Genotype Mixtures of Hepatitis B Virus in Patients Treated with Interferon

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Little is known about coinfection among several hepatitis B virus (HBV) genotypes, although previous reports of recombination and genotype shifts indicate that this should occur. In the present study, we designed a method to identify mixtures of genotype A and another genotype, regardless of whether one of them predominates. Using this method, signs of genotypic coinfection were found in 20 (67%) of 30 hepatitis B e antigen–positive patients treated with interferon (IFN). In 8 of these patients, coinfection or genotype shifts were detectable by direct sequencing or standard preS genotyping. In most of these cases, genotype changes were detected after a >2-log decrease or increase of the HBV DNA level. The presence of genotype mixtures did not significantly influence IFN response. Because quasi-species selection may occur at or shortly after transmission, patients might acquire HBV infection from subjects who appear to be infected with a different genotype. This should be considered when tracing the source of HBV infection.

Hepatitis B virus (HBV) is an important cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma, and >350 million people worldwide carry the virus. HBV has been classified into 6 genotypes, A–F [1, 2], and are primarily found in different geographical areas: A in Europe and sub-Saharan Africa, B and C in East Asia, D in the Mediterranean and Middle East regions, E in western Africa, and F in North and South America [3]. Recently, an additional genotype, G, has been observed in France and the United States [4]. Although the quasi species nature of HBV infection has been documented [5–7], reports about infection with >1 genotype are lacking.

We recently found that mutations in the core promoter region were rare before and after interferon (IFN) treatment [8]. However, we also observed genotype changes in patients showing relapse after IFN treatment, and we examined this phenomenon further in the present study. Genotype switches from A to D have been observed elsewhere by others after IFN response [9]. Such switches indicate that mixtures of 2 genotypes are present prior to treatment, but that the minor quasi species is not detectable by standard genotyping assays or direct sequencing.

To investigate the frequency of genotype mixtures/coinfection and to what extent they are revealed by IFN treatment, we analyzed serum samples from before and after IFN treatment using a genotype-specific polymerase chain reaction (PCR), followed by restriction fragment length polymorphism (RFLP) analysis and sequencing.

Patients, Materials, and Methods

Patients and serum samples. The patients in the present study originate from a European multicenter study of IFN treatment (with or without steroid priming) for hepatitis B e antigen–positive chronic hepatitis B (the INTERPRED study, 1987–1990 [10]). When analyzing HBV DNA in serum samples from 98 patients in that study by quantitative PCR (Amplicor; Roche), we identified virological response as a reduction of HBV DNA to <106 copies/mL, including also a 99% (1.5-log) reduction of viremia [11]. In the present study, we analyzed serum samples drawn at baseline (sample 1; S1), end of treatment (sample 2; S2), and follow-up (sample 3; S3) of 30 patients representing different response patterns: sustained responders (SR), relapser (Rel), late responders (LR), and nonresponders (NR). The SRs (n = 9) had a virological response both at end of treatment and follow-up 6 months posttreatment. The Rel (n = 4) had a response at end-of-treatment but not at follow-up. The LRs (n = 9) showed a response only at follow-up, and the NRs (n = 8) showed no virological response at all.

All the patients were given human lymphoblastoid IFN (Wellferon) at a dose of 10 MU daily during a 1-week induction phase, followed by 10 MU three times weekly for 11 weeks further. Prior to IFN treatment, 12 patients had been given placebo, whereas 18 had received a tapered pretreatment with prednisolone (2 weeks of 0.6 mg/kg/day, followed by 1 week each of 0.45 mg/kg/day and 0.25 mg/kg/day), followed by a 2 week rest phase prior to IFN treatment.

None of the patients admitted injection drug use. Twelve were male homosexuals.

Quantitative PCR. Serum samples were analyzed by Amplicor.
Figure 1. Primers designed for genotype-specific polymerase chain reaction. Primer A (nt 2356–2384) has 5 mismatches for non-A genotypes but only 1 mismatch for genotype A (5 nt from the 3' end). Primer non-A (nt 2350–2378) has 5 mismatches for genotype A but only 1 mismatch for non-A genotypes (5 nt from the 3' end). The 6-bp insertion typical for genotype A is boxed. Homologies are indicated by dots. Primer sequences are complementary.

HBV Monitor (Roche Diagnostic Systems), according to the manufacturer’s instructions. The linear detection range for this test is 10^3–10^7 copies/mL. To further expand this range, the samples were prediluted in HBV-negative serum up to 1:10^5.

