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Point Mutation in the *S* Gene of Hepatitis B Virus for a d/y or w/r Subtypic Change in Two Blood Donors Carrying a Surface Antigen of Compound Subtype adyr or adwr

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Genomes of hepatitis B virus (HBV) were cloned from the plasma of a blood donor who carried subviral particles of three distinct subtypes in the following proportions: adr, 25%; ayr, 63%; and adyr, 12%. HBV DNA clones were classified into two groups based on a difference at only one nucleotide in the *S* gene. Two clones had A as nucleotide 365 that formed part of the codon for lysine as amino acid residue 122 and produced a surface antigen of subtype adr in transfected NIH 3T3 cells. The remaining four clones had G determining the codon for arginine and produced a surface antigen of subtype ayr in transfected cells. Similarly, HBV genomes were cloned from the plasma of an individual who carried subviral particles of subtypes adr (71%) and adwr (29%). Two clones had T and A as nucleotides 476 and 479, respectively. The other seven clones had C and G as the respective nucleotides. Based on a comparison with previously reported HBV genomes of various subtypes, the mutation of nucleotide 479, forming part of the codon for lysine or arginine as amino acid residue 160, was deduced to determine the w or r subtype, respectively. When NIH 3T3 cells were transfected separately with the genome of subtype adw or adr, derived from plasma containing a surface antigen of subtype adwr, and then cocultured, they produced subviral particles of either subtype adw or adr. When cells were transfected with the genomes of subtypes adw and adr simultaneously, however, subviral particles were produced that possessed w and r determinants on the selfsame particles. These results attributed the d/y or w/r subtypic change to a point mutation in the *S* gene and favored coinfection of hepatocytes with an HBV genome and its mutant as the mechanism of compound subtypes.

Hepatitis B virus (HBV) produces a large excess of envelope protein in the host. The envelope protein, designated hepatitis B surface antigen (HBsAg), occurs on the surface of HBV and as small spherical or tubular particles with a diameter of 22 nm. HBsAg possesses the group-specific determinant called a and one each of the two sets of subtypic determinants named d/y and w/r (2, 9), thereby creating the four major subtypes adw, adr, ayw, and ayr. These subtypes have distinct geographical distributions (3, 13) and help in tracking down the route of infection in horizontal and vertical transmission of HBV (12, 16).

With the advent of recombinant gene technology that disclosed the entire nucleotide sequence of the HBV genome, subtypic variations can now be explained at the amino acid level in the translation product of the *S* gene (6, 21) and even at the nucleotide level in the sequence of the *S* gene (17). It has not yet been possible, however, to pinpoint a single nucleotide substitution in the *S* gene for the d/y or w/r subtypic change by comparing the sequences reported for HBV genomes of different subtypes.

The four major subtypes have their exceptions. HBsAg of a mixed or compound subtype has been reported that carries both allelic determinants (d and y or w and r), or even all four subtypic determinants, on the selfsame particles (14, 15, 28). The mechanism whereby such compound particles are created is not evident; either genomic recombination of HBV strains of different subtypes (15) or double infection (20) has been proposed to explain them. We propagated HBV genomes from two plasma samples containing HBsAg of subtype adyr or adwr and analyzed their nucleotide sequences. The results indicate a point mutation in the *S* gene for the d/y or w/r subtypic change and implicate coinfection of hepatocytes with HBV of a certain subtype and its mutant in the production of compound HBsAg particles.

MATERIALS AND METHODS

Plasma samples. One plasma sample was obtained from a Japanese blood donor. It contained HBsAg of subtype adyr, as determined by sandwich enzyme immunoassay with monoclonal antibodies (28), at a reversed passive hemagglutination titer of 2^{15}. It was positive for hepatitis B e antigen by enzyme immunoassay (1). The other plasma sample was obtained from an asymptomatic carrier in Papua New Guinea and contained HBsAg of subtype adwr, at a reversed passive hemagglutination titer of 2^{14}, along with hepatitis B e antigen. Thirty milliliters of each of them was used in the following experiments.

