Evolution of *Hepatitis B virus* in an acute hepatitis B patient co-infected with genotypes B and C

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The interactions between different genotypes of *Hepatitis B virus* (HBV) in co-infected patients remain largely unknown, especially in acute infection. Here, the evolution of HBV strains was studied in an acute, self-limited hepatitis B patient co-infected with genotypes Ba (B2) and C. Virological analyses were performed at four time points after admission: T1 (5 days), T2 (11 days), T3 (22 days) and T4 (260 days). A dominant-genotype change from genotype C to Ba was found after anti-HBV e antigen (anti-HBe) seroconversion. Further clonal and phylogenetic analyses of the pre-S and pre-core/core regions of HBV were carried out to clarify the interactions between genotypes Ba and C. All clones propagated from T1 and T2 were of genotype C. In contrast, clones propagated from T3 (after anti-HBe seroconversion) were of genotype Ba, C and/or recombinant within the pre-S region. At T4, all clones were of genotype Ba with a 123 bp (from nt 3147 of the pre-S1 region to nt 54 of the pre-S2 region) in-frame pre-S deletion and had lost the start codon of the middle envelope protein and the nucleocapsid-binding site. Phylogenetic analysis showed that genetic distance was greater at T3 after seroconversion to anti-HBe. By using SimPlot, the breakpoint of one pre-S recombinant was located at nt 3069–3100 and the other two at nt 49–87. In conclusion, HBV genotype Ba may overtake genotype C as the predominant strain after anti-HBe seroconversion in acute hepatitis B. Recombination within the pre-S region emerged transiently and the pre-S deletion mutant was finally cleared.

**INTRODUCTION**

*Hepatitis B virus* (HBV) is a small, enveloped DNA virus that causes acute and chronic liver disease. The majority of acute HBV infections are self-limiting, whereas chronic HBV infection usually pursues a lifelong course that may lead to the development of liver cirrhosis and hepatocellular carcinoma (HCC) (Seeger & Mason, 2000; Kao & Chen, 2002). There are at least eight genotypes of HBV, designated A–H, based on divergence of >8% over the entire genomic sequence (Stuyver et al., 2000; Arauz-Ruiz et al., 2002; Norder et al., 2004). In addition, subgroups of the same HBV genotype have been reported in genotypes A (Aa or A1, and Ae or A2) (Kimbi et al., 2004), B (Bj or B1, and Ba or B2) (Sugauchi et al., 2002) and C (Ce or C1, and Cs or C2) (Huy et al., 2004; Chan et al., 2005). In addition to the two described subgenotypes, genotypes B, C and D have also been divided into four subgenotypes each, following analysis of their complete genomes (Norder et al., 2004). The eight HBV genotypes are distributed within specific geographical locations. All genotypes can lead to progressive liver disease, but they have been shown to have varying clinical significance. For example, patients infected by genotype C (HBV/C) or D (HBV/D) usually have a higher frequency of core-promoter mutation, a lower response rate to interferon therapy and a faster progression to liver cirrhosis and HCC than those infected by genotype B (HBV/B) or A (HBV/A), respectively (Kao et al., 2000a, b, 2003; Hou et al., 2001; Orito et al., 2001; Thakur et al., 2002). However, conflicting reports still exist on the clinical differences among HBV genotypes. For example, HBV/Ba (HBV/B2) was found more often than HBV/C in young patients with non-cirrhotic HCC in Taiwan (Kao et al., 2000a), whilst in Spain, HBV/D was not associated more often with severe liver disease and HCC than HBV/A (Sánchez-Tapias et al., 2002). Taken together, these data suggest possible differences in pathogenicity and response to therapy among HBV genotypes.

Mixed HBV genotype infections in HBV carriers are not common (Kao et al., 2001, 2002; Chen et al., 2004b), especially in acute infection, and the interactions between
different genotypes in co-infected patients remain largely unknown. By using a newly developed line-probe assay, we previously determined the HBV genotypes in 325 HBV-infected intravenous drug users and detected mixed genotype infections in 16.3% (Chen et al., 2004b). In this report, an acute self-limited hepatitis B patient co-infected with HBV/B and HBV/C was identified. We followed up this patient for 334 days and studied the evolution of HBV strains to clarify whether mutations or recombination occurred within the hypervariable pre-S (Norder et al., 1994) and pre-core/core promoter regions.