Sequencing of X and precore regions. The entire X open reading frame and the precore region were analyzed by direct sequencing. After PCR of 2 overlapping regions using primer pairs 1351 (forward 5'-TCGCGGAAATATACATCGTTTCC) and 1798 (reverse 5'-ACCAATTTATGCCTACAGCC), and 1680 (forward 5'-ATGTCGACAACCGACCTTGA) and 1933 (reverse 5'-AGCTCCTAATTCTTTATAA), respectively, sequencing was done by the chain-termination method. Each amplicon was analyzed in both sense and antisense direction, with the same primers as in PCR in a cycle-sequencing reaction using fluorescent dye terminators (dNTPs). The sequence was read in an ABI Prism 310 automated capillary sequence reader (Applied Biosystems) and then processed using the Sequence Navigator software (Applied Biosystems).

Core RFLP. To investigate the possible presence of genotype mixtures, core-region primers specific for A and non-A genotype were designed. These antisense primers (for A, reverse P2384, 5'-TTCTTCTTCTTAGGGACCTCGCTCAGTCC and for non-A, P2384 reverse 5'-TTCTTCTTCTTAGGGACCTCGCTCAGTCC) were located at nt 2356–2384 (nt 2350–2378 in non-A genotypes), with the 3' end of the primer annealing at the position of the 6 bp insertion specific for genotype A. As shown in figure 1, the specificity of the 3' end of these primers promotes amplification of even a minor fraction of a non-A strain in the presence of predominant A strains or vice versa. Each sample was run in PCR in 2 parallel reactions with either reverse primer P2384 (A) or reverse primer P2384 (non-A) as antisense primers and primer P1865 (forward 5'-CAAGCCTCAAGCTGCTGCTTGAGG) as sense primer, using a relatively high annealing temperature (62°C) to enhance specificity. The amplicons were then incubated with the restriction enzyme Tsp509I, and the observed patterns (figure 2) were compared with the expected fragment sizes (table 1).

Core sequencing. To confirm the accuracy of the core specific PCR-RFLP and to further analyze the strains in mixed infections, amplicons from the core PCR were directly sequenced. The sequences were aligned and phylogenetically compared with database sequences representing genotypes A–F using the MEGA2 software [12]. The GenBank accession numbers of the sequences are AF474378–AF474403.

preS PCR and RFLP. Assessment of the proportion of genotypes present in individual samples was done using 2 strategies. In samples that by core RFLP had shown mixtures of genotypes A and D, the preS region was amplified by primers 2823 (forward 5'-TCACCATATTCTTTGGAGAACAGA) and 3098 (reverse 5'-GCAGGGAGGCCGATTTCG). By this PCR, HBV of genotype A produces amplicons of 276 bp, and genotype D produces amplicons of 243 bp. The relative HBV DNA concentration of genotypes A and D in the sample could thus be approximated from the volume intensity of each of these bands on gel electrophoresis. For analyzing mixtures of genotype A and C or E, which produce indistinguishable band sizes after PCR alone, the relative proportions were estimated after further analysis of the preS amplicons by RFLP [13]. These methods allow detection of minor strains constituting >10% of the total viral population.

preS cloning. In 4 serum samples from 2 patients, genotype mixtures were evaluated after cloning. Introduction of preS amplicons into the pCR4-TOPO vector (Invitrogen) and cloning by use of the TOPO TA Cloning Kit (Invitrogen) were done according to the instructions given by the manufacturer. Colonies were dissolved in buffer and subjected to preS PCR. The genotype was assessed directly from the agarose gel by the size of the amplicon

Figure 2. Tsp509I cleavage of core-region polymerase chain reaction product showing (A) typical patterns representing genotypes A, C, D and E and (B) atypical patterns with multiple bands indicating the presence of 2–3 quasi species.
were seen in 15 samples (table 2, figure 2).

D when HBV DNA had risen by 2.4 log. In addition to these
E strains, respectively, were no longer detected at follow-up
2 patients, there were only minor changes in viremia (SR2 and
(6 D and 1 E) appeared. In 4 of these (LR3, LR8, SR1, and
in serum samples in which core RFLP indicated mixed infection, amplicons derived from PCR using
the reverse primer P2384 (A) or the reverse primer P2384 (non-
A) were further analyzed by sequencing and phylogenetic anal-
ysis (figure 4). The results closely agreed with the RFLP inter-
pretation and confirmed that the minor strain really belonged
to a different genotype. Moreover, the presence of quasi species,
suggested by RFLP, was supported by the identification of am-
biguous nucleotide positions on direct sequencing (figure 3). In
addition to the differences at the Tsp50I sites, the quasi species
were found to differ at 1 to 15 nt positions.

