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## Surface protein mutations in chronic hepatitis B patients who received hepatitis B vaccine therapy

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### ABSTRACT

**Objective (s):** The aim of this study was to determine the correlation between vaccine therapy and appearance of mutations in hepatitis B surface antigen (HBsAg)-positive chronic hepatitis B virus (HBV) patients.

**Materials and Methods:** 16 patients received the HBV vaccine and another 16 individuals from the control group did not. The surface gene was amplified and directly sequenced from samples prior to vaccination and six months after the third dose.

**Results:** Only one patient lost HBsAg. 48 and 44 amino acid mutations were found before and after vaccine therapy in the vaccine group respectively, 51 of which (55.4%) occurred in immune epitopes: 5 were in B cell, 21 in T helper (Th), and 25 in cytotoxic T-lymphocyte (CTL) epitopes. In the control group, 35 and 41 amino acid substitutions were found before and after therapy, respectively. 32 (42%) of 76 amino acid changes occurred within immune epitopes. There were no differences in age, gender, and duration of chronicity in both patient and control groups in terms of the frequency and the patterns of mutations.

**Conclusion:** In chronic carriers who already had HBsAg variants selected by the host-immune response, any immune stimulation by the vaccine had no effect on the chronic state of these patients or selected any remarkable escape mutants. Newer strategies should be considered based on third generation or the use of DNA vaccines or new adjuvants.

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### Introduction

Despite the presence of an effective prophylactic vaccine since 1982, more than 370 million people in the world are now chronically infected with hepatitis B virus (HBV). A considerable number of these chronic HBV carriers would eventually develop serious complications like liver cirrhosis and hepatocellular carcinoma (HCC). Chronic HBV carriers are a permanent source of HBV infection, and can transmit HBV to uninfected, healthy individuals. Taken together, chronic HBV infection represents a major global public health problem, especially in the developing nations of Asia and Africa where most of the chronic HBV carriers reside.

Nucleoside analogues such as lamivudine have exhibited highly effective antiviral activity, but

mutations in the viral polymerase protein are frequently associated with a recurrence of HBV replication (1, 2). In this context, an alternative approach to HBV treatment has been proposed, consisting of immunotherapy using vaccination with recombinant envelope proteins (3-5), and a new field of immunological research and clinical application of therapeutic vaccines (vaccine therapy) has been started in chronic HBV carriers (6-9). In recent trials, therapeutic vaccines have been associated with antiviral agents in an attempt to favor T-cell restoration (10, 11). Basically, four types of therapeutic vaccines are being developed to fight chronic HBV infection. They include: (i) vaccines based on injection of recombinant HBV proteins, (ii) HBV-envelope subviral particles, (iii) naked DNA eventually combined with viral vectors and (iv)

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vaccines based on T-cell peptide epitopes derived from different HBV proteins (12).

It is well-established that the HBV surface antigen (HBsAg) is a major target of the humoral and cellular immune response against HBV. Within the HBsAg, the 'a' determinant is an important target of the humoral immune response (13). In recent years, envelope mutants have been detected following vaccination or hepatitis B surface antigen (anti-HBs) immunoglobulin (HBIG) therapy. Vaccine-associated HBsAg mutations have been identified principally in the 'a' determinant (14-16). The sequence variation in antigenic regions is one of the most powerful viral strategies for escaping recognition by both the B and T cell-mediated immune system of the host and facilitates viral persistence (17). These mutants, possibly selected under vaccine pressure, may escape neutralization by vaccine-induced anti-HBs (vaccine-escape) (15). However, such HBV mutants are also present in chronic, asymptomatic HBV carriers (natural immune-escape) (18-20). Not surprising, the success of vaccine-therapy strategies, has now been challenged by the recent discovery of mutant hepatitis B viruses showing amino acid exchanges in HBsAg, which might lead to reduced or even abolish binding of vaccine-induced neutralizing antibodies (15).

The aim of this study was to determine the correlation between vaccine therapy and mutation patterns in chronic HBV patients given a recombinant hepatitis B vaccine.

## Materials and Methods

We enrolled 32 persons with biopsy-proven chronic active HBV replication, as shown by the presence of HBV DNA in sera, in a controlled study of vaccine therapy. None had prior anti-HBV therapy, or co-infection with HIV, HCV and HDV. The inclusion criteria were chronic hepatitis which was defined as HBsAg positivity with or without the presence of HBeAg and moderate to high HBV DNA levels (<100,000 copy/ml; mean 15,000), persistent or intermittent elevation in the serum ALT levels ( $85 \pm 109.2$  (mean  $\pm$  SD)), and documented hepatitis by liver biopsy. The subjects were randomized into two groups: those given Engerix-B (Glaxo Smith Kline, Belgium) (16 patients), and those given no vaccine as control (16 patients). Subjects were given three standard injections (each dose 40  $\mu$ g to the left deltoid muscle) at zero, one- and six-month intervals. All patients gave informed consent and the study protocol was approved by the local ethics committee (No 3954). Blood samples were collected at various times before, and 6 months after, vaccination. Vaccine efficiency was defined as a sustained loss of, or a 50 % prevaccination-level decrease in HBV DNA.

