ing but no cytolysis, thus, no eradication of virus. Bertoletti et al show that hepatitis B virus (HBV) is capable of doing just that. The authors studied two patients who had a strong immunodominant response to Hepatitis B core peptide AA 18-27. They found one or two amino acid substitutions within the contact residues for the TCR. When these mutant peptides were used in a T-cell cytolytic assay, they failed to stimulate a response and even inhibited the response to normal wild-type peptides. HLA recognition by the mutant peptides was the same as for wild-type peptides. Therefore, those residues that interact with HLA were unaltered. However, alteration of the TCR contact residues with amino acid substitutions led to an altered peptide ligand that was structurally modified but still recognized by the TCR.

How does this occur? Viruses mutate to evade host defense. Patients with chronic hepatitis B have been described to have mutations to important or immunodominant peptides.[7] These mutations may no longer be recognized by the host immune system. In this case, the virus mutates a peptide that is still capable of binding to antigen-presenting cells and to the TCR but, rather than stimulate the T cell, leads to antagonism. The TCR also recognized that these ligands were structurally different. It is also clear that these altered TCR ligands are important in maintaining viral chronicity and counteracting the host immune response to virus.

Conventional wisdom has explained chronic hepatitis B as the failure of the host to eliminate infection because of failure to generate specific T-cell cytolysis. After exposure, the risk of developing chronic disease is age-dependent. Neonates see HBV peptides as "self" and >95% of them develop chronic disease. Children have a 40% to 80% chance of developing chronic infection. Adults have a <10% chance, the majority of adults clearing HBV within 6 months. Why do some not recover? Perhaps they are incapable of initiating CTL-specific responses to eradicate infection. Ferrari has shown that, in fact, individuals with chronic HBV infection have CTL present in peripheral blood and liver.[8] So why do these patients not clear virus and recover from their infection? This article shows that, although there are CTLs present, there are also mutations of viral peptides. These mutant peptides bind but do not cause T-cell cytolysis. Thus, these chronic patients have hepatocytes infected with mutant viruses that do not induce a host immune response. These mutant viruses will preferentially survive. Hepatocytes infected with both mutant and wild-type virus will also survive without immune clearance because the mutant inhibits the stimulation by wild-type viral peptides. Hence, the HBV has developed a brilliant mechanism to evade immune clearance by the host. HBV is not cytopathic, that is, it does not kill its host cell. Rather, it adapts itself to engage the T cell in paralyzing the normal immune effort to eradicate the infection. This mechanism may be very important in establishing chronic viral infections by other noncytopathic viruses.

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HEPATITIS B VIRUS ESCAPE MUTANTS: "PUSHING THE ENVELOPE" OF CHRONIC HEPATITIS B VIRUS INFECTION

Yamamoto K, Horikita M, Tsuda F, Itoh K, Akahane Y, Yotsumoto S, Okamoto H, et al. Naturally occuring escape mutants of hepatitis B virus with various mutations in the S gene in carriers seropositive for antibody to hepatitis B surface antigen. J Virol 1994;68:2671-2676.

ABSTRACT

Hepatitis B virus (HBV) DNA was extracted from sera of six carriers with hepatitis B e antigen as well as antibody to hepatitis B surface antigen and sequenced within the pre-S regions and the S gene. HBV DNA clones from five of these carriers had point mutations in the S gene, resulting in conversion from Ile-126 or Thr-126 of the wild-type virus to Ser-126 or Asn-126 in three carriers and conversion from Gly-145 to Arg-145 in three of them; clones with Asn-126 or Arg-145 were found in one carrier. All 12 clones from the other carrier had an insertion of 24 bp encoding an additional eight amino acids between Thr-123 and Cys-124. In addition, all or at least some of the HBV DNA clones from these carriers had in-phase deletions in the 5′ terminus of the pre-S2 region. These results indicate that HBV escape mutants with mutations in the S gene affecting the expression of group-specific determinants would survive in some carriers after they seroconvert to antibody against surface antigen. Carriers with HBV escape mutants may transmit HBV either by donation of blood units without detectable surface antigen or through community-acquired infection, which would hardly be prevented by current hepatitis B immunoglobulin or vaccines.

