Impaired Virion Secretion by Hepatitis B Virus Immune Escape Mutants and Its Rescue by Wild-Type Envelope Proteins or a Second-Site Mutation

Karen Kwei,* Xiaoli Tang,* Anna S. Lok,b Camille Sureau,c Tamako Garcia,* Jisu Li,a Jack Wands,a Shuping Tonga

Liver Research Center, Rhode Island Hospital, Brown University, Providence, Rhode Island, USA; Division of Gastroenterology and Hepatology, University of Michigan Health Systems, Ann Arbor, Michigan, USA; Laboratoire de Virologie Moleculaire, INTS, Paris, France

Hepatitis B virus immune escape mutants have been associated with vaccine failure and reinfection of grafted liver despite immune prophylaxis, but their biological properties remain largely unknown. Transfection of 20 such mutants in a human hepatoma cell line identified many with severe impairment in virion secretion, which can be rescued to various extents by coexpression of wild-type envelope proteins or introduction of a novel glycosylation site. Consistent with their role in maintaining intramolecular disulfide bonds, cysteine residues within the “a” determinant are critical for virion secretion.

Infection by hepatitis B virus (HBV) can be prevented by vaccination with the small (S) envelope protein, the most abundant envelope protein on the virion surface and the primary component of the empty envelope protein particles, or subviral particles (1). The neutralizing antibodies thus elicited target the immunodominant loop of the S protein (residues 101 to 163), especially its “a” determinant (residues 124 to 147), with residues 141 to 146 being the most dominant epitope (2). However, breakthrough infection despite induction of neutralizing antibodies has been documented and is often attributed to single amino acid substitutions within the “a” determinant (3–8). Such immune escape mutants are also responsible for reinfection of grafted liver despite passive prophylaxis with hepatitis B immunoglobulin (HBIG) (9–14). The mutations render the S protein poorly recognizable by antibodies raised against the wild-type (WT) virus, which forms the structural basis for both immune escape and false-negative diagnostic test results. An important serological marker of HBV infection is hepatitis B surface antigen (HBsAg), the collective term for viral envelope proteins present on virions and the large excess of subviral particles. The immune escape mutations can render HBsAg undetectable or poorly detected by immunoassays based on monoclonal antibodies against wild-type virus (15–18), contributing to some cases of “occult HBV infection” (lack of detectable HBsAg despite positive HBV DNA) (8, 19–23).

There are concerns that the universal vaccination program will provide the driving force for the spread of immune escape mutants and gradually render the current vaccine ineffective in eradicating HBV infection (24, 25). The prevalence of immune escape mutants in HBV DNA-positive children in Taiwan increased from 7.8% before to 22.6% 20 years following the nationwide vaccination program, but the prevalence of chronic HBV infection among the pediatric population decreased from 9.6% to 0.5% during this same period, indicating that while immune escape does occur, the HBV vaccine continues to be highly effective (5, 26). The classic G145R immune escape mutant reverted to the wild type when HBIG was discontinued in liver transplant recipients and during follow-up of mutant-infected children (10, 26) or when the mutant was transmitted to naïve chimpanzees (27, 28). These observations strongly suggest that in the absence of immune pressure, these mutants have reduced fitness compared to the wild-type virus. In this regard, the classic G145R immune escape mutant was found to be severely impaired in virion secretion (29, 30). Similarly, we reported that the I110M and G119E mutations hamper virion secretion (29, 31). In the present study, we characterized the virion secretion impact of 20 immune escape mutations or other mutations inside the immunodominant loop (Table 1). The escape mutations chosen for the current study, including those from HIV- and hepatitis C virus (HCV)-coinfected women with alcohol abuse, have been associated with vaccine failure, breakthrough infection of transplanted liver despite HBIG prophylaxis, and occult HBV infection.