Affinity column chromatography. HBsAg particles carrying the d or y determinant or both were separated from the plasma containing HBsAg/adyr by the following procedure. Plasma (4 ml) was subjected to an affinity column (bed volume, 10 ml) in which monoclonal antibody against the y determinant (anti-y, monoclonal antibody no. 3457) was immobilized on Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) by means of BrCN. It was washed and eluted with 4 M $MgCl_2$. The eluted fractions were dialyzed against saline and subjected to an affinity column of anti-d (no. 3423). The captured HBsAg particles were eluted.

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Pass-through fractions from the anti-y column (containing HBsAg/adr), as well as pass-through (HBsAg/ayr) and retained (HBsAg/adyr) fractions from the anti-d column, were dialyzed against saline.

Similarly, HBsAg particles carrying the w or r determinant or both were isolated from the plasma containing HBsAg/adwr by use of affinity columns to which monoclonal anti-w (no. 4111) or anti-r (no. 313) antibody was conjugated. Preparation of monoclonal antibodies against each subtypic determinant of HBsAg was described previously (28).

Molecular cloning of HBV genomes and determination of nucleotide sequence. Dane particles representing HBV were isolated from plasma by a method described elsewhere (27). HBV genomes were cloned in the plasmid vector pSP65 (Promega Biotec, Madison, Wis.) at the *Bam*HI site by the method described for the cloning of HBV of subtype ayr (17). Appropriate restriction fragments were subcloned in M13 mp10 and mp11 phage vectors (Amersham, Buckinghamshire, England). The nucleotide sequences of both plus and minus strands were determined by the dideoxy chain termination method (22).

Transfection of NIH 3T3 cells. A tandem dimer of each cloned HBV genome was ligated to the *Bam*HI site of plasmid pSP65. Four kinds of recombinants were obtained. They were pDYR110D carrying the cloned HBV DNA called pDYR110 (subtype adr; see Results), pDYR73D carrying pDYR73 (ayr), pRWB594D carrying pRWB594 (adw), and pRWB741D carrying pRWB741 (adr). NIH 3T3 cells were transfected with the individual clones or the mixture thereof, along with plasmid pSV2-neo (24), by the calcium phosphate technique (25) and cultured in Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) containing 10% (vol/vol) fetal calf serum and a neomycin analog, G418 (GIBCO). The culture medium was harvested after 4 weeks and tested for HBsAg and its subtype by enzyme immunoassay (28).

RESULTS

Heterogeneity of HBsAg particles in plasma containing HBsAg of a compound subtype. When plasma containing HBsAg/adyr was subjected to anti-y and anti-d columns sequentially, three HBsAg preparations with different subtypes were obtained. By quantification of the common determinant, a, adr, ayr, and adyr were found to constitute 25, 63, and 12%, respectively, of the total HBsAg. The presence of d and y determinants on the selfsame particles in an HBsAg/adyr preparation was ascertained by sandwiching them between anti-d on the solid support and anti-y labeled with horseradish peroxidase.

All HBsAg particles in plasma containing HBsAg/adwr carried the r determinant. HBsAg particles without the w determinant (subtype adr) represented 71% and those with it (adwr) represented 29% of the total HBsAg; no HBsAg particles of subtype adw were detected.

Molecular cloning of HBV genomes and comparison of their nucleotide sequences. Six clones of HBV DNA were propagated from plasma containing HBsAg/adyr. All of them had a genomic length of 3.2 kilobases and shared the 33 restriction sites for the 11 endonucleases, including *Acc*I, *Ava*I, *Bam*HI, *Bgl*II, *Bst*EII, *Hin*cII, *Hpa*II, *Rsa*I, *Taq*I, *Xba*I, and *Xho*I. Their sequences of the *S* gene differed only in nucleotide 365 (Fig. 1). Two of them (pDYR72 and pDYR110) had A and were deduced to code for HBsAg of subtype adr on the basis of the results of transfection studies (see below), while the remaining four (pDYR50, pDYR70, pDYR73, and

