.[22]

Using a mouse model, Milich *et al* have analysed the fine specificity of T lymphocyte recognition of the nucleocapsid protein.[16] HBcAg specific T cells from a variety of murine strains recognised multiple, but distinct 12 to 21 peptide fragments within the HBcAg/HBeAg sequence. Most importantly, although several strains recognised multiple sites, each strain was found to recognise a predominant T cell epitope and the fine specificity of this determinant was dependent on the H-2 haplotype of that particular strain.

In a series of experiments C Ferrari *et al* from Parma, in collaboration with F Chisari in La Jolla, California analysed the fine specificity of cytotoxic T cell epitopes of HBV nucleocapsid protein in patients with acute hepatitis B.[23–26] So far, two epitopes for CD8 positive cytotoxic T lymphocyte have been identified on HBcAg – an epitope located between amino acid residues 18–27 from the beginning of the nucleocapsid protein, which is HLA-A2 restricted and an epitope between residues 141–151, which is dually restricted by HLA-A31 and HLA-AW68. In addition, it has been shown that the HBcAg specific cytotoxic T lymphocyte response in acute, self limiting hepatitis B is directed to multiple viral epitopes – that is, it is polyclonal and multispecific.[26] In stable chronic hepatitis B it was not possible to show the cytotoxic T lymphocyte response, suggesting one possible defect to permit virus persistence in these patients. In the studies in acute hepatitis B it was shown that endogenously synthesised precore and core proteins are equally good targets for the nucleocapsid cytotoxic T lymphocyte response to a shared epitope 141–151, similar to the shared 18–27, thus implying that the HBV variant with a precore stop codon mutation, is unlikely to arise as a cytotoxic T lymphocyte escape mutant, as hepatocytes infected by this strain will still produce core protein and express the cytotoxic T lymphocyte epitope 141–151. At present, it is unknown whether the peptides expressed on the surface of hepatocytes infected with a wild type HBV differ from the peptides presented on the surface of hepatocytes, infected with the HBe minus variant of the virus. Proteins that are present in different cellular compartments (HBeAg in the membranes of endoplasmic reticulum and particulate HBcAg in the cytosol) may be processed differently and result in different peptides being inserted into the HLA groove.

Thus, recent data support the notion that helper and cytotoxic T lymphocyte response to HBV antigens and HBcAg in particular, play an important part in the pathogenesis of liver cell necrosis and for viral clearance in HBV infection.

Variations in the virus genome – the chicken or the egg in chronic active hepatitis B

Molecular cloning and nucleotide sequencing have clearly established the organisation of the HBV genome and its four open reading frames.[27] Wider application of the molecular biological techniques to clinical samples showed considerable variation and heterogeneity in the virus population among different patients with chronic HBV infection, as well as during different phases of this infection, thus emphasising variations in the HBV genome as another important determinant in the pathogenesis of liver damage.

An HBV variant with a point mutation at position 1896 (G to A substitution) of the precore region has been identified, which creates a stop codon and hampers the translation of HBeAg.[28 29] As translation from the second start codon in the core gene is possible, HBcAg can be produced and this mutation thus does not interfere with virus replication. After the initial description, this precore mutant type of HBV was thought to be associated with severe liver damage in chronic hepatitis B seropositive for anti-HBe, and in patients with acute fulminant hepatitis B.[30–33] We studied the nucleotide sequence of the HBV precore region in 29 patients, seropositive for anti-HBe, with a wide spectrum of underlying liver damage to analyse the pathogenicity of this mutant variant and its relation to liver disease.[5] HBV variants with a precore stop codon were found both in patients without hepatic inflammation, as well as in those with chronic active hepatitis. The fact that the precore stop codon A_{1896}HBV is not invariably pathogenic was subsequently confirmed. This mutant strain emerges during seroconversion to anti-HBe in patients treated with interferon and is also detectable in

asymptomatic HBsAg, anti-HBe seropositive blood donors without evidence of liver damage.[34 35]

In about 20% of cases with recurrent HBV infection in a liver transplant a novel syndrome has been described, fibrosing cholestatic hepatitis, characterised by serpiginous liver fibrosis, severe cholestasis, massive expression of viral proteins in hepatocytes, and progressive liver failure.[36–38] The pathogenesis of liver cell necrosis in this syndrome is probably due to a direct cytotoxic effect of HBV, as there is minimal inflammatory reaction in the liver. To analyse the impact of HBV mutants in the development of this syndrome, 11 patients with HBV recurrence in the graft, six of whom progressed to fibrosing cholestatic hepatitis, have been studied at the Institute of Liver Studies.[39] The results showed that the presence of precore stop codon A_{1896}HBV was not associated with the development of this syndrome, while multiple mutations in the core gene, resulting in considerable amino acid alterations in viral nucleocapsid have been identified only in those patients who developed fibrosing cholestatic hepatitis.

