Infectivity and Pathogenicity in Chimpanzees of a Surface Gene Mutant of Hepatitis B Virus that Emerged in a Vaccinated Infant

Norio Ogata, Alessandro R. Zanetti, Minshu Yu, Roger H. Miller, and Robert H. Purcell

Hepatitis B virus (HBV) variants with amino acid mutations in the α epitope of the major protein have been identified, and questions have been raised regarding their biologic properties. Dilutions of serum that contained the first such described HBV mutant, with an Arg-for-Gly substitution at codon 145 of the S gene, were inoculated into 6 seronegative chimpanzees. Five of the animals developed serologic and/or biochemical evidence of hepatitis B. A polymerase chain reaction–based assay that discriminated between the wild type and mutant viral genomes revealed that a pure population of the mutant genome was present in the 10⁻⁶ and 10⁻⁷ dilutions of the index serum, resulting in infection of the chimpanzees receiving these dilutions only with the mutant virus. A clone of the mutant virus replicated normally following transfection in vitro. Thus, this HBV surface gene mutant is viable, infectious, and pathogenic in chimpanzees.

Hepatitis B virus (HBV) infection remains a serious worldwide problem. Vaccines against HBV, which consist of HBV surface antigen (HBsAg), either purified from the plasma of chronically infected persons or expressed in yeast from cloned HBV DNA, have proven to be effective in preventing HBV infection and disease when administered alone [1–4] or in combination with hepatitis B immune globulin (HBIG) [5–9]. Antibody responses to the common epitope of HBsAg, termed the α determinant, are considered to confer protection against infection with HBV regardless of viral subtype, since subtype cross-protection was demonstrated by challenge studies in chimpanzees [10] and by controlled studies in a population at high risk for HBV infection [2].

Although an adequate amount of serum antibody to HBsAg (anti-HBs) is generally regarded as a marker for protection of persons against HBV infection, there are several instances in which HBV infection occurs despite the presence of anti-HBs. Some of these instances have been attributed to the fact that certain anti-HBs antibodies lack activity against the α epitope [11–13] or that the patient was infected with a variant of HBV [14, 15]. Zanetti et al. [16] identified 44 persons who became HBsAg-positive after receiving immunoprophylaxis, with successful development of serum anti-HBs. Of these, 32 had other markers of HBV infection such as antibodies to HBV core antigen (anti-HBc) or to e antigen (anti-HBe) [17]. Some of these patients had HBsAg with reduced antigenic reactivity of the α epitope, and 1 patient was found to carry a mutant virus with an amino acid substitution of Arg for Gly within the α epitope, at codon 145 of the S gene [17]. Similar HBV variants harboring amino acid mutations in the α epitope, in which an Arg substitution at codon 145 was most commonly observed, have been identified in vaccinated infants in Singapore [18] and in Japan [19–22]. Also, such variants were found in several patients who had received liver transplants for end-stage liver disease associated with HBV infection and who had received human monoclonal anti-HBs antibody [23] or HBIG [24] in an attempt to prevent recurrent hepatitis B. Thus, HBV S gene mutants, possibly generated by immune pressure of anti-HBs, may pose a potential threat to vaccinated persons.

To investigate the infectivity and pathogenicity of one such mutant, we experimentally infected HBV-seronegative chimpanzees with serum obtained from the child who had acquired chronic infection with the HBV S gene mutant containing Arg at position 145 despite perinatal administration of HBIG and hepatitis B vaccine as originally described by Zanetti et al [16] and Carman et al. [17].

Materials and Methods

Source of clinical material. A serum sample was obtained from an Italian child who harbored an HBV mutant with Arg instead of Gly at codon position 145 of the S gene [16, 17]. This child was born to an HBV carrier mother who was positive for serum hepatitis B c antigen (HBcAg). Although the infant was thought to have acquired a protective titer of serum anti-HBs by passive immunization with HBIG and active immunization with a licensed plasma-derived hepatitis B vaccine, the infant developed hepatitis B and has been a chronic virus carrier for at least 9 years (Zanetti AR, unpublished data). The serum used in this study was...
obtained on 21 April 1990, when the child was 7 years old. This serum was positive for HBsAg, HBeAg, and anti-HBc, but anti-HBs was no longer detectable.

Animals. Six chimpanzees (nos. 1384, 1396, 1406, 1434, 1500 and 1516) were used for transmission studies. The chimpanzees had never been experimentally infected with HBV and were seronegative for all HBV markers.