Serum HBV DNA was measured by COBAS Amplicor version 2 (ROCHE, Heidelberg, Germany).

### DNA extraction

HBV DNA was extracted from a 200  $\mu$ l aliquot of serum from the pre-vaccination period and 6 months post-vaccination, using the Qiagen Mini Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In brief, 20  $\mu$ l of protease was added to the serum in a 1.5 ml tube. Then, 200  $\mu$ l of AI buffer was added to each tube, vortexed and incubated for 10 min at 56°C. For DNA precipitation, 200  $\mu$ l of ethanol was added to the mixture, and centrifuged for 1 min. Components were transferred to a collection tube containing a filter tube. Trapped DNA was washed in two steps with AW1 and AW2 buffers to eliminate impurities, together with centrifugation after each step. Finally, DNA was eluted using 100  $\mu$ l of elution buffer, and stored at -20°C.

### Polymerase chain reaction

The surface gene was amplified using two pairs of primers as described previously (26). A nested PCR was carried out in 100  $\mu$ l of a mixture containing 5  $\mu$ l of DNA using HotStart Taq PCR (Qiagen, Hilden, Germany). 5  $\mu$ l extracted HBV DNA and 1  $\mu$ l of the first round amplicon were used as template for the first and the second round PCR reactions, respectively. Finally, 3  $\mu$ l of second- round PCR product was analyzed by electrophoresis in 1% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light.

### DNA sequencing

The HBsAg subtype of the sequences was defined by substitutions in the 'a' determinant between codons 122 and 160, inclusive. Direct sequencing of surface genes was carried out (Perkin Elmer ABI-3130XL DNA Sequencer, Fostercity, CA, USA) using 0.5  $\mu$ l of an appropriate internal primer for the surface gene (26). The results were analyzed using Chromas and BioEdit software. Genotyping was carried out on samples using the region of surface gene specifying HBV genotypes/subtypes.

### Sequence analysis

After allocating a sequence to an HBV genotype by analysis of the S gene, the surface gene amino acid/nucleotide variations found were compared with a reference sequence obtained from Okamoto (1988, accession number, AB033559) and HBsAg sequences from Iranian isolates obtained from GenBank and NCBI. Compared to the former, any amino acid changes were defined as "variant" (host HLA-determined). As regards the latter (Iranian database sequences), amino acid differences were defined as "mutation".

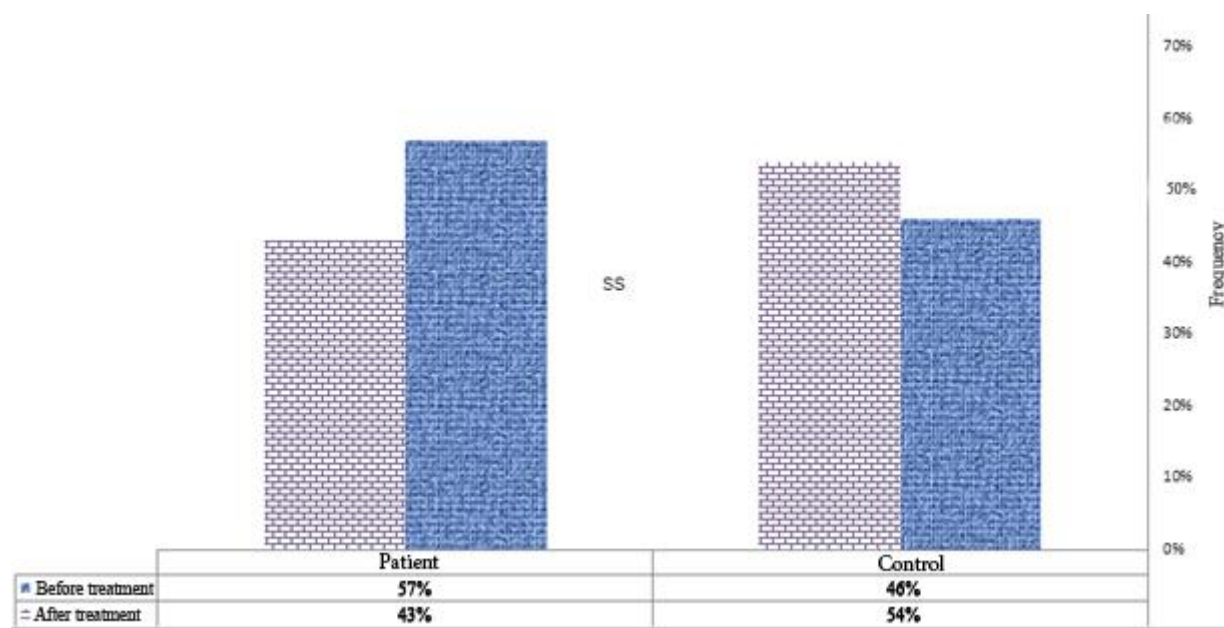


Figure 1. Diagram shows percentage of mutations frequency in recipient vaccine and control group at the same time