METHODS

Patient. A 20-year-old female with acute HBV infection was followed at the gastroenterological clinic of the National Taiwan University Hospital. Acute HBV infection was defined as acute-onset elevation of serum alanine aminotransferase (ALT) levels, as well as seropositivity for IgM antibody to HBV core antigen (anti-HBc). The patient was seronegative for antibodies to hepatitis A virus (anti-HAV), hepatitis C virus (anti-HCV) and hepatitis D virus (anti-HDV) and had no serological markers suggestive of autoimmune disease. She did not have a history of alcohol abuse (>50 g day⁻¹), parenteral drug abuse or hepatotoxin exposure. No specific antivirals or immunomodulators were given before or during the study period. The infection was self-limited and she recovered from hepatitis activity with normalization of serum ALT during the study period. The infection was self-limited and she recovered from hepatitis activity with normalization of serum ALT levels, loss of HBV surface antigen (HBsAg) and appearance of anti-HBs. No specific antivirals or immunomodulators were given before or during the study period. The infection was self-limited and she recovered from hepatitis activity with normalization of serum ALT levels, loss of HBV surface antigen (HBsAg) and appearance of anti-HBs (anti-HBs) during the follow-up period. Serial serum samples were collected at four time points after admission: T1 (5 days), T2 (11 days), T3 (22 days) and T4 (260 days). Serum samples were stored at −20 °C until use.

Detection of serological markers of hepatitis virus. The presence of serum HBsAg, anti-HCV, anti-HAV and anti-HDV was tested by using commercially available assays (Ausria-II, HCV EIA II, anti-HAV and anti-delta, respectively; Abbott Laboratories). Levels of serum IgM anti-HBc were measured by using an AxSYM II, anti-HAV and anti-delta, respectively; Abbott Laboratories). Serum HBV e antigen (HBeAg) and antibodies to HBc (anti-HBc) were detected by using the IMx HBe 2.0 assay (Abbott Laboratories).

Table 1. PCR primers for HBV DNA used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence (5'→3')</th>
<th>Position (nt)</th>
<th>Polarity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pre-S gene</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS1</td>
<td>GGGTCACCTTATTCCTGGGA</td>
<td>2814–2833</td>
<td>Forward</td>
</tr>
<tr>
<td>PS2</td>
<td>CCCCGGCTGTAACAGGCA</td>
<td>208–189</td>
<td>Reverse</td>
</tr>
<tr>
<td>PS3</td>
<td>TTGGGAAACAAGTCTACAGC</td>
<td>2828–2847</td>
<td>Forward</td>
</tr>
<tr>
<td>PS4</td>
<td>GTCCCTGATGGATGTCCTCC</td>
<td>176–157</td>
<td>Reverse</td>
</tr>
<tr>
<td>PS-B1</td>
<td>ATTTCAAGGCAACTCAGAAA</td>
<td>2946–2965</td>
<td>Forward</td>
</tr>
<tr>
<td>PS-B2</td>
<td>ACAGTATTCTGAGCCGGCAGC</td>
<td>105–85</td>
<td>Reverse</td>
</tr>
<tr>
<td><strong>Pre-core/core region</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC1</td>
<td>ACATAAGAGGAGCTCTGGAC</td>
<td>1652–1671</td>
<td>Forward</td>
</tr>
<tr>
<td>PC2</td>
<td>GAAGGAAGAAGAGTCAAGGAGC</td>
<td>1977–1957</td>
<td>Reverse</td>
</tr>
<tr>
<td>PC3</td>
<td>TACTTCAAGACAGTGTGTGTTTA</td>
<td>1704–1725</td>
<td>Forward</td>
</tr>
<tr>
<td>PC4</td>
<td>GTCAAGAGGCAAAAAAAGAGA</td>
<td>1966–1947</td>
<td>Reverse</td>
</tr>
</tbody>
</table>

Extraction of serum HBV DNA and quantification of HBV DNA. Serum viral DNA was extracted by using a commercial kit (QIAamp DNA Blood Mini kit; Qiagen). The extracted DNA was used for amplification in both genotyping and clonal analyses.