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    ATGGAGAGCACAACATCAGGATTCCTAGGACCCCTGCTCGTGTTACAGGCGGGGTTTTTCTTGTTGACAAGAATCCTCACAATACCACAG  90
adr MetGluSerThrThrSerGlyPheLeuGlyProLeuLeuValLeuGlnAlaGlyPhePheLeuLeuThrArgIleLeuThrIleProGln
    ------------------------------------------------------------------------------------------
ayr  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

    AGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGAGCACCCACGTGTCCTGGCCAAAATTCGCAGTCCCCAACCTCCAATCAC  180
    SerLeuAspSerTrpTrpThrSerLeuAsnPheLeuGlyGlyAlaProThrCysProGlyGlnAsnSerGlnSerProThrSerAsnHis
    ------------------------------------------------------------------------------------------
     - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

    TCACCAACCTCTTGTCCTCCAATTTGTCCTGGCTATCGCTGGATGTGTCTGCGGCGTTTTATCATATTCCTCTTCATCCTGCTGCTATGC  270
    SerProThrSerCysProProIleCysProGlyThrArgTrpMetCysLeuArgArgPheIleIlePheLeuPheIleLeuLeuLeuCys
    ------------------------------------------------------------------------------------------
     - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

    CTCATCTTCTTGTTGGTTCTTCTGGACTACCAAGGTATGTTGCCCGTTTGTCCTCTACTTCCAGGAACATCAACTACCAGCACGGGACCA  360
    LeuIlePheLeuLeuValLeuLeuAspTyrGlnGlyMetLeuProValCysProLeuLeuProGlyThrSerThrThrSerThrGlyPro
    ------------------------------------------------------------------------------------------
     - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

    TGCAAGACCTGCACGATTCCTGCTCAAGGAACCTCTATGTTTCCCTCTTGTTGCTGTACAAAACCTTCGGACGGAAACTGCACTTGTATT  450
    CysLysThrCysThrIleProAlaGlnGlyThrSerMetPheProSerCysCysCysThrLysProSerAspGlyAsnCysThrCysIle
    ----G-------------------------------------------------------------------------------------
     - Arg - - - - - - - - - - - - - - - - - - - - - - - - - - - -
      (122)
    CCCATCCCATCATCCTGGGCTTTCGCAAGATTCCTATGGGAGTGGGCCTCAGTCCGTTTCTCCTGGCTCAGTTTACTAGTGCCATTTGTT  540
    ProIleProSerSerTrpAlaPheAlaArgPheLeuTrpGluTrpAlaSerValArgPheSerTrpLeuSerLeuLeuValProPheVal
    ------------------------------------------------------------------------------------------
     - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

    CAGTGGTTCGCAGGGCTTTCCCCCACTGTTTGGCTTTCAGTTATATGGATGATGTGGTATTGGGGGCCAAGTCTGTACAACATCTTGAGT  630
    GlnTrpPheAlaGlyLeuSerProThrValTrpLeuSerValIleTrpMetMetTrpTyrTrpGlyProSerLeuTyrAsnIleLeuSer
    ------------------------------------------------------------------------------------------
     - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

    CCCTTTTTACCTCTATTACCAATTTTCTTTTGTCTTTGGGTATACATT  678
    ProPheLeuProLeuLeuProIlePhePheCysLeuTrpValTyrIle
    ------------------------------------------------
     - - - - - - - - - - - - - - - -
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FIG. 1. Nucleotide sequence and translation product of the *S* gene of HBV DNA clones propagated from plasma containing HBsAg of compound subtype adyr. They were grouped into adr clones (pDYR72 and pDYR110) and ayr clones (pDYR50, pDYR70, pDYR73, and pDYR177), which differed only in nucleotide 365. Substitution of G for A resulted in the change of amino acid residue 122 from lysine to arginine.

pDYR177) had G for encoding HBsAg of subtype ayr. Consequently, amino acid residue 122 from the N terminus of the *S* gene product was lysine for the genomes with a nucleotide 365 of A (adr), while it was arginine for those with G (ayr).