### Statistical analysis

Descriptive statistics were used such as frequency; mean and standard deviation. Comparisons between groups were made using the chi-square test and the Fisher's exact test.

### Results

All HBV patients were assigned to genotype D, subtype ayw2 (results not shown). One out of sixteen patients was responsive to vaccine, i. e. became HBV DNA and HBsAg negative. Overall, at the nucleotide level, 310 and 272 point mutations (before and after vaccine therapy) were found in the patient and the control group, respectively (Figure 1). 92 (29.6%) and 218 (70.3%) of those changes seen in the patient group were missense and silent substitutions, respectively (results are not shown). In the control group, 76 (24.3%) and 195 (75.7%) of nucleotide changes were missense and silent mutations, respectively (results are not shown). The nucleotide mutation frequencies in the patient and control groups were 2.3 and 2.5, respectively. 28 and 44 amino acid mutations were found before and after vaccine therapy in the patient group (Figure 1). In the control group, 35 and 41 amino acid substitutions were found before and after therapy, respectively (Figure 1). A summary of amino acid changes within the surface proteins is shown in Tables 1 and 2.

### Correlation of gender and age with occurrence of mutants

The Fisher's exact test showed no correlation between gender ( $P=0.516$ ) and age ( $P=0.65$ ) of participants and amino acid changes within the

immune epitopes inside the surface protein in the patient and the control groups (results are not shown).

### Mutations within immune epitopes

#### Patient groups

Forty-eight and forty-four amino acid mutations were found before and after vaccine therapy. 51 (55.4%) out of 92 amino acid changes occurred in different immune epitopes within the surface protein (Table 1, Figure 2), of which, 5 (9.8%) occurred in B cell epitopes [27] affecting 4 residues; 21 (41.1%) occurred in T helper epitopes [28] affecting 19 residues and 25 (52.9%) affected 5 residues inside known HLA-A2-restricted CTL epitopes [29-30] (Table 1, Figure 2).

#### Control group

Thirty-five and forty-one amino acid mutations were found before and after the period of vaccine therapy. Similarly, 32 (42%) of 76 amino acid changes which occurred within immune epitopes, 4 (12.5%) occurred in B cell epitopes affecting 4 residues; 14 (43.7%) occurred in the T helper epitopes affecting 9 residues and 14 (43.7%) occurred in 3 residues inside the CTL epitopes (Table 2, Figure 3).

### B cell epitopes

Within the "a" determinant, only one mutation (S143L) was found in one patient before vaccine administration which was absent after therapy (Table 1). 76.4% and 87.5 % of sequences had no mutation before and after therapy within the B cell epitopes in patient and control groups, respectively (Tables 1 and 2, Figure 2 and 3). Statistical analysis showed no association between vaccine therapy and the occurrence of mutations before and after therapy, in