A trans-complementation assay to study virion secretion. Experiments were performed on a genotype A clone. As previously reported (32), we used a subgenomic HBV DNA fragment to express the large (L), middle (M), and S envelope proteins. The 2.3-kb (0.7mer) fragment (nucleotides 2721 to 3221/1 to 1770) was inserted upstream of the SV40 polyadenylation signal and cloned into the pBluescript vector. Mutations were introduced to the 0.7mer construct by replacement of its AvrII-EcoRV restriction fragment with PCR products. To examine the impact of immune escape mutations on virion secretion, the 0.7mer construct was cotransfected with a 1.5mer (4.8-kb) HBV genome (nucleotides 1044 to 3221/1 to 2600) cloned into the pBluescript vector (32). The latter construct, which was rendered unable to express the envelope proteins through a G261A nonsense mutation at the 5′ end of the S gene, served as the source for core and polymerase protein translation and genome replication. Since the genome
thus packaged inside virions is unable to express envelope proteins, this approach minimizes biological hazard. Using a 1.5mer construct as the common source for all functions other than envelope protein expression also simplifies data interpretation, because some immune escape mutations induce missense or even nonsense mutations in the overlapping polymerase gene (Table 1) that might affect HBV DNA replication and, consequently, the amount of virions secreted.

Envelope protein expression and HBsAg secretion. The Huh7 human hepatoma cell line grown in 6-well plates was cotransfected with the 1.5mer replication construct (1.5 μg) and the 0.7mer WT or mutant envelope protein construct or 0.5 μg of vector DNA. Alternatively, cells were transfected with 1.5 μg of N16, a 1.5mer construct capable of envelope protein expression. Cells and culture supernatant were harvested at day 5 posttransfection.

(A and B) One aliquot of cell lysates was used for a Western blot analysis with a monoclonal anti-preS2 antibody (S26; Virogen) (A) and, following stripping, with a horse polyclonal anti-S antibody (anti-Ad/Ay; Abcam) (B). The two bands for L, M, and S proteins are gp42/p39, gp36/gp33, and gp27/p24, respectively. Please notice the lack of gp42, gp36, and gp27 bands in the N146S mutant. (C) Another aliquot was used for a Western blot analysis of core protein. (D) A third aliquot was used for a Southern blot analysis of replicative DNA. (E) Virions were immunoprecipitated from culture supernatant using a monoclonal anti-preS2 antibody (Virogen), followed by DNA extraction and

![FIG 1](impact of amino acid substitutions in the immunodominant loop on virion secretion. Huh7 cells grown in 6-well plates were cotransfected with 1.5 μg of the 1.5mer replication construct and 0.5 μg of the 0.7mer WT or mutant envelope protein construct or 0.5 μg of vector DNA. Alternatively, cells were transfected with 1.5 μg of N16, a 1.5mer construct capable of envelope protein expression. Cells and culture supernatant were harvested at day 5 posttransfection. (A and B) One aliquot of cell lysates was used for a Western blot analysis with a monoclonal anti-preS2 antibody (S26; Virogen) (A) and, following stripping, with a horse polyclonal anti-S antibody (anti-Ad/Ay; Abcam) (B). The two bands for L, M, and S proteins are gp42/p39, gp36/gp33, and gp27/p24, respectively. Please notice the lack of gp42, gp36, and gp27 bands in the N146S mutant. (C) Another aliquot was used for a Western blot analysis of core protein. (D) A third aliquot was used for a Southern blot analysis of replicative DNA. (E) Virions were immunoprecipitated from culture supernatant using a monoclonal anti-preS2 antibody (Virogen), followed by DNA extraction and

![TABLE 1](mutations in the immunodominant loop for functional characterization)

<table>
<thead>
<tr>
<th>S domain substitution</th>
<th>Nucleotide change</th>
<th>Polymerase substitution</th>
<th>Other substitution(s) of the same site</th>
<th>Association(s) and/or purpose(s)</th>
</tr>
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<tbody>
<tr>
<td>T114R</td>
<td>G495G</td>
<td>N122K</td>
<td>T (T114T)</td>
<td>B, C</td>
</tr>
<tr>
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<td>N123S</td>
<td>I, N</td>
<td>C</td>
</tr>
<tr>
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<td>A506G</td>
<td>Y126C</td>
<td>K, M, R, S, V</td>
<td>A, B, D</td>
</tr>
<tr>
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<td>C507A</td>
<td>Y126stop</td>
<td>A, M, R, S, V</td>
<td>A, B, D</td>
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<td>514insAGCS15</td>
<td>128insK/M129L</td>
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<td>B</td>
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<tr>
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<td>C512A</td>
<td>T128N</td>
<td>L, Q, R, S</td>
<td>A, B, C, D</td>
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<td>M129S</td>
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<td>E</td>
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<td>R138T</td>
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<tr>
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<td>S</td>
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<td>I, Q, W</td>
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<sup>a</sup> A, vaccine failure; B, graft reinfection despite passive immunoprophylaxis; C, occult infection; D, anti-HBs-positive infection; E, to test the role of cysteine residues.
Southern blot analysis. We did not use anti-S antibodies for immunoprecipitation in case immune escape mutations weaken antibody binding to mutant envelope proteins. The G130R, C138Y, D144G, N146S, and C149R mutants were completely defective in virion secretion (Fig. 1E), while the T114R, T115A, P127S, Q129H, C147R, and K141E mutants secreted very few virions. In contrast, the T118A, P120T, and N131S mutants displayed efficient virion secretion. Very similar results were obtained when virions were immunoprecipitated with R254, a rabbit polyclonal antibody targeting the preS1 domain of the L protein (data not shown) (33).