The nine clones of HBV DNA propagated from plasma containing HBsAg/adwr were 3.2 kilobases long and possessed in common the 31 restriction sites for the 11 endonucleases. They were classified into two groups based on substitution of nucleotides at positions 476 and 479 (Fig. 2). Seven of them (pRWB193, pRWB220, pRWB382, pRWB741, pRWB1731, pRWB2068, and pRWB2330), deduced to have subtype adr (see below), had C and G at the respective positions, while the remaining two (pRWB594 and pRWB807), of subtype adw, had T and A. This resulted in coding for alanine and arginine as amino acid residues 159 and 160, respectively, by the former group of genes, in contrast to valine and lysine by the latter. Besides these differences, three clones (pRWB594, pRWB741, and pRWB807) had C as nucleotide 444 in place of T in the remaining six clones. Since these nucleotides were at position 3 of the codon for threonine, they did not affect the translation product of the *S* gene. All of the remaining 675 nucleotides of the *S* gene were identical for the nine clones.

The complete nucleotide sequence of the HBV genome was determined for two representative HBV DNA clones derived from plasma containing HBsAg/adyr. They were pDYR110, with A as nucleotide 365 of the *S* gene and of the adr subtype, and pDYR73, with G and of the ayr subtype. They differed from each other only in 10 (0.3%) of 3,215 nucleotides. Three representative HBV DNA clones propagated from plasma containing HBsAg/adwr were different only in 5 to 8 (up to 0.2%) of 3,212 nucleotides. They were pRWB594 (subtype adw), with nucleotides 476 and 479 of T and A in the *S* gene, respectively, pRWB193 (adr), and pRWB741 (adr), with the respective nucleotides of T and G; pRWB193 had nucleotide 444 of T in place of C in pRWB741 or pRWB594. All three clones had a deletion of nucleotides

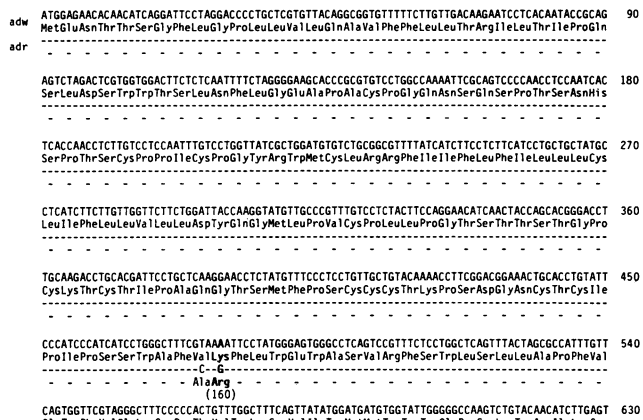

FIG. 2. Nucleotide sequence and translation product of the *S* gene of HBV DNA clones propagated from plasma containing HBsAg of compound subtype adwr. They were grouped into adw clones (pRWB594 and pRWB807) and adr clones (pRWB193, pRWB220, pRWB382, pRWB741, pRWB1731, pRWB2068, and pRWB2330), different in nucleotides 476 and 479. The substitution of G for A in nucleotide 479, accompanying the change in amino acid residue 160 from lysine to arginine, is deduced to be essential for the w or r subtype.

1513 to 1515 (three base pairs). Since none of them had an *Eco*RI site, the nucleotides were numbered with reference to reported HBV genomes (5, 17, 18).

HBsAg particles produced by NIH 3T3 cells transfected with the cloned HBV DNA. NIH 3T3 cells were transfected with either of the two representative clones propagated from plasma containing HBsAg/adyr. The cells transfected with pDYR73D, named TD403, produced HBsAg/adr, while TC302 cells transfected with pDYR110D produced HBsAg/ayr. Similarly, NIH 3T3 cells were transfected with the two representative clones from plasma containing HBsAg/adwr. TA207 cells transfected with pRWB594D produced HBsAg/adw, and TB19 cells transfected with pRWB741D produced HBsAg/adr.