COMMENTS

The hepatitis B virus (HBV) shows greater mutability than previously appreciated.[1,2] Like human immunodeficiency virus, HBV uses reverse transcription as an essential step in the replication cycle. Calculations suggest that this error-prone replication leads to 2×10^{-4} base substitutions per site per year, which is four orders of magnitude higher than other DNA viruses but lower than RNA viruses. Variability in the virus was first demonstrated through classical serological subtyping studies of hepatitis B surface antigen (HBsAg).[3] By applying modern molecular biological procedures, which include amplification of the viral DNA by polymerase chain reaction and rapid DNA sequencing procedures, the molecular basis of these HBV subtype differences have been identified.[4] This article by Yamamoto et al and other recent findings support the thesis that there is ongoing but restricted immune surveillance of HBV envelope proteins in chronic carriers that could provide selective pressure for the emergence of HBV envelope escape mutants.

The envelope of the hepatitis B virion consists of three proteins and their glycosylated derivatives. These proteins, termed small (S), middle (M), and large (L) HBs proteins, are encoded by a common reading frame and differ only with respect to the length of their amino terminus. In addition to the S gene sequence, the MHBs contains the preS2 sequence (55 amino acids) and the LHBs protein contains the preS1 sequence (108 or 119 amino acids, depending on subtype) plus the preS2 sequence. The preS1 and preS2 regions and the region 100 to 160 of SHBs are exposed on the surface of the viral particles, have been shown to be highly immunogenic, and are potentially under selective pressure by the immune system. The common or group-specific epitopes, termed the "a" determinants, are contained within the region of approximately 120 to 160 of the S gene. The "a" determinants are complex, conformational, and dependent on disulfide bonding among highly conserved cysteine residues. Although the "a" epitope(s) had traditionally been defined by reactivity to polyclonal anti-sera, use of monoclonal antibodies has shown that the "a" region consists of at least four nonoverlapping epitopes.[5] A single point mutation in the "a" determinant leading to immune escape has been described in newborn vaccinees in Italy and Japan, in liver transplant patients receiving monoclonal anti-"a" antibody therapy, and in a lymphoma patient after successful cytotoxic therapy.[6,7] In this mutation, a single nucleotide change leading to the substitution of glycine with an arginine at position 145 (G-R145) destroys some but not all "a" epitopes.[8,9]

Yamamoto et al[6] provide only the second report of this G-R145 mutation occuring during the course of a natural HBV infection. Yamamoto et al found that three of six chronic HBV patients who were reactive for hepatitis B e antigen (HBeAg), anti–hepatitis B core (HBc) and anti-HBs had S gene sequences coding for the G-R145 mutant. It is likely, although not proven in this article, that the G-R145 mutants emerge during chronic disease as the result of immune selection. Serial bleeds in which development of anti-"a" antibody coincident with emergence of G-R145 would provide compelling evidence for immune escape. In fact, this was shown by the same investigators in transmission of HBV infection from a carrier mother to her newborn who was treated with hepatitis B immunoglobulin and vaccine.[9] S gene sequences derived from the infant's serum contained exclusively the G-R145 mutant. This infant became chronically infected with HBV despite the presence of ongoing anti-"a" antibody production. The mother had no detectable G-R145 mutant even after examination of 99 independent clones suggesting that the G-R145 arose *de novo* in the infant or that the mutant was selected from a virus population in the mother in whom wild-type viruses far outnumbered the mutant.

Yamamoto et al found a unique S gene mutation in one patient who was seronegative for HBsAg but reactive for HBeAg. All 12 S gene clones derived from this patient's serum had a 24-bp insertion leading to an 8-amino acid insertion at codon 123. Interestingly, the nucleic acid sequence of this insertion is nearly identical to the sequence of S gene codons 116 to 123, suggesting the possibility that this sequence was duplicated during replication to give rise to the insertion. This 8-amino acid insert contains a potential N glycosylation signal as well as an additional cysteine residue. The mutation is likely to significantly disrupt the "a" determinant, thereby destroying its ability to be detected by conventional HBsAg immunoassays. A 6-bp insertional S mutant was reported previously in a highly viremic, fulminant HBV patient who was also seronegative for HBsAg.[6,7] This insertion of 2 amino acids, asparagine and threonine, at codon 122 in the S gene also led to the creation of a new glycosylation site. These findings raise the intriguing possibility that amino acid insertions between Cys121 and 124 can be accomodated in the viral envelope. It is not known whether the new glycosylation sites were actually used or whether the efficiency of HBsAg secretion or relative infectivity of these insertional HBV mutants is affected.