Which other factors are then involved in determining the degree of liver damage in HBV infection with predominant A_{1896}HBV variant? The level of virus replication seems one of them, as we and others have found that the hepatic injury in anti-HBe positive chronic hepatitis B correlates with the serum HBV-DNA concentration.[5 40] Two other findings lend further support to this relation. In a liver transplant recipient with severe recurrence of precore mutant HBV infection, progressive increase of viraemia led to fibrosing cholestatic hepatitis five months after transplantation.[41] Effective inhibition of precore mutant HBV replication with nucleoside analogues in this patient resulted in a dramatic improvement of liver function and liver histology tests, while in another study, reactivation of the precore mutant HBV, after withdrawal of cytotoxic treatment in anti-HBe positive healthy carriers resulted in a high level of virus replication and led to fulminant hepatic failure.[42] This positive relation between the level of virus replication and the degree of liver damage in anti-HBe positive chronic hepatitis B contrasts with the findings in HBeAg seropositive carriers in whom there is an inverse relation between serum HBV-DNA concentration and the degree of liver cell necrosis.[2 43] HBeAg plays an important immunomodulatory part, which may explain this difference between HBeAg(+) and anti-HBe(+) chronic hepatitis.[44] A recent study in woodchucks has shown that after the inoculation of a wild type HBV or a precore mutant HBV strain in neonatal animals both virus strains have a comparable level of replication, but none of the five woodchucks infected with the precore mutant virus as neonates became chronic virus carriers.[45]

Mutations in the virus genome outside the precore region of the same HBV strain must also be considered, as they may play a significant part in changing virus-host interactions. A recent in vitro study compared the replication of wild type HBV, the same strain with an introduced precore stop codon, and an HBV strain, isolated from a patient with fulminant hepatitis, which in addition to the precore stop codon had numerous mutations in other parts of the genome.[46] The fulminant HBV strain showed much higher replication as well as a higher level of intracellular accumulation of HBcAg in comparison with the other two strains, which had similar replicative capacity. These data show convincingly that the precore stop codon in itself is not the cause of a more pathogenic HBV variant. Instead, higher replicative capacity and higher antigen expression, induced by other mutations in an HBV type with a precore stop codon may be the cause of the severe liver damage. A temporal relation between clustering missense mutations in the core gene of HBV and the A_{1896} stop codon mutation in the precore region has been noted in patients with chronic hepatitis B.[47]

Several studies have shown that patients with chronic active hepatitis B are often carrying HBV variants with mutations in the core gene, which are not present in HBeAg(+) carriers who despite the ongoing virus replication have minimal hepatic changes.[6 47–50] In a study from Japan, as well as from our Institute, large deletions in the middle of the core gene were identified and the deleted area among different clones of the virus was remarkably consistent between codons 80–110 from the start of the core gene.[6 48] The number of deleted bases was often a multiple of 3, suggesting that a core protein could be translated and this variant core peptide is probably both immunologically and functionally different from the full length nucleocapsid protein. In other reports the observed variations in the core gene were nucleotide substitutions, rather than deletions, but the missense mutations resulting in changes in the deduced aminoacids clustered in particular areas – between codons 84–101 and 48–60 of the core gene.[47 49] Thus, there is a substantial body of evidence that HBV variants with clustering mutations in the core gene comprise the predominant part of the virus population in patients with chronic active hepatitis.

As these studies analysed the variations in the core gene in a cross sectional aspect, it is uncertain whether these HBV variants are the cause of a more severe liver damage or whether these HBV strains have been left over by the host immune attack. Indeed, these may be escape mutant variants of the virus, accumulated as a result of a positive selection by the host immune response, focused on particular epitopes of the core protein during the inflammatory activity. In an attempt to answer this question Wan Chuang *et al* sequenced HBV core gene in serial serum samples from two HBV carriers seropositive for HBeAg and minimal hepatic changes – one of whom progressed to chronic active hepatitis while the clinical course in the other patient was uneventful.[51] HBV strains with missense mutations in the core gene were cloned from the former patient at a comparatively early phase of raised serum aminotransferases but the same strains were not found in the serum samples at the time of most severe liver damage. This was associated with concentrating point mutations between codons 84–101. In contrast, no missense mutations were found in the other patient who remained asymptomatic. The fact that liver damage in HBV infection is not related only to a particular virus strain is emphasised by the finding that an identical HBV type with no changes in the precore and with multiple mutations in the core gene, was isolated from a patient with fulminant hepatitis, as well as from the sexual partner who had chronic active hepatitis B.[50]