preS analysis of genotype mixtures. By preS analysis, which
aimed at estimating the relative proportions of genotypes,
mixed infection could only be detected in 8 samples from 6
patients (LR4, LR9, Rel1, SR1, SR3, and SR6; table 2). In one
other patient (Rel4), a genotype change was identified. The
proportions of genotypes A and D during treatment are de-
scribed in table 3. The disappearance of a genotype, as detected
by preS analysis, coincided with a decrease of 1.8 log (LR9) in
HBV DNA, or with an increase of 3.5 log (Rel1) or 2.4 log
(Rel4). The emergence of a new genotype coincided with a
1.1–3.5-log decrease (SR1 and Rel1) or a 0.4–2.4-log increase
(SR3, SR6, and Rel4) in HBV DNA. Genotypic coinfections
or shifts detected by preS RFLP or core RFLP, in relation to
IFN response, are shown in figure 5.

preS analysis after cloning. In patient LR9, direct PCR-
RFLP had indicated a genotype A/D mixture with a 60:40 ratio
before treatment. In this sample, 42 clones were genotype A,
and 12 clones were genotype D, as determined by preS amplicon
band size. In addition, 18 clones were sequenced, with results
confirming the interpretation by preS band size (9 A and 9 D).
In the sample collected after IFN treatment, in which only
genotype D was observed by direct PCR-RFLP, all 22 clones
showed genotype D.

In patient NR4, preS RFLP had shown only genotype A,
both before and after treatment, but genotype-specific PCR,
including confirmatory sequencing, had revealed a minor ge-
notype D strain. This genotype D strain could not be identified,
even by analysis of a large number of clones (110 before treat-
ment and 60 after treatment), indicating that it was present in
<1% of the viral population.

Discussion

The results of the present study suggest that genotypic coin-
fection is frequent in chronic HBV infection, but, in general,
one genotype predominates and is detected by direct sequencing
and standard genotyping. When analyzed by a genotype-spe-
cific core region PCR-RFLP, a mixture of or shift between
genotypes was observed in 20 (67%) patients before or after
IFN treatment.

In a majority of patients, coinfection was detectable only by
the core PCR-RFLP assay. The assay uses a 4-nt difference

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Amplicon</th>
<th>Nucleotide positions of Tsp50I sites</th>
<th>Size of fragments, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>520</td>
<td>2091, 2159</td>
<td>168, 226</td>
</tr>
<tr>
<td>B</td>
<td>514</td>
<td>2091, 2159, 2196</td>
<td>168, 183</td>
</tr>
<tr>
<td>C</td>
<td>514</td>
<td>2148</td>
<td>200, 231</td>
</tr>
<tr>
<td>D</td>
<td>514</td>
<td>2074</td>
<td>151, 256</td>
</tr>
<tr>
<td>E</td>
<td>514</td>
<td>2196</td>
<td>183, 200</td>
</tr>
<tr>
<td>F</td>
<td>514</td>
<td>2188</td>
<td>191, 200</td>
</tr>
</tbody>
</table>

* Obtained by primer pairs 1865/2384 in A strains and 1865/2378
in non-A strains.

b In addition, all genotypes have Tsp50I cleavage sites at positions
1923 and 2123.

c Only fragments >150 bp are shown.

Results

Sequencing of X and precore regions. By direct sequencing,
genotype A was found in 27 patients, genotype D in 2 patients,
and genotype C in 1 patient before treatment (table 2). At end
of treatment, a change of genotype was observed in 2 cases, from
C to A in Rel1 and from A to E in Rel2. At follow-up, when
viremia levels had returned to pretreatment levels in these 2 pa-
tients, the genotypes C and A, respectively, reappeared. In 1
patient, Rel4, genotype D emerged in the follow-up sample.