Most mutants with low HBsAg values in culture supernatant (suggestive of impaired HBsAg secretion or detection or reduced HBsAg stability) were also impaired in virion secretion. On the other hand, several mutants with severely impaired virion secretion had no major defect in HBsAg recognition, at least according to the three kits employed in this study (G130R, N146S). As we already reported, the N146S mutant is deficient in virion secretion due to ablation of the N-linked glycosylation site in the S protein (29). As for the G130R mutant, its S protein was easily detectable by Western blotting if preconcentrated from culture supernatant by immunoprecipitation with the same anti-S polyclonal antibody but not if precipitated by polyethylene glycol (PEG) (data not shown). Thus, this mutation might disrupt particle formation or trigger its disassembly.

The observation that the C138Y, C147R, and C149R mutants were all severely hampered in virion secretion is consistent with the importance of cysteine residues in maintaining the proper intramolecular or intermolecular disulfide bonds in the immunodominant loop (34,35). Analysis of additional mutations (C121A, C124A, C147A, and C149A) revealed that they severely reduce virion secretion as well (Fig. 3C), although HBsAg secretion was unaltered by the C147A mutation and only moderately reduced by the C149A mutation when analyzed by the Abbott kit (Fig. 3D).

**Rescue of virion secretion by coexpression of wild-type envelope proteins.** Immune escape mutants often coexist with a small amount of wild-type virus. We cotransfected a mutant 0.7mer construct with a wild-type 0.7mer construct at a 5:1 (0.5 μg/0.1 μg) or 1:1 (0.3 μg/0.3 μg) ratio to examine whether the wild-type envelope proteins can rescue virion secretion through phenotypic mixing. At a 5:1 ratio, virion secretion was rescued efficiently only for the P127S and K141E mutants and moderately for the G145R mutant (Fig. 4A). It is interesting to note that the K141E mutant has been associated with vaccine failure in two Gambian children despite protective levels of anti-HBs antibody (7). Coexpressing mutant and wild-type envelope proteins at a 1:1 ratio led to effi-
cient rescue of virion secretion for all the mutants except C138Y (Fig. 4C). However, at such a ratio, a high level of HBsAg was detectable in culture supernatant, especially according to PEG precipitation followed by Western blots (Fig. 4E), suggesting a reversal of the immune escape phenotype or restoration in HBsAg secretion. It remains to be determined whether virions produced by coexpression of mutant and wild-type envelope proteins contain a mixture of the two types of proteins or just the wild-type proteins (if the mutant proteins have a dominant negative effect on virion formation or secretion). In the latter case, viral infectivity will still be neutralized by anti-S antibodies raised against wild-type envelope proteins.

Rescue of virion secretion by an M133T mutation. We recently found that a virion secretion defect associated with two missense mutations in the viral envelope proteins (I110M and G119E) as well as the classic G145R immune escape mutation can be overcome by another naturally occurring mutation, M133T, which creates a novel N-linked glycosylation site (29, 31). In fact, M133T itself is frequently associated with occult HBV infection or failed HBIG prophylaxis and often associates with "a" determinant mutations such as G130N, F134L, D144A, D144G, G145A, G145K, and G145R (12,36–40). Introduction of the M133T mutation rescued virion secretion efficiently for the T118K, P127S, and N146S mutants (Fig. 5B). It also rescued virion secretion for the G130R, C138Y, C147R, and C149R mutants but much less efficiently. As expected, the M133T mutation is associated with an