Quantification of HBV DNA was performed by using real-time PCR as described previously (Yeh et al., 2004a), with a sensitivity of 10³ copies ml⁻¹.

HBV genotyping. HBV genotyping was determined primarily by using a line-probe assay (INNO-LiPA HBV Genotyping Assay; Innogenetics) according to the instructions of the manufacturer (Chen et al., 2004b).

The pre-S gene and pre-core/core region of the HBV genome in each sample were also amplified by nested PCR, sequenced directly with an automatic sequencer (model 3730; Applied Biosystems) and compared with published sequences of various genotypes to ascertain the HBV genotype, as described below.

Amplification, sequencing and cloning of the HBV pre-S gene and pre-core/core region. We performed direct sequencing and clonal analysis of the pre-S gene as described previously (Chen et al., 2004a). Briefly, this segment of HBV pre-S DNA was amplified by nested PCR using two sets of HBV/B and HBV/C co-positive primers (PS1/PS2 for the first-round of PCR and PS3/PS4 for the second round; see Table 1) and KlenTaq DNA polymerase (Ab Peptides). PCR was performed for 36 cycles of 94 °C for 1 min, 58 °C for 30 s and 72 °C for 1 min in a thermal cycler. To avoid false-positive results, the precautions described by Kwok & Higuchi (1989) were followed strictly.

To detect the presence of HBV/B in a mixed population, we performed a genotype B-specific PCR using HBV/B genotype-specific inner primers (PS-B1 and PS-B2; Table 1). To determine the specificity of the HBV/B-specific PCR in mixed genotype infections, we mixed two plasmids corresponding to genotypes B and C in varying ratios ranging from 1:1 to 1:2000 with a total concentration of 0.1 ng DNA ml⁻¹. The detection sensitivity was 0.1 % for the minor HBV/B population (data not shown).

As HBV replication and HBeAg expression are associated with mutations of the core-promoter, pre-core and core regions, sequential changes in the pre-core/core region were also investigated to confirm that HBeAg negativity and low HBV DNA titre were not caused by
relevant mutations (T1762/A1764 and G1896→A). Direct sequencing and clonal analysis of the pre-core/core region were carried out as described above. HBV pre-core/core DNA was amplified by nested PCR using two sets of HBV/B and HBV/C co-positive primers (PC1/PC2 for the first round of PCR, and PC3/PC4 for the second round; Table 1). PCR was performed for 36 cycles of 94 °C for 1 min, 45 °C for 30 s and 72 °C for 30 s in a thermal cycler.

**Sequence alignment and phylogenetic analysis.** The viral genotype of each sample was further ascertained by comparing the nucleotide sequence with reference HBV strains representing each of the eight genotypes, A–H, obtained from GenBank. Alignment analysis was performed by using the Biology WorkBench 3.2 CLUSTAL_W software program (http://workbench.sdsc.edu) (Thompson et al., 1994). Phylogenetic trees were constructed by using the neighbour-joining method implemented in MEGA3 (Kumar et al., 2004) based on the nucleotide sequences of the amplified pre-S gene and the pre-core/core region of the HBV genome. Genetic distances were estimated by using the six-parameter method and phylogenetic trees were constructed by the neighbour-joining method (Saitou & Nei, 1987). To confirm the reliability of the phylogenetic-tree analysis, bootstrap resampling and reconstruction were carried out 1000 times.

**Identification of recombination sites.** Recombination was identified by using SimPlot, as described previously (Chen et al., 2004a), to calculate and plot the percentage identity of a query sequence to a panel of reference sequences in each nucleotide position and locate the informative sites (Lole et al., 1999).

**RESULTS**

**Clinical characteristics**

Results of the detection of serial serum ALT levels, hepatitis virus markers, dominant viral genotype and the titres of HBV viral DNA before and after seroconversion to anti-HBe are shown in Table 2 and Fig. 1. Seroconversion to anti-HBe and normalization of serum ALT levels occurred 22 and 44 days after admission, respectively. Finally, the patient recovered, with loss of HBsAg and HBV DNA, as well as the appearance of anti-HBs during follow-up (334 days after admission) (Fig. 1).

