Two sequential hepatitis B virus (HBV) strains obtained before and during an icteric flare-up of an occult HBV infection in a patient coinfected with human immunodeficiency virus revealed HBV surface antigen (HBsAg) test escape mutations, although the patient had never received hepatitis B–specific immunoglobulin. In contrast to the high HBV DNA loads, recurrence of HBsAg, and resulting icteric hepatitis, phenotypic analysis of the mutated HBV strains revealed significantly reduced replication efficacies in vitro, compared with wild-type HBV. Therefore, immune escape in the transiently anti–HBs–positive patient appeared to be crucial for persistence and reactivation. Immune escape mutants evolved even without exogenous selective pressure, hampered detection in HBsAg screening, and might be transmitted during reactivation with high HBV loads.

Hepatitis B virus (HBV) infection is a major cause of acute and chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma worldwide. Using its own encoded reverse transcriptase, HBV exhibits a high replication error rate [1] and thus is able to react rapidly to selective pressure, which can be either host related (endogenous) or iatrogenic (exogenous; e.g., receipt of vaccination, hepatitis B immunoglobulin [HBIG], or antiviral therapy). As the open reading frames (ORF) of the polymerase gene and the HBsAg gene overlap in the HBV genome, mutations in the polymerase gene may also affect antigenic sites within the envelope protein HBsAg and vice versa.

Polymerase and/or envelope protein mutations are frequently found in patients receiving extensive antiviral therapy, for example, patients who have undergone liver transplants and been treated with HBIG and nucleoside and/or nucleotide analogues. In the majority of cases, these polymerase mutations display reduced viral replication efficacy, which can be compensated for by additional mutations [2–4].

The serological diagnosis of active HBV infection is made by detecting HBsAg in different kinds of ligand assays. However, HBV vaccination and the administration of HBIG can result in diagnostic HBsAg immune assay test escape mutants, as well as in vaccine and/or immunoglobulin treatment failures [5]. In the present article, we report HBsAg escape mutations in strains recovered from an HBV–HIV–coinfected patient who had never been vaccinated or received any HBIG.

Materials and methods. Serological markers for HBsAg, HBV surface antibody (anti-HBs), HBV e antigen (HBeAg), and HBV core antibody (anti-HBc) were determined by use of commercial assays (AxSym and Architect, Abbott Laboratories; Enzygnost, Dade Behring). HBV DNA was quantified using the Cobas Taqman48 Instrument (Roche).

For sequence analysis, HBV DNA was isolated from the patient’s serum samples by use of the QIAamp Blood Kit (Qiagen) and amplified by polymerase chain reaction (PCR) using the Amplicon system (Perkin Elmer), with a 0.1 μmol/L final concentration of primer pair HBV-S6s and HBV-S6as [6]. The amplified products were cloned to the vector pGEM-Teasy (Promega) and sequenced in a 310 Genetic Analyser (Applied Biosystems). Five clones of each sample were sequenced, and mutations found in all 5 isolates were regarded as significant. Phylogenetic and molecular evolutionary analyses were conducted by using Molecular Evolutionary Genetics Analysis software (MEGA version 3.1) as well as BioEdit (version 7.0.4.1) [7, 8].

To generate replication-competent HBV vectors, an Earl-
Hpal fragment containing the patient’s target sequence was introduced into the 1.28 HBV replication-competent HBV vectors (genotype A2, subtype adw2) [9]. To achieve this, subcloning via an intermediate subcloning vector pACYC184 (Stratagene) de-

Received 6 February 2008; accepted 12 June 2008; electronically published 9 October 2008.

Potential conflicts of interest: none reported.


Financial support: German Network of Competence for Viral Hepatitis (BMBF; grant 123 to C.T. and F.T.); EU VIRGIL grant (to C.T. and F.T., partner no. 60); German Research Foundation (DFG; to C.T. and F.T.); Iranian Ministry of Health (Ir2006 to S.A.).


Potential conflicts of interest: none reported.

Reprints or correspondence: Albert Heim, PD Dr. med, Institute of Virology, Medical School Hannover, Carl-Neuburg-Str. 1, D-30625 Hannover, Germany (Heim.Albert@mh-hannover.de).