Experimental infection of chimpanzees. Serum obtained from the infant was diluted in 10-fold increments with fetal calf serum to produce a standard challenge pool and was inoculated intravenously into chimpanzees using 1 mL of dilutions of 10^{-1}, 10^{-2}, 10^{-3}, or 10^{-4}. Liver function of the chimpanzees was assayed with tests for alanine aminotransferase (ALT), isocitrate dehydrogenase, and γ-glutamyltransferase.

Serologic tests. Serum samples were obtained from the 6 chimpanzees weekly. Serum HBsAg, anti-HBs, anti-HBe, and anti-HBc were assayed by solid-phase RIA (Austria II for HBsAg, Ausab for anti-HBs, Corab for anti-HBe, and Abbott-HBe for HBsAg and anti-HBe; Abbott Laboratories, Abbott Park, IL). Antibodies to hepatitis A virus (HAV) and hepatitis delta virus (HDV) were assayed by solid-phase RIA (Havab and Anti-Delta, respectively; Abbott). Antibodies to hepatitis C virus (HCV) were assayed by ELISA for anti-C100-3 antigen (Ortho Diagnostics, Raritan, NJ) and by a second-generation assay consisting of three different antigens (Abbott).

Polymerase chain reaction (PCR) and direct sequencing of HBV DNA. Serum obtained from the donor, and sera collected from chimpanzees 1434, 1384, 1406, 1500, and 1516 at several points during viremia, were tested by PCR for detection of a portion of the S gene of the HBV genome. Serum HBV DNA was extracted by a method used for the extraction of tissue DNA [25, 26]. In brief, 10 μL of serum was digested in a lysis buffer containing 10 mM TRIS-HCl (pH 7.4), 1 mM EDTA, 50 μg/mL proteinase K, and 0.1% (wt/vol) SDS at 37°C for 3 h, followed by phenol-chloroform extraction and ethanol precipitation. The DNA sample was dissolved in distilled water and was prepared for the first round of PCR in a reaction volume of 100 μL containing 10 mM TRIS-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.001% gelatin, 200 μM each dNTP, 0.2 μM sense and antisense primers, and 2.5 U of Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). A thermal cycle of 94°C for 1 min, 37°C for 2 min, and 72°C for 3 min was repeated for 35 cycles. The second-round PCR was carried out under the same conditions as first PCR by using 1 μL of first PCR product. The nested primer set within the S gene was synthesized to encompass the a determinant of the S gene and was as follows: The outer sense primer was 5′-CGAGAATTCAGTAGCTGCCTTGAGTT-3′, the outer antisense primer was 5′-TGGAGATCTAGCAAGACGATAATGAACTGCA-3′, the inner sense primer was 5′-CCGCGATTCCTAGACTGCGT-3′, and the inner antisense primer was 5′-CTTGTCCACTGCAACAATGAACTGCA-3′. A portion of the S gene that covered the a determinant was sequenced directly on both DNA strands as previously described [27].

PCR-based nucleotide analysis specific for the first nucleotide at codon 145 of the S gene. To discriminate between wild type and mutant HBV genomes in a given serum sample, a PCR-based nucleotide assay was devised so that amplification of DNA with the nucleotide G (wild type) or A (mutant), at the first position of codon 145 of the S gene, would yield products with different molecular weights after digestion with restriction endonucleases. The sense primers used were as follows: PBS1: 5′-(376) TGGATGTGCTGCGGTGTATTAT (398)-3′; PBS2-Bgl: 5′-(448) CTGGAGATCTCAAGGGATGGTGGTTTGTGCC (479)-3′; PBS2-Bam: 5′-(448) CTGGAGATCCTCAAGGGATGGTGGTTTGTGCC (479)-3′. The antisense primers were as follows: MBS-Bgl: 5′-(627) AGCCGGAGGGTAGGTGGTTAATTACAGGGTACGATC (590)-3′; MBS-Bam: 5′-(627) AGCCGGAGGGTAGGTGGTTAATTACAGGGTACGATC (590)-3′; MBS: 5′-(627) AGCCGGAGGGTAGGTGGTTAATTACAGGGTACGATC (590)-3′. The number in parentheses indicates the nucleotide position at the 5′ or 3′ terminus of the primers [28]. Nucleotide positions were counted by numbering the second residue (i.e., A) of the EcoRI site as number 1 in the subtype ayw genome. The underline denotes the recognition site of a restriction endonuclease, either BglII or BamHI, which was created by introducing mismatches into the primers. Mismatched nucleotides are in boldface type. A diagram of the analysis is shown in figure 1. The conditions of the PCR assay were the same as described above except first PCR was performed for only 20 cycles to reduce the possible accumulation of in vitro-generated mutations caused by the lack of a 3′-to-5′ proofreading activity for Taq polymerase [29]. One-twentieth of the first PCR products was digested with 40–50 U of restriction endonuclease using a buffer and a temperature recommended by the supplier (Boehringer Mannheim, Indianapolis). Second PCR was carried out using an amount equivalent to one one-hundredth of the product from first PCR, the same conditions as for first PCR, and 35–40 cycles, followed by digestion with the restriction endonuclease. One-half of the digested DNA was visualized under ultraviolet light after electrophoresis in a 2.5% agarose gel containing 0.5 μg/mL ethidium bromide.