**Evolution of HBV genotype**

By using a line-probe assay, genotype changes in this patient were investigated (Table 2 and Fig. 1). HBV/C was found to be predominant shortly after the onset of acute hepatitis B (T1 and T2). Co-dominance with HBV/B was detected at T3. At T4, the predominant HBV/C was replaced by HBV/B.

Genotype changes were confirmed by PCR amplification of the pre-S gene and pre-core/core region followed by direct sequencing and phylogenetic analysis, respectively. The results were concordant with those obtained by using the line-probe assays (based on the S region) except for that obtained at T3 (Table 2). By direct sequencing, only HBV/B sequences from the pre-S region and HBV/C sequences from the pre-core/core region were detected at this time point.

HBV/B was not detected at T1 and T2, but emerged later at T3. HBV/B might thus have co-existed as a minor population within the viral pools at the onset of illness. To
test this hypothesis, we used a pair of HBV/B-specific primers as an inner primer set (Table 1; PCR conditions were the same as those used for pre-S gene amplification) to examine the possible co-existence of HBV/B at T1. This HBV/B-specific PCR could detect HBV/B DNA at a level of 0.1% when mixed with HBV/C DNA. After HBV/B-specific PCR, direct sequencing and alignment, HBV/B was indeed detectable at this time point and the sequence (T1-BB) is shown in Fig. 2.

Evolution of the HBV pre-S gene and pre-core/core region

Clonal and sequence analysis of the pre-S region was performed to investigate sequential changes in nucleotide sequences. The results are shown in Table 2. Before seroconversion to anti-HBe, all clones obtained at T1 and T2 wereHBV/C. After seroconversion, a remarkable change occurred in the distribution of HBV/B and HBV/C at T3, with 12 (63.2%) of the 19 clones being HBV/B, four (21.0%) being HBV/C and the remaining three (15.8%; T3-1S, T3-13S and T3-21S) having both HBV/B and HBV/C sequences and being possible recombinants between HBV/B and HBV/C. At T4, all 18 clones were HBV/B with a 123 bp in-frame deletion (from nt 3147 of the pre-S1 region to nt 54 of the pre-S2 region). The sequence alignments of the representative clones are summarized in Fig. 2.

Genotype changes and mixed populations of HBV infection were confirmed by cloning and sequencing the pre-core/core region (Table 2). The results were in agreement with those obtained from the pre-S region. Mixed populations were detected at T3, with 13 (76.5%) of the 17 clones being HBV/C and four (23.5%) being HBV/Ba, having recombinant with genotype C. The sequence alignments of the representative clones are summarized in Fig. 3, which shows that, with the exception of clone T3-3C, the clones did not contain the pre-core G1896A and basal-core promoter A1762T and G1764A mutations.

Phylogenetic analysis of HBV DNA clones from the four time points

To confirm co-infection with HBV/B and HBV/C, phylogenetic trees were constructed based on two partial sequences from the pre-S gene and pre-core/core region, respectively (Fig. 4). HBV DNA clones from the four time points, T1–T4, clustered with representative HBV isolates that were genotype B or C or were recombinant within the pre-S region (Fig. 4a). Likewise, clones from different time points clustered with representative HBV isolates of genotype Ba or C within the pre-core/core region (Fig. 4b).

Recombination between HBV/B and HBV/C

We used SimPlot to confirm and localize the crossover points of the three recombinants obtained from the pre-S gene region and the two representative HBV/Ba (B2) subgenomes from the pre-core/core region at T3 and T4 (Fig. 5) (Lole et al., 1999). The most likely breakpoints were located at nt 3069–3100 in the pre-S1 region for clone T3-1S, at nt 49–87 in the pre-S2 region for clones T3-13S and T3-21S, and at nt 1730–1740 for T3-1C and T4-4C, respectively.