The accumulation of HBV variants with precore/core gene mutations may change virus – host interactions in several aspects. Firstly, the peptides derived from the virus nucleocapsid presented within HLA molecules on the surface of hepatocytes will have a different composition and may trigger new sequences of T cell reactions. Secondly, mutations may affect the intracellular transport or packing of the core particles. It is known from earlier studies that there is an increased cytoplasmic and membranous expression of core protein in HBsAg positive patients with active hepatitis.[52 53] While the activation of cytotoxic T lymphocytes is believed to require the expression of no more than 100 viral peptides, the lysis of infected cells by antibodies require much stronger expression of the target antigen.[12 54 55] Thus, the enhanced expression of HBcAg in chronic active hepatitis may involve other immune mechanisms in addition to the cytotoxic T lymphocyte response, for example antibody dependent cytotoxicity or

cytokine release, or both which will also contribute to the progression of liver damage. Thirdly, some of the newly emerging HBV strains may have direct cytopathic effects on the infected cells or the accumulation of unenveloped core particles could be damaging to hepatocytes. Supporting evidence comes from in vitro studies using HBV transfected liver cells in culture. In HepG2 cells, non-productive HBV infection was associated with accumulation of core protein and direct cytopathic effect.[56]

In conclusion, current data suggest that two closely related factors – the characteristics of the virus population and the repertoire of the host immune response – will determine the outcome of the infection with HBV. A prompt polyclonal and multispecific response by the T lymphocytes – entailing both the cytotoxic and the helper T cells – will clear the virus in an acute self limiting hepatitis B. In some subjects infection with wild type HBV will become chronic as a result of a less efficient immune response and virus replication will continue with minimal liver damage. The precore region of the virus genome is highly conserved both in human and in woodchuck hepatitis virus. HBeAg encoded by this region and secreted in the circulation may act as an active mechanism for inducing immune tolerance. A gradual loss of immune tolerance occurs with time in HBeAg seropositive patients, but it is of limited clonality, thus permitting viral mutations to evade this limited response. It is possible, however, that mutants arise spontaneously because of errors in virus replication and this could trigger loss of tolerance as modified target peptides will be presented to the immune system.

During the HBeAg positive phase, those B lymphocytes that can bind HBeAg through their surface immunoglobulin will internalise this antigen and present on their cell surface the relevant peptides, derived from the proteolytic cleavage of HBeAg, in the groove of HLA class II molecules. While tolerance is maintained, T helper cells cannot signal the humoral or the cellular effector arms of the immune response by secreting interleukin 4, interferon gamma, etc. Therefore, the loss of helper T cell tolerance would not only be a fundamental factor in mounting the immune attack to HBV and a pivotal point during the course of chronic HBV infection, but would also provide the T cell help to permit B lymphocytes to produce antibody to circulating HBeAg. Indeed, HBeAg/anti-HBe immune complexes have been detected in chronic HBV carriers.[57] The immune pressure against HBeAg and a successful T cell attack against hepatocytes expressing HBV nucleocapsid peptides may lead to elimination of virus replication and cessation of liver damage. In some patients, however, the production of anti-HBe antibody may lead to the selection of HBV strains with mutations in the precore region, which will prevent the translation of HBeAg, the commonest of which is the A_{1896} precore stop codon. The lack of HBeAg and the release in the circulation of a particulate HBcAg from damaged hepatocytes will then accelerate the loss of immune tolerance to nucleocapsid peptides. The longer the duration of the inflammatory activity in the liver, the more genomic variations will accumulate in the dominant HBV strain or viral quasispecies will evolve in the individual patient. During this process HBV strains with mutations, which permit them to escape the immune pressure will be selected and will maintain chronic virus infection. Some HBV strains with point mutations or deletions may have direct cytotoxic effects on infected hepatocytes. Genomic variations may also affect the replicative potential of the virus and a selection of strains replicating at high level will cause exacerbation of hepatitis in a chronic HBV carrier. If such HBe minus variants with high replicative potential are transmitted from a chronic carrier to a new host, whose T cell response is not impaired and there is no secretable tolerogen like HBeAg, liver damage may be severe and could present clinically as acute fulminant hepatitis.

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