Determination of the sensitivity of PCR-dependent nucleotide analysis. An HBV DNA fragment of 1493 bp covering the whole preS/S gene was amplified by PCR from the serum of chimpanzee 1434, which was inoculated with a 10^{-1} dilution of serum from the donor. The chimpanzee serum contained a mixed population of wild type and mutant viral genomes (see Results). Primers for PCR amplification were as follows: 5′-CACCTGCAGCCTCATGAATCTCAAGGTATGTTGCCCGTTTGTCC (479)-3′; 5′-TGCTGCAGTTTGTGGGTCACATA-3′ (sense) and 5′-ATAAGCTTCTGGCGGTTTGTCC (479)-3′; 5′-TGCTGCAGTTTGTGGGTCACATATATC (479)-3′; 5′-CTGGAGATCTCAAGGGATGGTGGTTTGTGCC (479)-3′. The PCR fragments were cloned into the PsI1 and HindIII recognition sites of plasmid pGEM-3Z (Promega, Madison, WI), propagated in Escherichia coli (Epicurean Coli XLI-BLUE; Stratagene, La Jolla, CA), and sequenced [30] to identify clones containing either a wild type or a mutant nucleotide (A) at the first position of codon 145 of the S gene. The HBV DNA insert from each clone was purified by electroelution. After quantification of the DNA using a UV spectrophotometer (DU-50; Beckmann Instruments, Irvine, CA), wild type or mutant DNA was diluted in 10-fold or 2-fold increments with distilled water and mixed to produce standards containing different molar ratios of wild type and mutant DNAs. These DNA standards were used to determine the sensitivity of the PCR-based nucleotide assay for the detection of wild type and mutant viruses in the mixed population.
Amplification and cloning of a full-length HBV genome containing the S gene mutation for direct transfection. Chimpanzee serum (150 μL) containing mutant HBV particles was heated in a boiling water bath for 20 min. The denatured proteins were pelleted by centrifugation at 4°C for 20 min. The supernatant was adjusted to 20 mM TRIS-HCl, pH 8.0, 10 mM EDTA, and 0.1% SDS, followed by addition of proteinase K to 0.8 mg/mL. The reaction was incubated at 60°C for 4 h. Nucleic acids were purified by phenol-chloroform (1:1) extraction and ethanol precipitation after adding tRNA to 0.1 mg/mL. The full-length HBV genome (plus three extra restriction enzyme sites, SapI, SacI, and HingIII, in each flanking region) was amplified by PCR as previously described by Gunther et al. [31]. The amplified fragment was cloned into the vector PSP65. The entire cloned HBV DNA sequence was confirmed by sequence analysis.

Cell culture and transfection. The human hepatoma cell line HuH7 was used for transient expression of HBV genome DNA. The HuH7 cells were maintained in Dulbecco’s modified Eagle medium-F12 (Life Technologies GIBCO BRL, Gaithersburg, MD) in a ratio of 1:1 and supplemented with 10% fetal bovine serum. The calcium phosphate coprecipitation procedure was carried out to transfect DNA into the HuH7 cells as previously described [32] with modifications. Briefly, the plasmid PSP65/HBV was digested with either SapI (mutant) or EcoRI (wild type). The digested HBV DNA was gel-purified, and 10 μg of linear HBV DNA monomer was directly added to a 60-mm dish containing 1.5 × 10⁶ cells in fresh medium. The medium was changed at 16 h after transfection. The cells were incubated at 37°C with 3% CO₂ for 4 days until transfected cells were harvested.