Sequential changes in the derived pre-S amino acid sequence

The amino acid sequences deduced from the corresponding nucleotide sequences of the pre-S region obtained at the four time points, as well as the reference sequences, are shown in Fig. 6. Notably, amino acid conservation within the B- and T-cell epitopes of the pre-S1 region (aa 12–32 and 72–78) and the pre-S2 region (aa 1–6 and 21–30) was found among these clones. In contrast, there were many differences among clones of HBV/C (before anti-HBe seroconversion) and those of HBV/Ba (B2) (after anti-HBe seroconversion) within the hepatocyte-binding site (aa 21–47), the T- and B-cell epitopes (aa 29–53 and 94–117) of the pre-S1 region and the B-cell (aa 3–15 and 38–48) and T-cell (aa 29–48) epitopes of the pre-S2 region. The 123 bp deletion mutant led to an in-frame deletion from aa 100 to 140 of the large envelope protein, encompassing the 20 C-terminal amino acids of the pre-S1 region and the 21 N-terminal amino acids of the pre-S2 region and loss of the nucleocapsid-binding site and the start codon of the pre-S2 gene. As the pre-S region

![Fig. 1. Clinical course of a 20-year-old woman with acute, self-limited HBV infection. The four time points (T1–T4) for genotype analysis and direct sequencing/clonal analysis of the pre-S gene are marked by arrows. At 5 (T1) and 11 (T2) days after admission (HBsAg-positive), HBV/C predominated. An HBV/B virus strain emerged and co-existed with the originally predominant HBV/C at 22 days after admission (T3, anti-HBe-positive) and later became the dominant genotype at 260 days after admission (T4, HBsAg-negative). Finally, the patient recovered, with loss of HBsAg and the appearance of anti-HBs, as well as undetectable levels of HBV DNA, at the end of the follow-up period. The sequential changes in viral titre of HBV DNA are indicated.](image-url)
is located in the spacer region of the polymerase gene, the in-frame deletion of the pre-S region has no effect on polymerase function.

**DISCUSSION**

The interactions between different HBV genotypes are complicated and it is not easy to clarify this complex relationship without a chronological follow-up. By following an HBV/Ba (B2) and HBV/C co-infected patient manifesting as a case of acute self-limited hepatitis, we had a unique chance to examine the dynamic relationship between different HBV genotypes. We found that HBV/Ba (B2) co-existed as a minor population with HBV/C during the early course of acute HBV infection and then emerged and gradually became the dominant genotype. Remarkable changes in the relative distribution of HBV/Ba (B2) and HBV/C clones and the transient emergence of HBV/Ba (B2) and HBV/C recombinant clones within the pre-S region and the pre-core/core promoter mutation HBV/C subgenomes, as well as a 123 bp in-frame deletion (nt 3147 of the pre-S1 region to nt 54 of the pre-S2 region) of the pre-S gene were also noted during the process of anti-HBe seroconversion and subsequent loss of HBsAg.

The mechanisms responsible for the evolution and change of viral genotypes remain largely unknown. Both host and viral factors are likely to be involved in this process. Previous studies have found that the dominant HBV genotype changed from A (HBsAg- or HBeAg-positive) to D (after seroconversion) in chronically infected children (Bahn et al., 1997; Gerner et al., 1998) and from A to G in chronically infected adults after spontaneous seroconversion to anti-HBe (Kato et al., 2002). Likewise, we found that, after transmission to an HBV-naïve subject, the composition of viral pools evolved after seroconversion to anti-HBe, and HBV/Ba (B2) gradually replaced HBV/C as the predominant genotype in the late-acute phase. Numerous amino acid differences occurred in the pre-S gene sequence after HBeAg seroconversion (shown in Fig. 6). These variations might