Genotype-specific core PCR and RFLP. By this assay, ge-
notype mixtures were found, in at least 1 sample, in 20 (67%)
of the 30 patients (table 2). Before treatment, mixtures were
detected in 9 samples from patients (30%): LR4, LR5, LR9,
NR3–4, NR7, and Rel2–4. At the end of treatment, one of the
genotypes was no longer detectable in 4 patients. In 3 of them
this coincided with a 1.4-log (LR5), 1.8-log (LR9) or 3.1-log
(Rep3) reduction of viremia, whereas, in NR3, only minor (0.3-
log) reduction of viremia was seen. In 4 other patients (LR6,
Rel1, SR6, and SR7), an additional genotype was detected at
the end of treatment in parallel with a radical reduction of
viremia (3.3, 3.5, 5.4, and 4.3 log). At follow-up, further changes
were seen in 10 patients. In 7 patients, an additional genotype
(6 D and 1 E) appeared. In 4 of these (LR3, LR8, SR1, and
SR5), viremia was reduced by 3.0, 2.0, 1.1, and 2.1 log, re-
spectively; in another (NR8), viremia increased by 3.1 log to 4
× 10^6 copies/mL as a genotype D strain emerged, whereas in
2 patients, there were only minor changes in viremia (SR2 and
SR3). In contrast, in SR7 and NR7, the minor genotype D and
E strains, respectively, were no longer detected at follow-up
when HBV DNA had decreased by 1.0 log or increased by 1.5
log. In 1 patient (Rel4), the minor strain changed from E to
D when HBV DNA had risen by 2.4 log. In addition to these
genotype mixtures, RFLP patterns with multiple bands indi-
cating the presence of 2–3 quasi species of a single genotype
were seen in 15 samples (table 2, figure 2B, and figure 3).
between genotype A and non-A at the 3' end of the antisense primer to identify minor non-A strains in a background of genotype A (or vice versa). Genotype mixtures were not detected by direct sequencing in any of the subjects before treatment. However, in 3 patients, all of whom had relapsed, genotype shifts were identified by direct sequencing when IFN dramatically changed the levels of viremia. In addition, radical changes in the relative proportion of viral genotypes were observed in 6 patients with sustained response.

Similar findings were reported elsewhere, in a German study, in which genotype shifts were observed by direct sequencing when IFN completely took over.

Because none of the patients admitted injection drug use, sexual contact, or transfusion as a mode of transmission, it is possible that some of the patients became superinfected with the additional strains. For example, the proportion of genotype A in LR9 changed from ~60% to undetectable at follow-up, at which point the minor genotype D strain had completely taken over.

The very high frequency of genotypic co-infection suggests that chronic HBV carriers often become superinfected with additional strains or that de novo infection with genotype mixtures is common. Although past infection with one genotype confers immunity against other genotypes, protection against superinfecting strains may well be absent in chronic infection, which is characterized by a weak and ineffective immune response against HBV. That genotypic co-infection has rarely been observed may be because it is difficult for a superinfecting strain to establish itself when HBV infection is already present throughout the liver. In our series of patients, genotype A, which is considered to be the genotype originally prevailing in northern and western Europe, was observed in all patients. In most cases, the coinfecting strain was of genotype D, which has increased in Europe as a result of immigration [3]. Genotype E, which originates from western Africa, was found in 4 subjects. The coexistence of genotype A and D or E in these patients, most of whom were from southern France, probably reflects immigration to this region of HBV carriers from the Mediterranean area and western Africa.

It is not clear whether the high frequency of genotypic co-infection is representative of chronic HBV infection in general. The frequency of coinfection (or superinfection) could depend on the routes through which the HBV infection is acquired, and the mode of transmission was not known for most patients.
Figure 3. Core nucleotide alignment of samples directly sequenced after genotype-specific polymerase chain reaction using antisense primer specific for genotype A or non-A. Reference sequences of genotypes A, C, D, and E, as well as sequences from samples representing different strains and all observed cleavage patterns, are shown. International Union of Pure and Applied Chemistry codes are used at positions in which double curves indicated quasi-species mixtures (Y, CT; R, A/G; M, C/A; W, A/T). Tsp509I sites (AATT) are shaded.
transmission seems most probable. However, coinfection might first have been acquired by injection drug use in subjects who then introduced it into HBV carrier population by other routes, for example, sexually. Of note, 12 patients were male homosexuals, but coinfection was not more common in that group than it was among the other patients.

We were concerned that the finding of small fractions of genotype D in samples with genotype A might be due to contamination from samples with high levels of genotype D. However, this was ruled out by sequencing of the minor genotype D strains, which showed that these were not identical but differed by a mean of 2.7% (range, 1.3%–3.8%).

The results may be relevant for contact tracing (i.e. for identifying the source of HBV infection). Selective transmission of minor quasi species has been reported for human immunodeficiency virus [14], hepatitis C virus (HCV) [15], and HBV [5]. In a study by Ngui et al. [16], a strain representing only 7% of the circulating HBV clones in a surgeon was found to be transmitted to (and selected in) a patient. In another study, HBV from 3 surgeons was analyzed by sequencing of a large number of clones [6]. In 2 of these surgeons, the described quasi species did in fact include strains of a different genotype (although this was not commented). In both of these cases, only the major quasi species was found in the patients. Still, the data from these studies and the present study indicate that an individual might acquire infection from a subject who appears to be infected with a different genotype. This has, to our knowledge, not yet been reported.