Extraction and assay of viral DNA from transfected cells. Cells transfected with the surface gene mutant were frozen at −70°C for 30 min and lysed by adding 0.5 mL of lysis buffer (50 mM TRIS-HCl, pH 8.0, 1 mM EDTA, 1% NP-40) and incubated at 37°C for 10 min. The nuclei were pelleted by centrifugation. The supernatant was treated with DNase I (100 μg/mL) in the presence of 10 mM MgOAc at 37°C for 1 h. Then, the nucleocapsids were digested with Pronase at 0.5 mg/mL in the presence of 15 mM EDTA, 100 mM NaCl, and 0.5% SDS at 37°C for 1
Results

Quantification of HBV DNA in the donor serum. The amount of HBV DNA in the donor’s serum was determined on serially diluted samples by nested PCR with a primer set specific for the S gene. The DNA was detected in as little as $10^{-8}$ mL of serum (figure 2A). Since the detection limit of this PCR assay on cloned, purified HBV DNA of 3182 bp [28] was $\sim 10^{-6}$ ag (3 genomes) (figure 2B), the amount of HBV DNA in the donor serum was estimated to be 1 ng/mL.

Hepatitis B in experimentally infected chimpanzees. The course of HBV infection in 6 experimentally infected chimpanzees is shown in figure 3. Chimpanzees 1434, 1384, 1406, and 1516, which were inoculated with the donor’s serum at dilutions of $10^{-1}$, $10^{-3}$, $10^{-6}$, or $10^{-7}$, respectively, developed serologic and biochemical evidence of clinical hepatitis B. However, chimpanzee 1500, inoculated with a $10^{-6}$ dilution of serum, developed an inapparent infection, and chimpanzee 1396, inoculated with the serum at a dilution of $10^{-7}$, did not develop evidence of infection during the observation period of 25 weeks after challenge. All 6 animals were negative for markers of infection with HAV, HCV, and HDV. Thus, the infectivity titer of the serum from the donor was calculated to be $\sim 10^7$ CID$_{soi}$/mL. Serum HBsAg in chimpanzee 1434 ($10^{-1}$ dilution) was detectable for $\sim 40$ weeks, whereas detection of serum HBsAg in the other 4 infected chimpanzees was transient, with durations of 3–13 weeks.

Nucleotide and deduced amino acid sequences of the a epitope of the HBV S gene. The nucleotide sequence of a portion of the HBV S gene encompassing the a epitope was determined after PCR amplification of HBV DNA from the donor’s serum as well as that from the 5 infected chimpanzees. Except for the Arg substitution at codon 145, the predicted amino acid sequence spanning nucleotides 487–666 of the HBV DNA amplified from the serum of the donor was that of HBsAg subtype ayw [28,33–41]. The sequences of HBV DNA amplified from sera of chimpanzees 1434 ($10^{-1}$ dilution) and 1384 ($10^{-3}$ dilution) had two nucleotides at the first position of codon 145 (i.e., G [wild type nucleotide coding for Gly] and A [mutant nucleotide coding for Arg]) (figure 4). These mixtures of nucleotides did not change among serum samples collected at different time points over the period of viremia for chimpanzees 1434 and 1384 (table 1). The detection of codons for both Arg and Gly at position 145 of the S gene in chimpanzees 1434 and 1384 suggested that a small population of wild type HBV was also present in the inoculum.

In contrast, chimpanzees 1406 and 1500, each of which received the $10^{-6}$ dilution of the donor’s serum, had only nucleotide A at the first position of codon 145. However, serum from chimpanzee 1406 had both Pro (consensus) and Gln at
Figure 3. Course of experimental HBV infection in 6 chimpanzees. Chimpanzees were inoculated with serum obtained from donor who harbored surface gene mutant of HBV with mutation of Arg for Gly at codon 145 of S gene. Chimpanzee 1434 was inoculated at dilution of $10^{-1}$; chimpanzee 1384, $10^{-3}$; chimpanzees 1406 and 1500, $10^{-5}$; chimpanzees 1516 and 1396, $10^{-7}$. HBV antigens (HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen) or antibodies (anti-HBs, antibody to HBsAg; anti-HBc, antibody to hepatitis B core antigen; anti-HBe, antibody to HBeAg) in serum were regarded as positive when sample/negative control ratio was $\geq 2.1$ by RIA.

codon 120. Surprisingly, chimpanzee 1516 had a second mutation in codon 145: In addition to the G-to-A mutation in the first position of the codon, the second position was also changed from G to A, converting the encoded amino acid to Lys. The sequence of the wild type virus (GGA at codon 145) was not found in serial serum samples from chimpanzees 1406, 1500, and 1516, which received $10^{-6}$ or $10^{-7}$ dilutions of the donor’s serum.