When TA207 cells and TB19 cells were cocultured, they released HBsAg/adw or HBsAg/adr into the culture medium; no HBsAg particles of a compound subtype (adwr) were produced.

NIH 3T3 cells were transfected with a mixture of pRWB-594D (adw) and pRWB741D (adr). They produced HBsAg particles of a compound subtype, adwr. The presence of the w and r determinants on the selfsame particles was ascertained by sandwiching them between monoclonal anti-w antibody on a solid support and anti-r antibody labeled with horseradish peroxidase.

DISCUSSION

The four subtypes of HBsAg generated by the combination of either d or y (9) and either w or r (2) are accepted as the phenotypic expression of the four major genotypes of HBV (10). Since d and y, as well as w and r, are mutually exclusive, an allelic regulation is presumed for the coding of amino acid sequences responsible for the subtypic determinants of HBsAg. Rare sera display both of these allelic determinants, in which d and y (or w and r) occur on the selfsame particles. The mechanism by which HBsAg particles of a compound subtype are created is not clear as yet. Nordenfelt and Le Bouvier (15) and Mazzur and colleagues (14) were the first to demonstrate the presence of d and y on the same particles by two-dimensional immunodiffusion and counterelectrophoresis. Both groups discussed the various possibilities, and the former favored the genomic recombination of two HBV strains of different subtypes for production of compound HBsAg particles. Lately, Paul et al. (20) examined sera containing HBsAg of a mixed subtype. In one case, this represented a mixture of separate adw and ayw particles as in the serum reported by van Kooten Kok-Doorschodt (30). In the other, determinants d, y, w, and r were all present on the same particle. However, the finding of d and y on distinct HBsAg in a chimpanzee infected with this second serum led them to postulate phenotypic, not genotypic, mixing as the mechanism of generation of the original compound particles.

The results we obtained by molecular cloning of HBV genomes from two plasma samples containing HBsAg of a compound subtype, adyr or adwr, and transfection of NIH 3T3 cells with them are consistent with phenotypic mixing of surface antigen polypeptides in hepatocytes simultaneously infected with two distinct HBV genomes, rather than genomic recombination, as the mechanism for producing compound HBsAg particles.

HBsAg particles of three different subtypes were found in plasma containing HBsAg/adyr in proportions of 25% adr, 63% ayr, and 12% adyr. Plasma containing HBsAg/adwr, however, disclosed HBsAg of subtypes adr (71%) and adwr (29%); no particles of subtype adw were detectable in it. The reason why HBsAg/adw was not detectable was not evident. Since HBsAg/adwr would be produced by the hepatocyte coinfected with HBV/adw and HBV/adr, HBV/adw might have been defective and unable to replicate without help from HBV/adr. An HBV DNA clone of subtype adw (pRWB594), propagated from the same plasma, displayed the complete nucleotide sequence of an HBV genome, however.

HBV DNA clones obtained from plasma containing HBsAg/adyr, as well as those from plasma containing HBsAg/adwr, were classified into two groups on the basis of nucleotide substitutions in the sequence of the *S* gene. When NIH 3T3 cells were transfected with adr and ayr clones (or adw and adr clones) separately and then cocultured, they produced HBsAg particles of one or another regular subtype; no particles of a compound subtype were created. When the cells were transfected with the mixture of adw and adr clones, however, they produced HBsAg particles of subtype adwr. These results indicated that simultaneous infection of hepatocytes with HBV genomes of two distinct subtypes would be required for production of HBsAg particles of a compound subtype.