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**Fig. 2.** Nucleotide sequences of representative pre-S gene clones obtained at the four time points (T1-10S from T1; T2-7S from T2; T3-8S, T3-13S, T3-1S and T3-11S from T3; T4-5S from T4; and T1-BB from T1, using HBV/B-specific PCR) during the follow-up period of acute HBV infection. Reference sequences from HBV/B (Ba, GenBank no. AF121246) and HBV/C (GenBank no. AB031262) isolates are shown above. The positions of nucleotide sequences of the pre-S gene region, the start codon of the pre-S1, pre-S2 and S genes, and two control elements in the S promoter (CCAAT box and its 3' terminus) are indicated above or below the reference sequence. Primer sites are underlined.
cause alteration of immune target sites, leading to escape from immune surveillance or reduced binding affinity by major histocompatibility complex class I-mediated presentation of modified oligopeptides on the cell surface of hepatocytes (Milich et al., 1990). Previous studies have shown that genotype-specific antibody is induced and reacts with the variable pre-S1 and pre-S2 sequences (Milich et al., 1990; Sobotta et al., 2000). Based on these findings, it is possible that host immune pressure operated to select a different viral genotype in the present case. Furthermore, a recent study has shown that there are differences in the rate and nature of evolution of HBV in patients with acute resolving hepatitis compared with those who go on to develop chronic hepatitis (Whalley et al., 2004). Genetic divergence was greater in the resolvers. Consistently, we found that genetic diversity in the pre-S gene and pre-core/core region, as measured by genetic distances (Fig. 4), increased during acute infection, reaching a maximum at the T3 time point, and this divergence may lead to the development of broadly reactive host immune responses, resulting in genotype change and clearance of HBV. Nevertheless, determination of the mechanisms contributing to the change of genotype in co-infected patients requires further studies.

Wild-type HBV can direct the synthesis of HBeAg in hepatocytes. The production of HBeAg is abolished by mutations in the pre-core region (G1986A) that inhibit translation of the HBeAg precursor (Carman et al., 1989) and is reduced by mutations in the core promoter (A1762T, G1764A) that downregulate the transcription of pre-core mRNA for the HBeAg precursor (Buckwold et al., 1996). In this study, the wild-type pre-core sequence was detected in HBV DNA samples from all clones isolated at all time points (T1–T4) except for three HBV/C subgenomes at the T3 time point. One of these three clones had mutations in the basal core promoter (A1762T, G1764A) and in the pre-core region (G1986A) (Fig. 3), whilst the other two contained only the pre-core mutation. This finding is consistent with previous studies in which pre-core and core promoter mutations did not occur or were of low frequency in acute, self-limited hepatitis B (Imamura et al., 2003; Kobayashi et al., 2004). In addition, nucleotide variation (T or C) at nt 1858 was observed. A relationship between HBV genotypes and types of pre-core mutation, as well as nucleotide variability at nt 1858, has been reported previously (Li et al., 1993; Chan et al., 1999). For example, genotypes other than genotype A usually have a T at nt 1858 (T1858), which results in wobble pairing with G1896 in the encapsidation signal.

The A1896 mutation (pre-core stop mutation) tightens the stem–loop by making a T–A pair. In contrast, genotype A possesses C1858, making a C–G pair with G1896 in the wild-type. As the A1896 mutation breaks this stable pair, this does not occur except in combination with another mutation.
from C\textsubscript{1858} to T\textsubscript{1858} and this may explain why HBV/A rarely circulates as an HBe mutant and why HBV/D is the most frequent HBV genotype among HBeAg-negative, chronic hepatitis B patients in western countries (Li et al., 1993). Similarly, C\textsubscript{1858} is also often observed in Chinese patients with genotype C infection, whereas fewer pre-core stop-codon mutations (G\textsubscript{1896}A) were found in these patients (Chan et al., 1999). Recently, Chan et al. (2005) characterized genotype C into two subgenotypes, Ce (C1) and Cs (C2), by amino acid polymorphisms in the polymerase protein. The nucleotide at position 1858 was T in all ten patients with subgenotype Ce (C1) and in two patients with subgenotype Cs (C2). The remaining 37 patients with subgenotype Cs (C2) had C at position 1858.