The Journal of Infectious Diseases 2008;198:1620–4

© 2008 by the Infectious Diseases Society of America. All rights reserved.
0022-1899/2008/19811-0007$15.00
DOI: 10.1086/592987
Nevertheless, his immunological status was acceptable, with a CD4 cell count of 415 cells/μL, and he showed no signs of opportunistic infections despite a high HIV load of 2.7 × 10^5 copies/mL.

HBV infection was serologically confirmed as the causative agent of the presumed acute hepatitis (table 1, which is available only in the electronic version). However, there was a discrepancy in HBsAg test results: the AxSym and Architect HBsAg immune assay detected HBsAg, whereas the Enzygnost 5.0 HBsAg (Dade Behring) failed to do so. There was no serological evidence of hepatitis C or D virus infection. The patient’s clinical condition improved after receiving therapy with tenofovir, lamivudine and lobivinav.

However, 2 archival serum samples taken 8 and 5 years prior to the onset of presumed acute hepatitis were available and were analyzed retrospectively. The latter showed entirely convalescent HBV serological results, although there was a low HBV load detected, suggesting an occult infection (table 1, which is available only in the electronic version). However, the result pattern in which the AxSym test was positive for HBsAg while the Enzygnost test was negative was also detected in the sample obtained 8 years prior to the acute event. An anti-HBc positive and anti-HBc IgM–negative result pattern supported the view that the virus had already established a chronic infection 8 years prior to the onset of “acute” hepatitis (table 1, which is available only in the electronic version). Absence of clinically overt liver disease and a low HBV load (181 IU/mL) further substantiated this notion. Moreover, the patient remembered a history of hepatitis 30 years ago, however neither clinical nor laboratory records were available. This classifies the “acute” disease as a reactivation (HBV DNA 2.3 × 10^6 IU/mL) of an occult infection (table 1, which is available only in the electronic version).

For molecular analysis, we obtained ~600-bp fragments from the acute-phase (hereafter, “HBV flare-up”) and chronic-phase (hereafter, “HBV chronic”) serum samples (table 1, which is available only in the electronic version); both were diagnostic escape mutants in the Enzygnost HBsAg test. No sequences could be obtained from the 5-year-old sample for technical reasons. Phylogenetic analysis of both genomes grouped the virus within genotype A2 [11], a group frequently recovered in Germany (figure 1A). Interestingly, both strains show a K→R exchange (adw-to-ayw subtype) at position 122 of the HBsAg (ntA519G exchange with respect to the reference genome, accession number NC003977) (figure 1B). This site is known to confer d/y subtype specificity [11] and is only very rarely found in genotype A genomes, but occurs frequently in genotypes D and E. In addition, both sequences exhibit 2 other unusual conserved nucleotide mutations (nt C512A and A850G), which are uncommon in genotype A and which support their direct phylogenetic relationship. The former mutation results in a nonsynonymous mutation in the a-determinant in the HBsAg (P120T in HBV chronic and P120K in HBV flare-up). The latter mutation is silent in the polymerase and situated downstream of the HBsAg ORF.

In addition to these concordant mutations, HBV flare-up and HBV chronic had several unique mutations. Next to the K122R exchange, HBV flare-up revealed the following mutations in HBsAg: T118M, P120K, V167A, S173N, and P213L (figure 1B). Because of the overlapping ORFs of HBsAg and polymerase in the HBV genome, these mutations lead to a T128K exchange in the polymerase. HBV chronic showed the following unique mutations in HBsAg: G112R, P120T, F134S, and I212L. The corresponding mutations in the polymerase are as follows: R120K, T128N, and Y221F. Several other mutations were observed only in single clones because of the quasispecies nature of HBV (figure 1B).

The potential effect of the observed mutations on RNA and protein expression of HBsAg and on the efficacy of viral replica-
tion was further analyzed. Both strains, HBV flare-up and HBV chronic—including all mutations described above—were cloned into replication-competent 1.28-fold HBV vectors of the same genotype A2. After transient transfection of human hepatoma cells, normal transcription of pregenomic-RNA and S-RNA was evident by Northern blot analysis (figure 2A). This related to a regular, cytoplasmic HBs protein expression pattern as shown by immunohistochemical staining with a monoclonal anti-HBs antibody in transfected cells (figure 2B).