Yamamoto et al also showed that two of six chronic patients had a change of Ile or Thr at position 126 to an asparagine residue (I/T-N126), but it is less clear that this represents an escape mutant. Okamoto et al[9] had shown evidence that this mutant was reactive for three distinct monoclonal antibodies with "a" reactivity and was vertically transmitted across three generations. Because reactivity to known group-specific, anti-"a" monoclonal antibody was not affected, the T-N126 might be considered a subtypic variant rather than an escape mutant. One additional chronic patient had S gene coding for serine at codon 126 in all clones derived from sera collected over 10 years. These data argue against the concept that this amino acid substitution emerged as the result of immune selection.

Although Yamamoto et al described S mutations in

patients who were seropositive for both HBsAg and anti-HBs, the presence of both markers in the patient's sera does not usually indicate the existence of HBV escape mutants. In fact, both HBsAg and anti-HBs are present in 10% to 25% of chronic active, HBeAg-positive patients. There are two reasons for codetection of antibody and antigen. First, the commercial enzyme immunoassay for HBsAg and anti-HBs can detect HBsAg immune complexes when neither species is in large excess over the other. Second, the commercial anti-HBs test can detect a variety of subtypic antibodies, including anti-y, d, and w in addition to anti-"a." Shiels et al[10] showed that in most cases of coincident detection, HBsAg was of one subtype, d or y, and the anti-HBs was exclusively of the alternate subtype, y or d, respectively. Only in cases with high levels of HBsAg coincident with high anti-"a" antibody or in patients seronegative for HBsAg but seropositive for HBeAg and other HBV markers would an S gene escape mutant be strongly suspected. These findings also indicate that the presence of anti-HBs in sera cannot necessarily be construed as a good prognostic sign, especially when the patient remains HBsAg and HBeAg positive.

Another interesting finding in the Yamamoto article was the presence of in-frame deletion mutants in the preS1 and preS2 genes derived from the 6 HBV carriers as well as from 7 of 14 HBV patients with conventional HBV serological profiles in whom no S gene mutations were detected. These deletions varied from isolate to isolate, even within the same patient, but the most common one led to the deletion of 11 to 29 amino acids in the N terminal half of preS2 without disrupting the preS2 initiation codon. This region of preS2 is known to be highly immunogenic. Also, a 183-bp (61-amino acid) deletion corresponding to the C terminus of preS1 and the preS2 N terminus was commonly found. This mutation eliminates part of the spacer region of the HBV Pol gene, the putative surface gene promoter, and the start codon of the preS2 gene, thereby preventing synthesis of the MHBs protein. Interestingly, none of the mutations described by Yamamoto et al would prevent production of the N terminal portion of the preS1 region, which is required for binding to a hepatocyte receptor. Their findings are consistent with several recent articles documenting the emergence of deletions and point mutations in the preS1/preS2 region in 1 patient during acute exaccerbations of chronic infection, in 1 patient with rapid progression to cirrhosis, in 2 patients with chronic hepatitis who seroconverted to anti-HBe but whose hepatitis did not subside, and in 1 patient with chronic hepatitis during treatment with interferon.[11,12] Although HBV devoid of MHBs protein has been shown to occur *in vivo* as a dominant or exclusive virus and variant preS2 genomes were shown to be replication competent in cultured hepatocyte cells,[13] the effect of preS1/preS2 deletions on HBV infectivity or replication *in vivo* is unknown.

Yamamoto et al showed that four of six of the HBV DNA–positive patients had detectable HBsAg but two were borderline or negative. This raises the issue of the infectivity risk of HBV carriers who have no detectable HBsAg. Lack of HBsAg detectability in viremic carriers may occur for a number of reasons. First, the concentration of HBsAg produced may be too low to detect. This low concentration likely reflects a complex interplay between host and viral factors but may result from partial immune clearance, viral interference, e.g., hepatitis D virus or hepatitis C virus, or emergence of mutations that downregulate expression or secretion of HBsAg. Second, as described above, a mutation in the S gene may directly affect the antigenicity and detectability in an immunoassay. The two HBV viremic, HBsAg seronegative carriers described in the article by Yamamoto et al were strongly reactive for anti-HBc, suggesting that anti-HBc may continue to have a role in preventing transfusion transmitted hepatitis B.