Sensitivity of PCR-based nucleotide analysis. To distinguish wild type and mutant HBV genomes in the sera, we devised a PCR-based nucleotide assay for the specific detection of nucleotide G (wild type) or A (mutant) at the first position of codon 145 of the S gene (table 1, figure 1). The detection limit for wild type DNA (W-PCR) with primer pairs PBS1/MBS-Bgl (for first PCR) and PBS-Bam/MBS-Bam (for second PCR) was 50 ag (16 genomes) for both DNA templates (figure 5C and 5D). The detection limit for mutant DNA (M-PCR) with primer pairs PBS1/MBS-Bam (for first PCR) and PBS-Bgl/MBS-Bgl (for second PCR) was 500 ag (160 genomes) (figure 5E, 5F). Next, we determined the sensitivity of the
**Figure 4.** Direct sequencing of portion of HBV S gene amplified by PCR from sera of donor and infected chimpanzees 1434, 1384, and 1406. A, Sense direction; B, antisense direction. Nucleotide at 1st position of codon 145 is indicated at right-hand side of sequence.

**Table 1.** Identification of wild-type and mutant viruses in progeny virus recovered from chimpanzees experimentally inoculated with different dilutions of original donor serum.

<table>
<thead>
<tr>
<th>Chimpanzee</th>
<th>Dilution of donor serum inoculated</th>
<th>Weeks HBsAg-positive*</th>
<th>Weeks tested</th>
<th>Any HBV</th>
<th>Wild type</th>
<th>S gene mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1434</td>
<td>$10^{-4}$</td>
<td>5–45</td>
<td>5, 8, 18, 30</td>
<td>5, 8, 18, 30</td>
<td>5, 8, 18, 30</td>
<td>5, 8, 18, 30</td>
</tr>
<tr>
<td>1384</td>
<td>$10^{-3}$</td>
<td>7–11</td>
<td>7, 9, 11</td>
<td>7, 9, 11</td>
<td>7, 9, 11</td>
<td>7, 9, 11</td>
</tr>
<tr>
<td>1406</td>
<td>$10^{-5}$</td>
<td>12–22</td>
<td>12, 13, 15, 18, 21</td>
<td>12, 13, 15, 18, 21</td>
<td>None</td>
<td>12, 13, 15, 18, 21</td>
</tr>
<tr>
<td>1500</td>
<td>$10^{-5}$</td>
<td>11–13</td>
<td>11, 12, 13</td>
<td>11, 12, 13</td>
<td>None</td>
<td>12, 13</td>
</tr>
<tr>
<td>1516</td>
<td>$10^{-5}$</td>
<td>16–28</td>
<td>16, 17, 18, 20, 24, 28</td>
<td>17, 18, 20, 24</td>
<td>None</td>
<td>17, 18, 20, 24</td>
</tr>
<tr>
<td>1396</td>
<td>$10^{-7}$</td>
<td>None</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

NOTE. HBsAg, hepatitis B surface antigen; PCR, polymerase chain reaction; NT, not tested.

* Weeks after inoculation with indicated dilution of donor serum.

† All viruses recovered from chimpanzee 1516 contained, in addition to G-to-A mutation in 1st nucleotide of 145th codon (Gly→Arg), additional mutation (also G to A) in 2nd nucleotide of codon 145 (Arg→Lys), as determined by direct sequencing of PCR product amplified with primers capable of amplifying both wild type and mutant virus.
PCR-based nucleotide assay in detecting a small population of both wild type and mutant DNAs by following the procedure shown in figure 1. The templates were the pre-S/S gene DNA fragments, which were mixed to consist of different molar ratios of wild type and mutant DNAs. For a total of 1 ng of the mixed DNA templates prepared in 10-fold dilutions, both W- and M-PCR consistently detected the minor component down to one one-hundredth of the total DNA content of the mixed sample (figure 6A and 6B). This detection limit was reproducible when the total amount of the template was reduced to 1 pg (figure 6C, 6D). On a total of 1 ng of the mixed DNA templates separately prepared by 2-fold dilutions, W-PCR detected wild type DNA in the presence of a 512- to 1024-fold greater concentration of mutant DNA and M-PCR detected mutant DNA in the presence of a 256- to 512-fold greater concentration of wild type DNA (figure 7A, 7B). These detection limits were, again, reproducible on a total of 1 pg of the template DNA (figure 7C, 7D). The specificity of the 134-bp band in the final products was confirmed by cloning each DNA fragment into the BamH1 site (for wild type DNA) or the BglII site (for the mutant DNA) of the plasmid vector pSP72 (Promega), followed by sequencing 12 clones. Thus, our PCR-based nucleotide analyses could detect a minor wild type DNA population that was 0.1%–0.2% of the total DNA and a minor mutant DNA population that was 0.2%–0.5% of the total DNA.

