Infectious HBV wild-type and mutant clones were produced in vitro with three mutations in pol (rtV173L plus rtL180M plus rtM204V) or with a single mutation in the S gene (sG145R) and inoculated in treatment-naive chimpanzees. Intravenous inoculation of these mutants in chimpanzees resulted in HBV infection; the pol mutations remained stable, whereas the sG145R mutation reverted to wild type during viraemia. Additional hepatitis B vaccine efficacy studies conducted in chimpanzees showed lack of sterilizing immunity against the pol mutant. Whether such mutants can transmit to and infect vaccinated humans requires further investigation.

Hepatitis B vaccination has been very effective in the prevention of new HBV infections and the treatment of chronic hepatitis B has involved the use of various nucleoside/nucleotide analogues [1]. However, mutant HBV capable of escaping neutralization by vaccination or that has developed resistance to antiviral drugs has been identified in a number of clinical settings [2]. Mutations in the ‘a’ determinant region of hepatitis B surface antigen (HBsAg) have been identified in various individuals, including chronic hepatitis B patients, infants receiving post-exposure prophylaxis to prevent perinatal HBV infection, liver transplant patients receiving hepatitis B immunoglobulin (HBIG) [3,4] and individuals who receive pre-exposure hepatitis B immunization [5,6].

Antiviral drug-resistant mutations arise in the reverse transcriptase (rt) region of the HBV pol after prolonged treatment of chronic hepatitis B with nucleotide analogues, such as lamivudine and famciclovir [7–10]. The transmissibility and the potential of these mutants to cause breakthrough infections have been reported in several studies [11–13]. Mutations in pol can bring about concomitant mutations in the ‘a’ determinant region of HBsAg because of the overlap between open reading frames that encode the viral polymerase and envelope proteins. Such mutations have been observed in patients receiving lamivudine therapy [10,14,15], and markedly reduced binding of antibody against HBsAg (anti-HBs) to the mutated HBsAg has been demonstrated in vitro [9,10]. Occult HBV infections associated with these mutants have also been observed in vaccinated individuals [11,16,17]. Our experimental studies recently conducted in the chimpanzee model and reviewed here, suggest that HBV mutants have the potential to infect previously vaccinated individuals. These mutants could enter the blood supply undetected and infect vaccinated recipients of blood products.

S gene mutants

The first vaccine-escape mutant was detected in 1988 in a child born to an HBV-infected mother who was actively vaccinated and received passive vaccination with HBIG [18]. The breakthrough infection in this child, which was associated with a substitution in codon 145 from glycine to arginine (sG145R), resulted in persistent viraemia and antigenaemia for >12 years despite seroprotective anti-HBs levels [19]. Reports of the sG145R mutation in infants born to HBsAg-positive mothers who received the hepatitis B vaccine with or without HBIG subsequently appeared [13,19–22]. It was recently reported that HBV sG145R mutants are frequently selected in peripheral blood leukocytes, which can serve as a source of reactivation or transmission of hepatitis B [23]. Because of the overlapping nature of the HBV genome, any mutations in the S gene have the potential also to produce functional alterations in pol. The sG145R mutation brings about a concomitant...
in vivo in a murine model of HBV infection [59]. The kinds of anti-hepatitis B antibody were first studied interactions of the envelop protein of HBV with various regions including the ‘a’ determinant between amino acids 122 and 123 was observed in a case of fulminant reactivation of hepatitis B that was seronegative for HBsAg [34]. The sG145R mutation has also been described in conjunction with clusters of mutations around codons 40–45, 114–122 and 198–208 [44,51].

The S gene proline-to-threonine mutation in codon 120 (sP120T), as well as sG145R, are often seen in patients after liver transplant following lamivudine or HBIG treatment in combination with other lamivudine-associated resistance mutations [52]. These mutations produce concomitant mutations, rtT129N and rtW153Q, in the Pol protein and partially restore the in vitro replicative capacity of lamivudine-resistant HBV [53–55]. The HBV mutants, sG145R and sP120T have been shown to affect HBsAg levels to different extents. The sG145R mutant expresses generally very low levels of HBsAg because the glycine to arginine substitution in codon 145 directly alters the ‘a’ determinant epitope region (amino acids 124–147) [56]. By comparison, the antigenicity of mutant sP120T is reduced to a lesser degree because the proline-to-threonine substitution in codon 120 is located in the adjacent major hydrophilic region 2 (encompassing amino acids 120–123) [10,57].