Genotypic coinfection might also be clinically important if it influences the response to IFN. Our finding of major genotype changes in 3 of 4 patients who relapsed could indicate such an impact. However, in only one of them was the clinical relapse paralleled by increasing viremia of a new genotype to high levels. Thus, in general, the emergence of a new or additional genotype after IFN therapy did not reflect treatment failure. Rather, in several cases, the radical changes in viremia during treatment revealed the minor strain and, thus, the genotypic coinfection. Accordingly, genotype changes detectable by standard methods (direct sequencing or preS RFLP) were typically paralleled by a >1–2-log increase or decrease in HBV DNA level. However, changes in the relative proportion of 2 genotypes may take place also when the total HBV DNA level is largely unaltered. The genotype shifts suggest that IFN influences the genotypes differently. This could be because of genotype differences in the inhibition of viral replication. Alternatively, IFN may enhance the immune response toward the major strain more, thus allowing the minor strain to take over or increase.

Our findings agree with reports elsewhere on HBV recombination [17–19], for which genotypic coinfection is a prerequisite. Recombination has been found between both genotypes A and

### Table 3. Estimated relative proportions of genotypes in samples in which coinfection was detectable by preS polymerase chain reaction–restriction fragment–length polymorphism analysis.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sample</th>
<th>Genotype (%) of sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>LR4</td>
<td>S1</td>
<td>A (15), D (85)</td>
</tr>
<tr>
<td></td>
<td>S2</td>
<td>A (15), D (85)</td>
</tr>
<tr>
<td></td>
<td>S3</td>
<td>A (10), D (90)</td>
</tr>
<tr>
<td>LR9</td>
<td>S1</td>
<td>A (60), D (40)</td>
</tr>
<tr>
<td></td>
<td>S2</td>
<td>A (65), C (35)</td>
</tr>
<tr>
<td>Rel1</td>
<td>S2</td>
<td>A (65), C (35)</td>
</tr>
<tr>
<td>SR1</td>
<td>S3</td>
<td>A (90), D (10)</td>
</tr>
<tr>
<td>SR3</td>
<td>S3</td>
<td>A (85), D (15)</td>
</tr>
<tr>
<td>SR6</td>
<td>S3</td>
<td>A (80), D (20)</td>
</tr>
</tbody>
</table>

*LR, late responder; Rel, relaper; SR, sustained responder.*
Hepatitis B virus (HBV) DNA levels during interferon (IFN) treatment in patients with genotype mixtures detected by standard restriction fragment–length polymorphism (RFLP) genotyping or by sequencing before (A) and after (B) IFN therapy or detectable only by genotype-specific core-region polymerase chain reaction (PCR)–RFLP before (C) and after (D) IFN therapy. Strains detectable by standard RFLP or direct sequencing are in uppercase. Minor strains, detectable only by core PCR-RFLP (<5–10% of total population) are in lowercase. For Rel2, preS RFLP showed genotype E, whereas X-region sequencing showed genotype A. LR, late responder; NR, nonresponder; Rel, relapser; S1, S2, and S3, sample 1, 2, and 3; SR, sustained responder.

D, which coexist in Europe, and between genotypes B and C, which coexist in eastern Asia [18]. Genotypes B and C both occur at a high prevalence in large areas of eastern Asia, and it should be possible to identify coinfeciton with them, in particular, in patients treated with IFN. For this purpose, genotype-specific PCR would be useful. This technique has recently been used in a study of HCV infection and was found to be superior to cloning and sequencing for identifying minor viral populations [20]. The method used in the present study was designed to identify coinfection between genotype A and other genotypes. By using the genotype A–specific 6 bp insertion at nt 2351–2356 to create a 4–5-nt mismatch region at the 3′ end of the reverse primer, this method admitted amplification and identification of strains undetectable by standard methods, including cloning. Cloning was performed on a few samples and confirmed the coinfeciton detected by preS RFLP. However, coinfeciton with minor strains, detectable only by genotype-specific core RFLP, was not identified, despite analysis of >150 clones. This supports the finding that the genotype-specific core PCR identifies minor coinfeciton more efficiently than does cloning.

In summary, we found a frequency of genotypic coinfeciton as high as 67%. However, in 8 patients (27%), the coinfeciton was detectable by direct sequencing or by standard RFLP and in only 2 of them before IFN treatment. Further studies of genotypic coinfeciton in patients with chronic hepatitis B are warranted.

INTERPRED Trial Group Members


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References