The following questions remain unanswered: What is the prevalence of emerging escape mutants in chronic carriers? Is this prevalence rate dependent on length of time of carriership and hepatitic disease activity? What is the relative infectivity and transmissability of these mutants? Are individuals immunized with the current HBV vaccine at risk of infection from HBV mutants? What are the implications of these findings for the development of new HBV vaccines? In regard to this latter question, the current vaccines are highly immunogenic and efficacious and the vast majority of vaccinees develop a broad-based, polyclonal anti-HBs response. Therefore, most vaccinees are unlikely to be at risk of HBV infection from HBV mutants. However, some newborns, immunosuppressed individuals (patients who underwent transplantation), and other patients with muted vaccine responsiveness (either caused by disease, age, or genetic factors) may develop weak or only subtypic or monoclonal-type responses. In these cases there would be a higher probability of HBV breakthrough infections and emergence of HBV escape mutants. In these situations, a more complex vaccine containing a combination of S, preS2, and preS1 gene products might lead to more diverse, higher titer antibody response and, thus, provide greater protection than that generated by the current recombinant vaccine that contains only the S gene product.

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THE DEVELOPMENT OF ANTI-HEPATITIS C VIRUS AGENTS

Pizzi E, Tramontano A, Tomei L, La Monica N, Failla C, Sardana M, Wood T, et al. Molecular model of the specificity pocket of the hepatitis C virus proteinase: implication for substrate recognition. Proc Natl Acad Sci U S A 1994;91:888-892.

ABSTRACT

We have built a model of the specificity pocket of the protease of hepatitis C virus on the basis of the known structures of trypsin-like serine proteases and of the conservation pattern of the protease sequences among various hepatitis C strains. The model allowed us to predict that the substrate of this protease should have a cysteine residue in position P1. This hypothesis was subsequently proved by N-terminal sequencing of two products of the protease. The success of this "blind" test increases our confidence in the overall correctness of our proposed alignment of the enzyme sequence with those of other proteases of known structure and constitutes a first step in the construction of a complete model of the viral protease domain.

COMMENTS

Hepatitis C virus (HCV) is a main causative agent of blood born non-A, non-B hepatitis. Chronic HCV infection is also linked to the development of hepatocellular carcinoma.

According to analysis of the viral genome as well as the putative viral proteins, HCV is classified into a genus apart from flavivirus and pestivirus genera in the *Flaviviridae* group. A typical feature of viral protein production in this family is the production of a precursor polyprotein followed by proteolytic processing. For the processing of the HCV precursor polyprotein, it is now known that at least three independent pathways are involved, one via a signal peptidase associated with the membrane of the endoplasmic reticulum and two via virally encoded proteinases (Fig. 1). A signal peptidase is responsible for the production of putative viral structural proteins, C, E1, and E2.[1,2] One viral proteinase, designated viral proteinase-1[3] in Fig. 1, cleaves only at a site between NS2 and NS3, of which the active domain locates between the C-terminus of NS2 and the N-teminus of NS3. The other viral proteinase, a product of NS3 and classified as a serine proteinase, cleaves NS3/NS4A, NS4A/NS4B, NS4B/NS5A, and NS5A/NS5B sites of the HCV precursor polyprotein.[4-6] A functional domain of the serine proteinase is located in a region of 200 amino acid residues in the N-terminal half of NS3.[7] Amino acid sequences surrounding the NS3 cleavage sites are listed in Fig. 1. Characteristic of the amino acid sequences of these cleavage sites is the presence of Thr or Cys at the P1 position and Ser or Ala at the P1′ position. It is notable that the P5 and/or P6 positions are occupied by acidic amino acids. The fact that a difference (Thr or Cys) exists at the P1 position may be explained by the fact that the NS3/4A site, the P1 position of which is Thr, is cleaved in a cis-acting manner,[7,8] whereas cleavage at the other sites, which have cysteine residues at their P1 positions, is likely to be conducted in a trans-acting manner.

Serine proteinases have a conserved amino acid triad, His, Asp, and Ser, in their catalytic active domain. Detailed structural analysis of serine proteinases shows the presence of an important region known as the substrate binding pocket. This binding pocket consists of approximately 20 amino acid residues. From comparative analysis among several serine proteinases, a possible role of this region in the divergent cleavage specificities of substrates has been pointed out. Thus, the three-dimensional structure of this particular domain could predict the substrate cleavage specificity.

The investigators propose a model for the substrate binding pocket of HCV serine proteinase using information from the alignment of the domain sequence of this enzyme with the conserved core regions of the substrate binding pocket of various serine proteinases. From this model of the substrate binding pocket, the amino acid at the P1 position is predicted to be one with a short side chain, preferably cysteine or serine. Because phenyalanine in the pocket seems to be important for interaction with the amino acid side chain at the P1 position, the investigators prefer cysteine at the P1 position of substrates for HCV serine proteinase because of its favorable interaction with the aromatic ring system of phenylalanine.