PCR-based nucleotide assay of serum from the donor and from HBV-infected chimpanzees. Results of the PCR-dependent nucleotide analysis of codon 145 of the HBV S gene in serum samples from the donor and selected chimpanzees are shown in figure 8. Serum from the donor yielded a faint 134-bp band among the final products of W-PCR (figure 8A). This DNA fragment was confirmed to be from wild type DNA by cloning into the BamH1 site of plasmid pUC18 (Pharmacia LKB Biotechnology, Piscataway, NJ) and sequencing. Thus, the donor’s serum was shown to contain a small population of wild type virus in the presence of an excess amount of mutant virus that was clearly detected by the M-PCR (figure 8B). Serum from chimpanzees 1384 (10-1 dilution) and 1434 (10-3 dilution) had the 134-bp band in both W-PCR (figure 8A) and M-PCR (figure 8B), confirming the results obtained by direct sequencing and demonstrating that these sera consisted of a mixed population of wild type and mutant viruses. In contrast, in serum from chimpanzee 1406, the 134-bp band of wild type DNA could not be detected in W-PCR (figure 8A), while that of the mutant was clearly observed in M-PCR (figure 8B).

Identical data were obtained with sera from chimpanzee 1500 (data not shown). In contrast, W-PCR and M-PCR assays were both negative or irreproducible when sera from chimpanzee 1516 were tested. Sequencing of product obtained by PCR amplification of HBV sequences from the sera of chimpanzee 1516 with other primers (see Materials and Methods) revealed...
that a second mutation, in the second position of codon 145, had appeared, probably early in the infection of this chimpanzee. This mutation markedly diminished the sensitivity and specificity of the W-PCR and M-PCR assays, since they were based upon specific mismatches in codon 145. However, the finding of two other mutations associated with the mutant virus in the sequences obtained from chimpanzee 1516 confirmed that the chimpanzee was infected with the mutant virus, not the wild type virus. These data indicated that the sera from chimpanzees 1406, 1500, and 1516, which were inoculated with 1 or 10 CID₉₀ (32 or 320 genomes, respectively) of HBV from the donor, probably contained pure populations of mutant virus. These patterns were observed in serial serum samples collected throughout the viremia of the chimpanzees (table 1).

In addition, serially diluted serum samples from the donor were analyzed. As shown in figure 9, wild type HBV DNA was clearly detected in 10⁻³ mL of the serum, but at other dilutions as high as 10⁻⁶ mL of the serum, only the mutant virus DNA was detected. The results indicate that a small population of wild type virus existed in the serum, and this was confirmed by inoculation of chimpanzees 1434 (10⁻¹ dilution) and 1384 (10⁻³ dilution). However, only mutant virus was detected in chimpanzees inoculated with higher dilutions of the serum. Applying the correction factors found for the sensitivities of the PCR assays for wild type and mutant virus, respectively, the original donor serum can be calculated to contain 10⁸.2 genomes of any HBV, 10⁴.2 genomes of wild type virus, and 10⁸.2 genomes of mutant virus/mL. The latter figure is remarkably close to the titer of 10⁸.5 genomes of HBV/mL in the serum as measured by the standard PCR assay. Thus, the titer of mutant virus is calculated to be 10⁴ higher than that of the wild type virus.

Because 10⁻⁵ mL of the serum from the donor contained ~10 ag (3.2 genomes) of HBV DNA (figure 2), 10⁻⁷ mL of serum, in which only the mutant virus genome was detected, would be estimated to contain ~32 HBV molecules. Since this has been shown to be the minimum amount of virus required for the establishment of HBV infection [42], we are of the opinion that the pure genomic populations of mutant viruses in the sera of chimpanzees 1406, 1500, and 1516 are the consequence of infection with only the mutant virus (or its derivative) and that the mixed populations of wild type and mutant virus detected in sera from chimpanzees 1434 and 1384 are the result of coinfection with both viruses.

Replication in cultured cells of an HBV surface gene mutant isolated from an infected chimpanzee. To confirm that the HBV surface gene mutant was viable, its ability to replicate in vitro was determined. The full-length mutant viral genome was
amplified from the serum of inoculated chimpanzee 1406 and cloned into vector PSP65. Linear HBV genome monomers were released from the vector by digestion with SapI. The full-length HBV genome monomers were directly transfected to HuH7 cells. Thus, the linear HBV mutant monomers were able to recircularize in transfected cells through a ligation of the sticky ends of SapI at each end of the genome. Replication of the mutant in the transfected cells was compared with that of a wild type plasmid processed in a similar manner (figure 10). The HBV surface gene mutant produced a normal level of replicative intermediates within nucleocapsids, compared with those produced by a wild type HBV genome.