The replication efficacy of HBV constructs was compared to that of wild type (wt) HBV constructs of the same genotype A2. After transient transfection of human hepatoma cells, normal transcription of pregenomic-RNA and S-RNA was evident by Northern blot analysis (figure 2A). This resulted in a significantly decreased number of mature HBV virions released into the supernatant as well, which were quantified by use of real-time PCR (figure 2D).

Discussion. In the present article, we describe 2 novel hepatitis B virus mutants that caused persistent infection and an icteric hepatitis B flare-up despite a previous transiently positive anti-HBs titer in an HIV-positive patient. This may be related to the observation that HBV strains from different time points in disease progression displayed various HBsAg escape mutations. Three of the mutations uncovered at the nucleic acid level, which are rarely found in genotype A sequences, support a hypothesis...
of evolution of persisting HBV strains rather than superinfection with another strain.

Serological testing with the Enzygnost HBsAg ELISA revealed a diagnostic HBsAg test escape mutation for both samples—HBV flare-up as well as the HBV chronic, which was 8 years older. Sequence analysis of the HBV flare-up showed 5 amino acid substitutions in the HBsAg when compared with a typical A2 genotype sequence. HBV chronic revealed a partly different
set of mutations, but both strains showed mutations on positions within the α-determinant known to confer HBsAg test escape mutations [12]. Probably, the mutations P120T and P120K were essential for test escape because mutations at this position have been described as resulting in test escape [12]. The fixed mutation K122R (figure 1B) may have contributed to test escape in HBV with a genotype A background; in the genetic background of genotype D and E, it has never been described as test escape mutation. However, the most frequent mutation resulting in HBsAg test escape after HBIG administration and failed hepatitis B vaccination was a glycine-to-arginine exchange at position 145 [5, 13]. This mutation does not impair viral replication [14] and was not present in our patient, who had never received HBIG and had never been vaccinated against hepatitis B. Thus, the mutations observed in this study were solely driven by endogenous selective pressure, such as the patient’s own anti-HBs (table 1, which is available only in the electronic version), which suggests an increased rate of immune escape for HBV during the clinical course.

We assessed the replication efficacy of the mutants by transient transfections of human hepatoma cells with HBV constructs expressing the same α-determinant mutations as the patient during chronic infection or viral reactivation. Consistent with the benign long-term clinical course and the overlap of envelope protein and polymerase mutations, both mutants had significantly reduced viral fitness, as evidenced by the reduced amount of intracellular capsid DNA and a reduced viral copy number in the supernatant. Since the gene transcription of the envelope gene and intracellular HBsAg protein expression were not affected, this indicates an impediment at the virus packaging and/or secretion pathway, at least for (incomplete) HBs particles (figure 2). Taken together, these results indicate that a selective advantage was not gained by an increased viral fitness of the HBV flare-up virus but by an increase in the virus’s capacity to hide from the host’s immune system by modification of the α-determinant of HBsAg.

The reduced immunocompetence of an HIV-positive patient who did not take antiretroviral drugs for over a year might have favoured the flare-up. Notwithstanding, opportunistic infections were absent, and a CD4+ T cell count of 415 cells/μL was considered acceptable. It is possible that a slightly reduced immune response is conducive to viral immune escape, as it creates a strong but not overwhelming selection pressure for the virus. Nevertheless, we cannot excluded the possibility that the reduced viral fitness measured in our in vitro assays might have been overcome by compensatory mutations further downstream of the HBsAg ORF in vivo.

In conclusion, test and immune escape mutations do not develop only in chronically HBV-infected individuals who are treated with nucleoside analogues or are passively immunized; these mutations also develop in individuals who are not exposed to such an iatrogenic selective pressure. These mutations are associated with an increased immune evasive capacity in the virus and were able to cause a symptomatic flare-up and high virus loads. Thus, despite their reduced replication fitness, they may create the risk of transmission to vaccinated individuals [15]. Moreover, rapid diagnosis of infection with these mutants is complicated by the potential failure to detect HBsAg in immune assay systems.

References