**Infectivity and pathogenicity of S gene mutants, and efficacy of vaccination**

Experiments in the chimpanzee and the mouse have revealed that the sG145R mutant is pathogenic [58–60]. Interactions of the envelop protein of HBV with various kinds of anti-hepatitis B antibody were first studied in vivo in a murine model of HBV infection [59]. The model was developed by transfecting sG145R mutant HBV DNA into cultured hepatoma cells and livers of mice by liposome-mediated gene transfer. Examination of the interaction between the envelope protein and various preparations of anti-hepatitis B antibody revealed reduced binding of mutant envelope protein to human HBIG, rabbit polyclonal anti-HBs, and monoclonal anti-‘a’ antibody in vitro and in vivo. The data from this study suggested that there might be no correlation between antigenicity and immunogenicity of the sG145R mutant, and that it could cause hepatitis that is accompanied by the serological presence of HBsAg and anti-HBs [59].

The biological properties, infectivity and pathogenicity of sG145R mutant have been further investigated comprehensively in the only animal model of hepatitis B, the chimpanzee model. Six seronegative chimpanzees were inoculated with varying dilutions of serum that contained sG145R mutant. Five of the animals developed serological and/or biochemical evidence of hepatitis B. Using a PCR-based methodology, which discriminated between the wild-type and the sG145R mutant viral genomes, 10⁴ and 10⁷ dilutions of the index serum were shown to contain a pure population of the mutant genome. Inoculation of treatment-naive chimpanzees with these dilutions of the infected serum resulted in infection of the chimpanzees, showing that the sG145R mutant is viable, infectious and pathogenic [58].

We further investigated the virological, serological and clinical characteristics of infection with in-vitro-generated sG145R mutant in treatment-naive chimpanzees. The inoculum was generated in vitro because clinical isolates usually contain a heterogeneous mixture of HBVs composed of the mutated species of the virus, other quasispecies resulting from errors in replication by the HBV polymerase, as well as wild-type virus, so the infectivity and pathogenetic profile observed after an experimental inoculation cannot be fully attributed to the mutant per se. The inoculum was prepared by transfecting the sG145R mutant in HepG2 cells. Detection of HBV DNA and core protein in transfected cell lysates and HBsAg, and the electron microscopic demonstration of viral and subviral particles in the culture supernatants confirmed the success of these transfections in generating HBV particles. Intact hepatitis B viruses and subviral particles in culture supernatants were further purified through sucrose cushions and ultracentrifugation. Intravenous inoculation with the purified stock of sG145R HBV mutant produced infection in one of two inoculated animals. The infected animal showed HBV DNA and HBsAg in serum 14–24 and 19–24 weeks after inoculation, respectively. This animal had increased alanine aminotransferase (ALT) levels during weeks 21–23, and seroconverted to antibody...
against hepatitis B core antigen (anti-HBc) and anti-HBs (Figure 1A). Sequencing studies showed a mixed population of mutant and wild-type viruses in the first HBV-DNA-positive serum sample and complete reversion to the wild-type sequence coinciding with the development of acute hepatitis [60].

The data suggesting the stability of sG145R mutant are seemingly at variance with those from the previously reported study using the same mutant [58]. In our study, the majority of the clones in the very first HBV-DNA-positive sample from the infected chimpanzee had already reverted to the wild-type s145 sequence. Sequencing of a large number of clones from the inoculum, both at the time of preparation of the inoculum from tissue culture, as well as just before inoculation, ruled out any possibility of the presence of a wild-type sequence in the inoculum. It is also of significance to note that complete reversion to the wild sequence was concordant with a marked ALT increase in the chimpanzee [60]. The difference relating to the stability of sG145R mutation observed between the two studies may be due to the nature of the preparation of the inocula used and the methodologies applied for characterization of the mutations. The inoculum used in

Figure 1. Biochemical, serological and virological markers of HBV infection in two chimpanzees