How the blood donors carrying HBsAg/adyr or HBsAg/adwr became infected with HBV strains of different subtypes is not obvious. We propose infection with HBV of a certain subtype and its mutation to HBV of a different subtype as the mechanism for the following reasons. (i) The sequences of the *S* gene in the HBV DNA clones, propagated from plasma containing HBsAg/adyr, were different only in nucleotide 365, A for clones of subtype adr and G for those of subtype ayr. Furthermore, the representative adr clone (pDYR110) was different from the ayr clone (pDYR73) in only 10 (0.3%) of 3,215 nucleotides, but they were both different by 1.1 to 1.9% from other reported adr and ayr genomes (4, 8, 17, 18). Similarly, adw clones propagated

from plasma containing HBsAg/adwr were different in the *S* gene sequence from adr clones at only two or three positions. The representative adw clone (pRWB594) and two adr clones (pRWB193 and pRWB741) differed at only 0.2% in the entire genomic sequence but by 4.8 to 9.7% from other reported adw and adr genomes (4, 8, 18, 29). (ii) The HBV genome would be prone to mutation. The rate of nucleotide substitutions per site per year is estimated at 10^{-9} for DNA viruses and 10^{-5} for RNA viruses, including some retroviruses (7). HBV is a DNA virus, but it is peculiar in that it replicates by reverse transcription of an RNA intermediate (26), a process suspected of frequent errors due to a lack of proofreading enzymes for editing. (iii) Individuals carrying HBV of a certain subtype would be unlikely to harbor HBV of a different subtype introduced later in life because they can raise antibodies against the subtypic determinant(s) borne by newly presented HBV that is not shared by the HBV which they already carry. The consequence is the simultaneous presence of HBsAg particles of a certain subtype and antibodies against subtypic determinants not possessed by them (11, 23).

On the basis of comparisons of HBV genomes of various subtypes, it has not been possible to determine which of the amino acid residues 68 and 122 (or both) from the N terminus of the *S* gene product is relevant to the d/y change (17). Peterson et al. (21) have found that reductive methylation of amino acid residue 122 of lysine in HBsAg/adw decreases reactivity with monoclonal anti-d antibody. Their result is in agreement with our view that substitution of arginine for lysine, representing amino acid residue 122, would be the single essential difference between HBsAg subtypes d and y. Furthermore, this difference can be ascribed to substitution of G for A at nucleotide 365.

Likewise, substitution of amino acid residues 4, 47, 110, 113, 126, 160, and 207 has been found in association with the w or r determinant of HBsAg (17). Taken together with our finding that adw and adr clones differed only at amino acid residues 159 and 160, the lysine or arginine representing residue 160 can be deduced as the substitution responsible for the w/r subtypic change. This change can be explained further as a substitution of G for A at nucleotide 479. Pasek et al. (19) have reported the nucleotide sequence of the HBV genome considered to be of the compound genotype adyw. The present study, however, suggests that they may have been dealing with HBV of subtype ayw. It has arginine and lysine as amino acid residues 122 and 160, respectively, in the *S* gene product, which is indeed characteristic of subtype ayw. The clone they studied perhaps co-occurred with HBV/adw and collaborated in producing HBsAg of subtype adyw.

There was a striking resemblance between d/y and w/r subtypic changes. Both were attributable to substitution of G for A. In both situations, the nucleotides represented position 2 of a codon in the *S* gene, resulting in amino acid substitution. They constituted, however, position 3 of a codon in the *P* gene, coding for the putative DNA polymerase, without accompanying amino acid substitutions. A mutation such as this, without affecting replication, would be quite acceptable for HBV.

Given the evidence that the four major subtypes of HBsAg, and by inference those of HBV, can be generated by point mutations at nucleotides 365 and 479 in the *S* gene, one wonders whether d/y and w/r determinants of HBsAg may be taken as the most fundamental markers of viral genotypes. A good deal of variability is known among the nucleotide sequences of HBV genomes of the same subtype (4, 8, 17, 18). Conversely, there are genomes of different subtypes with a close similarity in the entire nucleotide sequence (17). In our observation also, the HBV/adr clone (pDYR110) was different by only 0.3% from the heterotypic clone (pDYR73) that was propagated from the same plasma but differed by as much as 4.5 and 4.6%, respectively, from two homotypic clones (pRWB193 and pRWB741) derived from the other plasma sample. Indeed, other criteria for subtyping HBV genomes, such as the degree of concordance of the entire nucleotide sequence, might prove to have virological relevance and find clinical as well as epidemiologic applications.

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