Discussion

In the present study, we demonstrated that an HBV mutant with an amino acid substitution of Arg for Gly at codon 145 of the S gene was infectious and capable of inducing hepatitis in seronegative chimpanzees. The mutation had emerged in an infant who was born to an HBV carrier mother and received combined active and passive immunoprophylaxis against HBV with subsequent development of anti-HBs [16, 17]. Because it is important to identify and characterize mutant HBV strains in nature, we devised a simple PCR-based method to distinguish between wild type and mutant viral genomes. Rapid and non-radioactive PCR-dependent methods for the detection of a specific nucleotide have been successfully applied to studies of the human genome. One such PCR-dependent method is selective amplification of DNA containing a specific nucleotide with primers that are perfectly complementary to the terminal 3' nucleotide of the template. In this case, the DNA with the complementary nucleotide is efficiently amplified, whereas that with a mismatched nucleotide is not amplified [43-45]. However, since templates can be amplified by primers with mismatched nucleotides even at the extreme 3' terminus [46], this approach has limitations.

One other PCR-dependent method is a selective enrichment of DNA possessing a specific nucleotide by using primers to create a novel restriction endonuclease recognition site in DNA not containing the specific nucleotide. By digestion with a restriction endonuclease, DNA not containing the specific nucleotide is digested, whereas that with the specific nucleotide
is not cleaved. This procedure is followed by the subsequent amplification of the uncut DNA molecules by a second round of PCR [47–49]. The limitation of this method is that noncleaved fragments are identified as containing a specific nucleotide and stringent PCR conditions are required so that in vitro-generated mutations, caused by the lack of 3'-to-5' exonuclease activity of Taq polymerase [29], will not occur.

To overcome these problems, we devised a method, reported herein, to detect a specific nucleotide in a restriction endonuclease-cleaved DNA fragment. Our method can be performed using standard PCR conditions and, therefore, is highly reproducible and reliable. However, it is slightly less sensitive for detecting a minor population (i.e., ≤0.1%–0.2% of wild type and ≤0.2%–0.5% of mutant virus) than the methods described above (i.e., <0.1%) [47–49]. These differences may be attributable to the number and position of the mismatched nucleotides introduced into the primers and to the molecular state of the final products, since those in our method are homoduplex DNA molecules whereas those in the other methods include heteroduplex DNA molecules [50, 51]. Also, as seen in the analysis

Figure 8. Polymerase chain reaction–based nucleotide analysis of serum from donor and infected chimpanzees 1434, 1384, and 1406 for detection of wild type (A) or mutant HBV DNA (B). Negative controls (distilled water) and positive controls of wild type (W) and mutant (M) 1496 bp-preS/S gene DNAs, either individually or mixed as indicated, were processed exactly as serum samples. Sizes of amplified DNA fragments are shown at right.

Figure 9. Polymerase chain reaction–based nucleotide analysis in serially diluted serum from donor (D.) for detecting wild type HBV DNA (A) or mutant HBV DNA (B). Sizes of amplified DNA fragments are shown at right.
of chimpanzee 1516, the addition of another mutation in the
codon of interest (G-to-A mutation in the second nucleotide of
codon 145) can render this PCR assay ineffective. However,
sequencing of the region of interest permitted a correct classiﬁcation
to be determined.

Our results, obtained by PCR-based nucleotide analysis,
showed that the distribution of viral genomes in the inoculum
was similar to that in the serum from infected chimpanzees.
Serum from the donor at dilutions of 10^{-3} and 10^{-4}, containing
a mixed HBV population, resulted in the replication of both
wild type and mutant viruses in the infected animals (chimpan­
zees 1434 and 1384). Inoculation of chimpanzees with the
donor’s serum at dilutions of 10^{-6} or 10^{-7} (the latter was at
the end point of infectivity), containing exclusively mutant
virus, resulted in the detection only of mutant viral genome in
serial serum samples from the infected animals (chimpanzees
1406, 1500, and 1516). Our results also showed that the ratio
of the mutant to wild type viral genomes did not change throughout
the course of infection in the other animals. Taken
together, we consider that the HBV mutant with an Arg substi­
tution at codon 145 of the S gene (or its derivative doubly
mutated virus with Lys at that codon) is infectious and does
not readily revert to a wild type genotype. Furthermore, it
is highly unlikely that wild type virus might have complemented
mutant virus infection of chimpanzees 1406, 1500, and 1516
because of the stoichiometric results obtained with the PCR
assays and infectivity titration, which clearly indicate a 10,000-
fold difference in the titers of wild type and mutant virus.
Finally, we PCR-ampliﬁed and cloned the S gene mutant virus.
We transfected HuH7 cells with the clone of the mutant virus
and compared its replication with that of a known infectious
clonal of wild type virus. The clone of the S gene mutant
virus was completely sequenced, and the only mutation was
the expected one in codon 145 of the S gene. Replication of
the two viruses in vitro was indistinguishable, conﬁrming that
both were viable. Thus, both by classical biologic and molecu­
lar biologic methods we have demonstrated that the S gene
mutant virus is capable of replication without a wild type virus.

