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Hepatitis B X open reading frame deletion mutants isolated from atypical hepatitis B virus infections

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The etiology of hepatitis in 32 renal dialysis patients, as indicated by elevated transaminases in one ore more serial serum samples, was examined by testing for HBV, HAV, and HCV markers. All patients had undectable HBsAg, anti-HBs, and anti-HBc in commercially available assays (Abbott Labs, North Chicago, IL). Ten of these patients developed anti-HCV (anti-C-100; Abbott Labs) at or after the rise in transaminases, suggesting acute HCV infection was responsible for the enzyme elevations. Several patients had anti-HAV IgM, but were positive long before the transaminase peak(s), suggesting prior infection. In addition, all patients had undectable HBV-DNA by slot blot hybridization using DNA extracted from 100 μl of serum, but 14 patients were anti-pol$^+$. Further analysis of selected serum samples from each of the 32 dialysis patients by polymerase chain reaction (PCR) showed the presence of HBV-DNA in 25 (78%). The primers used were as follows (using the HBV-DNA adw clone numbering system): MF03 (residues 1903–1929) and MF04 (residues 2436–2412) for amplification of the core region; MF24 (residues 1231–1247) and MF04 for amplification of the X plus core regions; MF16 (residues 1929–1903; complementary to MF03) and MF24 for amplification of the X region; MF06 (residues 2850–2873) and MF07 (residues 154–132) for amplification of the preS region; all of which were made by the oligonucleotide facility at the Fox Chase Cancer Center. Primers MD03 and MF06 were used for amplification of the preS/S regions (1) and were kindly provided by Dr. John Sninsky (Cetus). Amplification reactions using these different primer pairs showed the expected size of amplified products for the preS/S and core regions, but smaller than expected size for the X region. Possible artifacts due to secondary structure of the nucleic acid around the X region was excluded by showing that amplification of a recombinant plasmid containing full-length HBV-DNA resulted in the predicted sized band. The possibility that the smaller band(s) were due to the improper amplification of the nick and gap structure encompassing the X region was also excluded because HBeAg$^+$ sera from eight HBV carriers amplified to give only the expected wild-type band. Hence, many renal dialysis patients with unexplained peaks of transaminases appear to be infected with variants of HBV characterized by one or more deletions in the X region in the viral genome.

In addition to HBsAg negative cases, X deletion mutants have also been found in some HBsAg$^+$ patients with relatively high levels of viral DNA in serum, as detected by dot blot hybridization (using 1 μl serum samples). Among five renal dialysis patients who became HBsAg$^+$ during the course of regular treatments, two had detectable X deletion mutant(s) months or years prior to the appearance of HBsAg and wild-type HBV-DNA in serum, suggesting that these mutant strain(s) of HBV could infect liver and replicate at low levels relative to wild type. Usually the X region amplification products from the serum of such patients showed multiple bands, implying the existence of virus particles carrying different X region deletion mutations in a single infection. Moreover, the number and size of bands resulting from amplification changed in many patients during the natural history of infection. These observations are compatible with the possibility that such patients are being infected with different X deletion mutants while on dialysis and/or that two or more mutants infecting the same hepatocytes undergo genetic recombination. Among all the patients who had detectable X deletion mutant(s) months or years

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prior to the appearance of HBsAg, HBeAg and viral DNA (using dot blot hybridization), there was a strong correlation between the appearance of wild type X region within virus particles and the appearance of classical markers of HBV replication in serum. If the appearance of virus with a wild type X region in patients already infected by the X deletion mutant(s) represents initial exposure to wild type virus, then previous infection by the X deletion mutant(s) provide no protective immune responses against wild type virus infection. Alternatively, the appearance of wild-type virus in the serum of patients who had previously been infected by deletion mutants may be the consequence of genetic recombination among complementary mutants and emergence of the better replicating wild type strain. In three other infected patients, HBsAg and wild-type DNA became undectable, while one or more X deletion mutant(s) appeared in subsequent sera. The appearance of the X deletion mutant(s) after seroconversion from HBxAg to anti-HBx (2,3) in three out of five patients tested implies that immunological response(s) against X determinants associated with virus replication (4), may result in a shift in the host–virus relationship and the appearance of poorly replicating mutants. Hence, some immune responses which eliminate wild type HBV may be ineffective in removing X deletion mutants, and in some cases such immune responses may actually select for the appearance of X region deletion mutants in serum.