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**Fig. 4.** Phylogenetic-tree analysis of the subgenomic HBV sequences from time points T1–T4 compared with HBV strains representative of seven genotypes. HBV strains were retrieved from DDBJ/EMBL/GenBank and their accession numbers are indicated. Genetic distances were estimated by the six-parameter method and phylogenetic trees were constructed by the neighbor-joining method. Bootstrap values are shown along the main branch (values of 50 % and higher are shown). Bars indicate the number of nucleotide substitutions per site. (a) Phylogenetic tree constructed by using sequences of the pre-S gene region from representative clones from T1–T4, along with 27 HBV strains representative of seven genotypes. Representative clones were T1-2S, T1-10S and T1-22S from T1; T2-7S, T2-10S and T2-13S from T2; and T4-5S, T4-11S and T4-16S from T4. Representative clones from T3 were T3-4S, T3-6S and T3-8S for HBV/C; T3-1S, T3-13S and T3-21S for HBV/B; and T3-1S, T3-13S and T3-21S for recombinant genotypes. (b) Phylogenetic tree constructed by using sequences of the pre-core/core region from representative clones from T1–T4, along with 28 HBV strains representative of six genotypes. Representative clones were T1-2C, T1-4C and T1-9C from T1; T2-1C, T2-3C and T2-5C from T2; and T4-1C, T4-4C and T4-10C from T4. Representative clones from T3 were T3-2C, T3-3C, T3-9C, T3-10C, T3-11C and T3-12C for HBV/C; and T3-1C and T3-4C for HBV/Ba (B2).
Accordingly, it is possible that our patient was infected with the subgenotype Cs (C2) and may have had a lower frequency of the pre-core stop codon mutation.

Based on phylogenetic analysis of complete genomes supported by significant bootstrap values, genotypes B, C and D can be divided into four subgenotypes (Norder et al., 2004). Subgenotypes B and C differ in their geographical distribution, with B1 (Bj) dominating in Japan, B2 (Ba) in China and Vietnam, B3 confined to Indonesia and B4 confined to Vietnam. Subgenotype C1 (Ce) is common in Taiwan, Japan, Korea and China; C2 (Cs) in Hong Kong, South-East Asia and Bangladesh; C3 in Oceania and C4 in Aborigines from Australia. By phylogenetic analysis of the hypervariable pre-S subgenome (data not shown), our patient was infected with subgenotype C2 (Cs) and B2 (Ba). Different genotypes are shown to have different clinical significance. Subgenotype Bj (B1) also behaves differently from Ba (B2) in terms of the prevalence of HBeAg, the risk of HCC and the response to antiviral treatment (Akuta et al., 2003; Sugauchi et al., 2003). It is likely that different clinical significances may exist between subgenotypes C1 (Ce) and C2 (Cs) and this issue needs further examination.

In the present study, a subgenomic deletion between nt 3147 of the pre-S1 region and nt 54 of the pre-S2 region in the pre-S1/S2 region was identified after anti-HBe seroconversion. This mutation led to an in-frame deletion from aa 100 to 140 of the large envelope protein, encompassing the 20 C-terminal amino acids of the pre-S1 region and the 21 N-terminal amino acids of the pre-S2 region and thus would abolish translation of the pre-S2 gene and subsequent synthesis of the middle-envelope protein (Fig. 6). There is evidence that the pre-S2 protein is not essential for virion assembly, infectivity or secretion (Policino et al., 1997); thus, the virus appears to be able to dispense with it. Another important consequence of the deletion is the removal of the encapsidation site (aa 107–127, numbers adjusted to genotypes B and C used in this work) (Poisson et al., 1997; Le Seyec et al., 1998). In addition, as this mutant is deleted 3′ to the CCAAT box, it may change the steric position of the CCAAT box-binding factor in the S promoter and down-regulate its transcriptional activity (Bock et al., 1999). Therefore, genomes with pre-S deletions are no longer able to produce HBsAg and secrete viral particles efficiently. This phenomenon explains the finding of a low HBV DNA titre and the absence of HBsAg at the T4 time point (Table 2, Fig. 5. Similarity plots (generated by SimPlot) for a set of reference sequences compared with those of clones T3-1S (a), T3-13S (b), T3-21S (c), T3-1C (d) and T4-4C (e). The reference sequences involved in recombination were HBV/B [black line, GenBank no. AF121246 (a–c); HBV/Bj (B1), GenBank no. D00329 (d–e)] and HBV/C (grey line, GenBank no. AB031262) and an outgroup sequence of genotype F (dotted line, GenBank no. AB036910). Each curve is a comparison between the query sequence [pre-S region for (a–c); pre-core/core region for (d) and (e)] and a reference sequence. Each point plotted is the percentage identity within a sliding window of 100 bp centred on the position plotted, with a step size between points of 10 bp and with GapStrip off. The horizontal bars above the plots indicate the pre-S or pre-core/core regions of these recombinants. Shading is consistent with that used for the similarity curves and indicates the genotype to which that part of the genome is most similar based on the adjacent similarity plot.
Nevertheless, this mutant became predominant during follow-up, but finally disappeared. Its occurrence was probably due to immune escape and its disappearance may have resulted from the above-mentioned mechanism of enhanced immunological pressure on the HBV genome during the increase in genetic diversity.