Different clinical proﬁles were observed in the infected
chimpanzees. Chimpanzee 1434 had a lengthy serum ALT ele­
vation, while chimpanzee 1384 had a very high peak value of
serum ALT that persisted brieﬂy. It is at present unclear
whether coreplication of wild type and mutant virus in these
2 animals modiﬁed the clinical features of hepatitis B. It is
possible that phenotypic mixing [52] of the envelope proteins
of wild type and mutant viruses caused a biologic modiﬁcation
of the virus. However, the patterns observed in the other chimp­
zanzees were similarly variable. Overall, the incubation periods
and patterns of infection seen in these animals were no different
from those observed in chimpanzees inoculated with dilutions
of nonmutant HBV [53].

One other HBV S gene mutant with an Arg substitution at
codon 145, which emerged in a liver transplant patient during
treatment with a human monoclonal anti-HBs antibody, was
infectious in a seronegative chimpanzee [21]. However, that
HBV mutant seemed to possess a much lower infectivity and
a reduced ability to replicate than was the case with the HBV
mutant studied here. Genetic variation other than the Arg muta­
tion may account for such biologic differences among the S
gene mutants but did not appear to play a role in this study.

Most persons possessing the HBV mutant with Arg at codon
145 of the S gene who have been identiﬁed thus far received
postexposure immunoprophylaxis against HBV, either after
birth [16–20] or accompanying liver transplantation [21, 22].
This suggests that such mutant strains were generated de novo
from already established wild type virus infections in these
persons. The fact that such mutants show altered afﬁnities to
monoclonal anti-HBs antibodies directed against the a determi­
nant compared to wild type viruses [17, 20, 21] and the ﬁnding
that the HBV mutant studied here did not bind to anti-HBs in
convalescent sera or sera from vaccinees [54] would strongly
support this notion. However, although codon 145 resides
within a disulfide-linked protein loop, which is important in
maintaining the conformation of the a determinant [55–60],
the exact sequence(s) that is involved in interacting with protec­
tive or neutralizing antibodies against HBV has not been de­
deﬁned. Accordingly, the signiﬁcance of the substitution of the
amino acid 145 in the phenotypic alteration of the virus to
evade neutralization in the host is unclear. Therefore, the deter­
mination of whether or not such HBV S gene mutants are

Figure 10. Analysis of replication in cultured cells of HBV S mutant
genome isolated from infected chimpanzee 1406. Linear HBV ge­
nome monomers with Sapi site at each end were transfected into
HuH7 cells. Transfected cells were harvested 4 days after transfection.
Viral DNA replicative intermediates were isolated from nucleocapsids
and separated by gel electrophoresis in 1% agarose. Then, HBV DNA
was transferred to nylon membrane and hybridized with a ^32P-labeled
strand-speciﬁc probe. Hybridized blot was exposed for autoradiogra­
phy. Double-strand (DS) and minus-strand DNA (MS) are indicated.
Plus-strand ONAs, of various sizes, are distributed between DS and
MS. WT, wild type; MT, mutant; MOCK, negative control (mock-infected).
genuine neutralization-escape mutants should be made after evaluation of the infectivity of this strain in both susceptible and anti-HBs-positive hosts. Such a study is in progress.

In conclusion, we demonstrated that an HBV mutant with an Arg substitution at codon 145 of the S gene was infectious and pathogenic in seronegative chimpanzees. We also reported a simple method to distinguish between wild type and mutant viral genomes. This method may be useful for investigation of the biology and epidemiology of such mutants, especially in cases in which they exist in low abundance or encode an HBsAg that cannot be detected by conventional serologic assays.

Acknowledgments

We thank N. Caporaso for supplying clinical materials from the patient, Max Shapiro, Ronald Engle, Doris C. Wong, and Tatiana Tsareva for excellent technical assistance, Laurie L. Moore and Carmane Hutton for excellent clerical assistance, and Suzanne U. Emerson for thoughtful review of the manuscript.

References