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FIG 3 Critical role of cysteine residues in the immunodominant loop on virion secretion. Huh7 cells grown in 6-well plates were cotransfected with 1.5 μg of the 1.5mer replication construct and 0.5 μg of the 0.7mer expression construct as indicated or with vector DNA. Cells and culture supernatant were harvested at day 5 posttransfection. (A and B) An aliquot of cell lysate was used for a Western blot analysis of S protein (A), and another aliquot was used for a Southern blot analysis of replicative DNA (B). (C) Virions were immunoprecipitated from culture supernatant using an anti-preS2 monoclonal antibody, followed by Southern blotting. (D) Secreted HBsAg was determined by the Abbott ELISA kit.

FIG 4 Rescue of virion secretion by coexpression of wild-type envelope proteins at two different ratios. Huh7 cells grown in 6-well plates were transfected with 1.4 μg of the 1.5mer replication construct, together with 0.5 μg of the mutant 0.7mer expression construct plus 0.1 μg of vector DNA or the WT 0.7mer expression construct (A and B) or 0.3 μg each of the mutant 0.7mer expression construct and vector DNA or the WT expression construct (C to F). Cells and culture supernatant were harvested at day 5 posttransfection. (F) Cell lysate was used for a Western blot analysis of S protein. (A and C) The bulk of the culture supernatant was used for immunoprecipitation by an anti-preS2 antibody, followed by Southern blotting. (E) Another aliquot was used for PEG precipitation, followed by Western blotting with an anti-S antibody. A small volume was used for HBsAg detection by the Abbott kit (10 μl for panel B and 3 μl for panel D).
additional protein band consistent with the doubly glycosylated S protein (Fig. 5A).

Are S protein residues critical for virion secretion also important for infectivity? The S protein is involved not only in HBV virion secretion but also in viral infectivity. Interestingly, many immune escape mutations also severely impaired infectivity of hepatitis delta virus (41), which hijacks HBV envelope proteins for release from and entry into hepatocytes. The immune escape mutations were detrimental to infectivity when present on the S but not the L protein (33). Similarly, we found that introducing the I110M, G119E, and R169P mutations into the S protein impaired HBV virion secretion but that virion secretion was unaltered when the same mutations were introduced to L and M proteins only (29). Moreover, I110, G119, C124, K141, P142, C147, and C149 are critical for both virion formation and viral infectivity, with the notable exception that the G145R mutation did not compromise infectivity despite severely impairing virion formation (29, 33, 41). This maintenance of infectivity might explain its higher prevalence compared to other immune escape mutations. Further investigation is warranted to establish whether the same mutations were introduced to L and M proteins only (29).

The threat posed by immune escape mutants depends on the degree of immune escape as well as their biological fitness. Besides mutations in the “a” determinant, which may affect virion release from and entry into hepatocytes, missense mutations in the overlapping P gene (Table 1) might influence viral replication capacity, a topic which remains to be explored in the future. Antibodies made in response to the HBV vaccine mostly target an epitope composed of residues 141 to 146 (2), and mutations at this site will have the highest degree of immune escape. Nevertheless, as shown before and here, K141E, P142S, D144G, G145R, and loss of the N-linked glycosylation site at N146 severely impair virion secretion and are thus predicted to delay virus spread (29, 30). As these mutations (except G145R) probably also impair HBV infectivity (33, 41), they might not spread infection in the new host quickly enough to avoid clearance by the immune response. The deficiency in virion formation can be rescued by the WT virus at a 1:1 ratio, but that will most likely reverse the immune escape phenotype of the mosaic virus particle. A more worrisome scenario is the acquisition of a second-site mutation, such as M133T, which efficiently restores virion secretion of some immune escape mutants without reversing the immune escape phenotype (Fig. 5C). It should be pointed out that most non-A HBV genotypes contain T131 instead of N131, and for these genotypes, the M133T mutation alone is insufficient to create a novel N-linked glycosylation site. Nevertheless, a T131N/M133T double mutation creating such a glycosylation site in non-A genotypes is frequently observed (9, 42) (X. Zhang and J. Zhang, personal communications) and can be associated with G145R (38).

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