Reproduced, in part, from [60] with permission from John Wiley & Sons, Inc. Two chimpanzees, (A) CH1599 and (B) CH1611, were inoculated with tissue-culture-derived S gene sG145R mutant and triple pol mutant, respectively. The dashed line indicates the alanine aminotransferase (ALT) activity cutoff. Anti-HBc, antibody against hepatitis B core antigen; anti-HBe, antibody against hepatitis B e antigen; anti-HBs, antibody against hepatitis B surface antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; IgM, immunoglobulin M.
the previous study was directly sourced from the serum of a vaccinee and might have contained a mixture of wild-type and mutant viruses [58], whereas the inoculum used in our study was a pure monoclonal mutant. Furthermore, to characterize the stability of the mutation, we sequenced multiple clones from the inoculum as well as the samples from the viremic phase of the infected chimpanzee [60].

The protective efficacy of licensed hepatitis B vaccines against challenge with serum-derived sG145R mutant has been shown in other challenge studies in chimpanzees [61]. No evidence of HBV infection or hepatitis was observed in hepatitis B vaccinated chimpanzees after intravenous challenge with sG145R mutant [HBV strain AS]. Moreover serum anti-HBs in the vaccinated chimpanzees was shown to react not only with wild-type, but also with mutant HBsAg.

**pol mutants**

Antiviral drug resistance mutations have been described in chronic hepatitis B patients receiving prolonged treatment with approved nucleotide or nucleoside analogues, such as lamivudine, adefovir dipivoxil, entecavir, telbivudine and tenofovir disoproxil fumarate [62]. The emergence of antiviral drug resistance is associated with an increased risk of liver-related complications, such as hepatic flares in patients with compensated liver disease, worsening of the histopathology, decreased rates of hepatitis B e antigen seroconversion and severe exacerbation of hepatitis in patients with liver cirrhosis [62,63].

Lamivudine resistance mutations include rtM204V/I, with the compensatory mutation rtV173L [64,65]. The mutation M204V/I in the YMDD motif has been shown to confer lamivudine resistance with or without the rtL180M mutation in *in vitro* experiments. HBV with rtM204I alone is capable of replication under lamivudine therapy, whereas rtM204V is more often accompanied by rtL180M [63]. HBV with rtM204V and rtM204I mutations shows markedly reduced fitness compared with wild type. However, addition of rtL180M has been shown to restore replication [64,66]. There is experimental evidence that the mutations at rtV173L and rtL80IV do not alter the sensitivity of HBV to lamivudine, but enhance its replication efficiency [63,65]. Another mutation, rtA200V, which has been observed in combination with rtM204I, results in partially restored fitness without changing the sensitivity to lamivudine [67]. Several other mutations arising as a result of prolonged lamivudine therapy include rtL80IV, rtL82M, rtF166L, rtV173L, rtA200V and rtV207I [64,65,68–71].

The lamivudine-associated resistance mutations rtV173L, rtM204V/I and rtV207I could cause crucial antigenic changes in the HBsAg, as a result of producing the mutations sE164D, sW194stop, sI195M, sW196L/stop and sM198I or sW199stop [14,74–76]. Another mutation, rtA181T, found in lamivudine-treated patients, which was confirmed in an *in vitro* phenotypic assay to be responsible for lamivudine resistance, concomitantly generates a stop codon in HBsAg (sW172stop), resulting in the impaired secretion of the HBsAg and in reduced viral fitness [77]. The same mutation, rtA181T, has been described in a patient under famciclovir therapy [78]. Other lamivudine-associated resistance mutations can cause premature stop codons in the S gene, also resulting in impaired secretion of HBsAg [63]. A triple mutational pattern, rtV173L plus rtL180M plus rtM204V, associated with lamivudine resistance has enhanced HBV replication compared with rtL180M plus rtM204V alone [65]. This triple mutant brings about two amino acid changes in the overlapping S gene, sE164D plus sI195M, reducing its anti-HBs binding to levels seen only with the prototype vaccine-escape mutant, sG145R [53,79,80].