Our previous studies have indicated the occurrence of several novel recombinant pre-S subgenomes in intravenous drug users who were co-infected with HBV/B and HBV/C (Chen et al., 2004a). In this study, we also identified recombinants between HBV/B and HBV/C within the pre-S region. By using SimPlot, we estimated the recombination sites of these recombinants to be at nt 3069–3100 for clone T3-1 and at nt 49–87 for clones T3-13 and T3-21, within the junction between the pre-S1 and pre-S2 genes. In addition, recombination was also found within the pre-core/core region, with the cross-over sites estimated to be at nt 1730–1740 for clones T3-1C and T4-4C. This was consistent with a previous study in which HBV/B in Taiwan was shown to recombine with HBV/C over the pre-core region plus the core gene and was classified into genotype Ba (Sugauchi et al., 2002). Recently, ample evidence has revealed that recombination between different HBV genotypes could occur during the natural course of HBV infection (Georgi-Geisberger et al., 1992; Bollyky et al., 1996; Bowyer & Sim, 2000; Hannoun et al., 2000; Morozov et al., 2000; Owiredru et al., 2001; Sugauchi et al., 2002; Chen et al., 2004a, b). Recombination was shown to occur within the pre-core/core region, the S gene and the X gene/pre-core region, as well as in the pre-S region, but the frequency and mechanisms of recombination and their impact on the evolution and pathogenicity of HBV infection remained unclear within the different clinical settings.

The observed recombinant pre-S sequences in this study might be an artefact of in vitro manipulation. To exclude this possibility, great care was taken in designing the PCR assay. KlenTaq DNA polymerase was used for PCR amplification. This enzyme is an N-terminal deletion mutant of Taq DNA polymerase, analogous to the Klenow fragment of Escherichia coli DNA polymerase I. The error rate of KlenTaq is twofold lower than that of AmpliTaq (Applied Biosystems) and the extension rate of this enzyme is more than 1 kb min\(^{-1}\) (Barnes, 1992). The extension time allowed for the PCR in this study was 1 min to maintain the enzyme’s processivity. Therefore, the possibility of switching templates during PCR was quite low. In addition, we performed another nested PCR with unique sets of primers that only amplified the recombinant HBV from T3 (outer primers, PS1/PS2; inner primers, HBV/C-specific sense-strand primer and HBV/B-specific antisense primer). After direct sequencing, we found that the subgenomic sequence was 95 % similar to the original T3-1 sequence and 94 % similar to T3-13 and T3-21, but was only 90 % similar to HBV/B and 92 % similar to HBV/C. Alignment analysis again revealed that this sequence had changed to that of HBV/C at nt 3190 close to the breakpoint of the T3-1 clone.
(data not shown). Results and interpretation consistent with these results have been reported previously by Lole et al. (1999). Therefore, these recombinants were unlikely to be artefacts of PCR amplification.

In conclusion, our data indicated that the dominant HBV genotype changed from C to Ba in an acute, self-limited hepatitis B patient and that recombinants of HBV/Ba (B2) and HBV/Cs (C2), and pre-core/core promoter mutations, as well as pre-S1/S2 deletion mutations, emerged transiently during the process of anti-HBe seroconversion. The biological implications of these novel HBV recombinants and deletion mutants in the natural course of HBV infection require further examination. In addition, genetic divergence of the pre-S and pre-core/core regions was greatest at the T3 time point, which may induce broadly reactive host immune responses against HBV that result in control of virus replication, even in the presence of immune-escape mutants.

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