Similar experiments in WHV-infected woodchucks also showed the presence of X region deletion mutants in serum, and in one case (woodchuck 128, WC128) the nature of the deletion was characterized by direct cloning from serum and DNA sequencing. WC128 was a WHsAg$^+$ carrier with persistent WHxAg in serum. In this animal, viral DNA was readily detected by dot blot hybridization in 1 μl of serum. The serology associated with this infection appeared to be wild type. However, when the WHxAg polypeptide(s) in serum were characterized by Western blotting, there was no detectable 17 000 Da component, which is the size expected from the translation of the X region of WHV and found in the sera of many other WHV carrier animals. Instead, a 13 000 Da band was observed and when Western blotting was carried out with peptide antibodies directed against the amino terminal or carboxy terminal portion of the molecule encoded by the X open reading frame, binding was observed only with peptide antibodies made against the carboxy terminal portion of the protein. This result implied that the 13 000 Da polypeptide initiated translation at a start codon (AUG) within the X region. Further characterization of this polypeptide by amino terminal sequence analysis of the first 20 residues also supported the idea of internal initiation. However, when the template for this polypeptide was sought in virus particles from serum, PCR amplification showed only X region deletion mutants which, upon further characterization, did not contain sequences capable of encoding the 13 000 Da WHxAg polypeptide. However, when the X region template(s) was sought from the liver of WC128 by PCR, X region sequences with small deletions, capable of encoding the 13 000 Da component, were present. Hence, it appears that WHxAg could be made from one or more integrated templates, and that it could be found in serum during natural infections. Such infections can result in the development of the chronic carrier state with serology indistinguishable to those carriers infected with wild type virus. In such infections, however, X polypeptides made from an integrated and not episomal virus template may stimulate virus gene expression and replication of virus genomes from templates having deletions within the X region.

The findings above suggest that the production of X antigen polypeptide(s) during HBV or WHV infections could have profound consequences upon the nature of the host–virus relationship which evolves. Among infected individuals in which only X region deletion mutants are present in serum, most with detectable X antigen in serum are also positive for surface antigen, anticore, and virus DNA. Many are virus carriers. In contrast, those without evidence of X polypeptide production are usually negative for surface antigen, anticore, and virus DNA by conventional techniques. Some in the latter group have detectable anti-pol as the only serological marker of infection. The apparent stimulation of virus gene expression and replication among individuals making X antigen is compatible with the well-known properties of X as a *trans*-activating protein. However, the origin of wild type virus replication, which includes the direct repeat sequences (DR1 and DR2) within the X region, is not present within the deletion mutants. The core promotor and core enhancer are also absent in many of these deletion mutants. The presence of the X region promotor and adjacent enhancer sequences, however, were present in these mutants, and it is possible that core gene expression could be supported by stimulation of these elements by cellular transcription factors and by X. Most of the deletion mutants characterized so far do not extend into the highly conserved U5-like sequences (located near the preC/C junction), and it is possible that these sequences could serve as an alternate, although highly inefficient, origin for virus replication. It is well known that U5 sequences, located in the long terminal repeat of retroviruses, are important for retrovirus replication. The recent finding that X is capable of

trans-activating the long terminal repeat of human immunodeficiency virus (5), is consistent with the hypothesis that it may also *trans*-activate U5-like sequences in hepadnavirus genomes carrying X deletions. This model would be consistent with the observations that X antigen-positive individuals could support relatively high levels of replication of X deletion mutants.

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