Antiviral drug-resistant mutations have also been observed in patients treated with adefovir dipivoxil, entecavir, telbivudine and tenofovir disoproxil fumarate [62]. Resistance mutations after adefovir dipivoxil therapy are selected at a markedly reduced frequency compared with lamivudine therapy [81]. Mutations associated with resistance to adefovir dipivoxil include rtA181T or A181V, and rtN236T [82–84]. Recently, additional mutations such as rtV84M, rtS85A, rtA181T/V, rtV191I and rtL217R have been linked to adefovir dipivoxil failure [63,74,85,86]. Several of these mutations have been shown to result in decreased sensitivity to adefovir dipivoxil in *vitro* [87]. Entecavir resistance has been linked to either the rtM204V or rtM204I mutation. Recently, two different mutational patterns, namely rtL169T plus rtL180M plus rtM204V plus rtM204V, and rtL180M plus rtM204V plus rtT184G plus rtS202I, were reported in a small number of patients treated with entecavir who experienced viral breakthrough. These resistance patterns, when tested *in vitro*, were shown to confer decreased susceptibility to both lamivudine and entecavir [88]. A recent study has shown that approximately 50% of lamivudine-resistant patients develop entecavir resistance during 5 years of therapy [89]. The only mutation that has been shown to be associated with telbivudine resistance is rtM204I. Tenofovir disoproxil fumarate has been shown to be active against lamivudine-resistant HBV mutants *in vivo* and *in vitro* [87,90,91]. Several studies have shown no selection of mutations responsible for tenofovir disoproxil fumarate resistance within the first year of therapy [92,93].
Infectivity, pathogenicity and efficacy of vaccination against pol mutants

We recently reported virological, serological and clinical characteristics of infection with in-vitro-generated, lamivudine-resistance-associated, triple mutant, which contains the combination of rtV173L, rtL180M and rtM204V mutations [60]. The mutant was prepared as the sG145R mutant, described above (see Infectivity and pathogenicity of S gene mutants, and efficacy of vaccination). Three chimpanzees inoculated intravenously with the triple mutant developed similar serological profiles of HBV infection (Figure 1B). No increase in ALT levels was observed in any of the inoculated chimpanzees. Sequencing studies of multiple clones from the inocula and samples collected during viraemia in the chimpanzees showed the preservation and stability of the rt173L, rt180M and rt204V mutations. Challenge studies were then carried out in two young chimpanzees vaccinated with paediatric doses of hepatitis B vaccine to determine if vaccination could confer protection against the triple mutant. A robust T-cell immunity measured by the interferon-γ ELISPOT assay, and marked serum anti-HBs titres (>75 mIU/ml) were induced (Figure 2). HBsAg-specific T-cell lines derived from the blood of the vaccinated chimpanzees were exclusively CD4+; no CD8+ T-cells specific for HBsAg were detected. After challenge with the triple mutant, detection of anti-HBc in both the vaccinated chimpanzees provided immunological evidence of HBV replication [94,95]. Anti-HBc responses have been considered presumptive evidence of the breakthrough infection in other studies [96,97] and breakthrough infections linked to HBV mutants have been reported to occur among HBV-immunized populations, despite the presence of seroprotective levels of anti-HBs in the infected individuals [13,98]. Further evidence that breakthrough infection had occurred in our challenge experiment was provided by evidence of priming of T-cell responses against Pol, which is not a component of the vaccine (Figure 3). Together, these observations suggest that sterilizing immunity was not induced in the two vaccinated animals.

The efficacy of anti-HBV immunity induced by the commercial vaccine was further tested by re-challenging the same two chimpanzees (previously vaccinated and challenged with the triple mutant) with serum-derived wild-type HBV (HLD1). This experiment showed a subsequent boost of anti-HBs (6- to 10-fold increase) in both chimpanzees (Figure 2) and a surge of T-cell responses against both envelope- and polymerase-derived peptides (Figure 3). This pattern of cellular and humoral immune responses against HBV antigens strongly implicated viral replication occurring in the presence of very high levels of circulating anti-HBs, suggesting breakthrough infection by wild-type HBV [60].
The animal studies reviewed here, particularly those involving chimpanzees, suggest that HBV mutants have the potential to infect previously vaccinated individuals. These mutants could enter the blood supply undetected and infect vaccinated recipients of blood products. Whether such infected individuals clear the infection expeditiously or develop chronic infection remains unknown. Further studies of the efficacy of hepatitis B against S gene and pol mutants, and the natural history of people undergoing breakthrough infection with these mutants are warranted.

Disclosure statement

The author declares no competing interests.

References