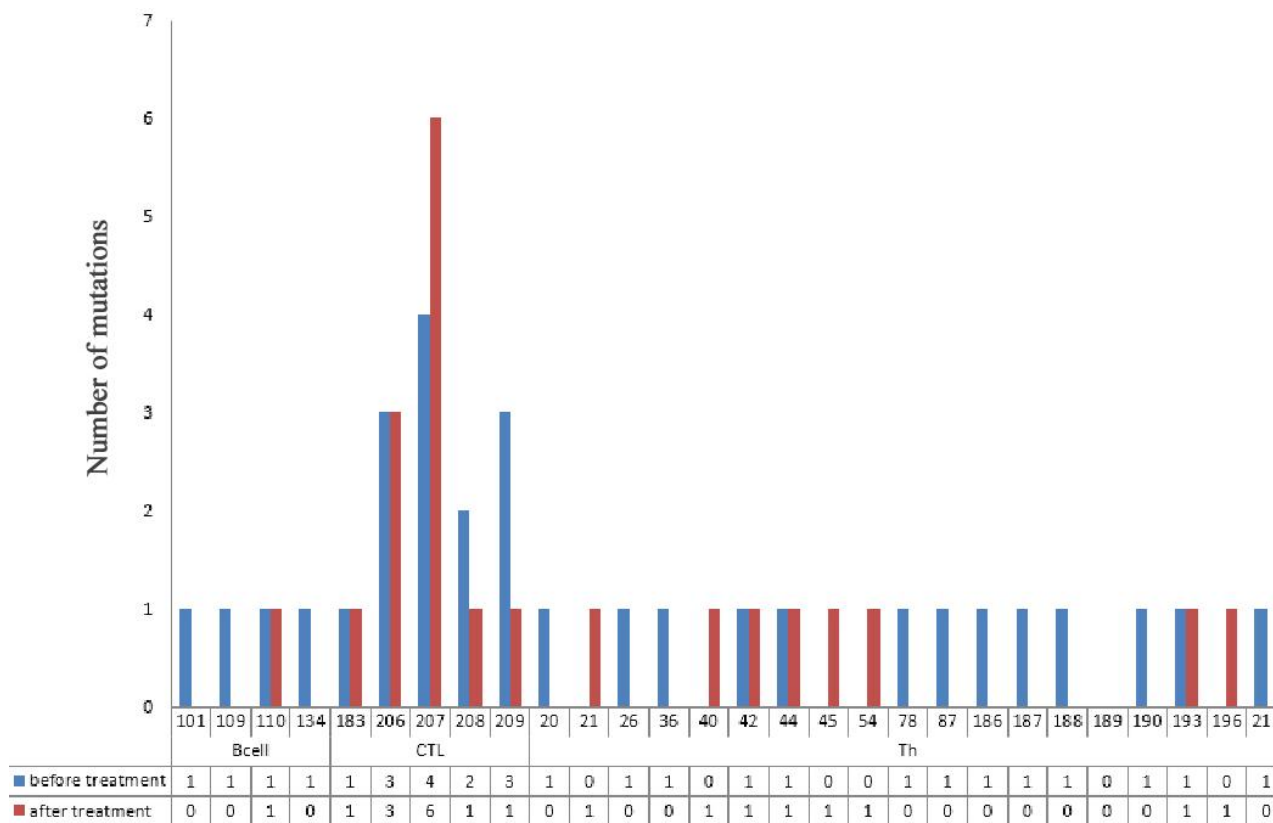


Figure 2. Position of amino acid and number of mutations at immune epitope in recipient vaccine group before and after therapy

both patient and control groups ( $X^2=0.12$ ).

**T helper epitopes**

Nine and seven mutations occurred after vaccine administration in patient and control groups, respectively. 20.8% and 21.9 % of sequences showed the same pattern of mutation distribution before and after therapy in the patient and control groups, respectively. 16.6% of the sequences contained no mutations before therapy, but showed at least one mutation after therapy. 37.5% and 65.8% of the sequences showed no mutation before and after therapy in patient and control groups, respectively (Tables 1 and 2, Figures 2 and 3). Statistical analysis showed no association between the occurrence of mutations before and after therapy in Th epitopes in both patient and control groups ( $X^2=0.4$ ).

**CTL epitopes**

Twelve and eight mutations occurred after vaccine administration in patient and control groups, respectively. 26% and 53.15 % of the sequences contained no mutation before and after therapy in patient and control groups, respectively (Tables 1 and 2, Figures 2 and 3).

Statistical analysis showed no association between the occurrence of mutations before and after therapy in the CTL epitopes in both groups ( $X^2=0.12$ ).

Overall, in 16 patients, 5 cases showed a decrease in number of mutations after therapy (15 mutations), 3 showed an increase in the number of mutations (8 mutations) and in 8 cases there were no changes before and after therapy (6 mutations). In the control group, one patient showed a decreased number of mutations with no therapy and 4 cases showed an increased number of mutations (8 mutations) and no differences in the pattern of mutations before or after no therapy were seen in 11 patients.

**Discussion**

Although there was considerable optimism regarding the therapeutic efficacy of vaccine therapy in patients with chronic HBV infection, most studies did not find any significant benefit from the administration of such a vaccine. Vaccine therapy has inspired optimism as an alternative therapeutic approach; however, the efficacy of vaccination in patients with end-stage liver diseases and transplant recipients is disappointing.

In this case control study, we investigated the efficacy of HBV vaccine in two groups of patients. The aim of this study was to determine the correlation between vaccine therapy and mutation pattern in patients given a recombinant hepatitis B vaccine in HBsAg- positive chronic patients with HBV DNA>10<sup>3</sup> copies/ml. The results showed that only one patient responded and became HBV DNA- negative.

**Table 1.** Amino acid mutations (described by single-letter code) within HBsAg of patient groups before and after therapy. Only positions at which changes occurred are shown. The wild type sequences aligned according to Okamoto's reference, accession number AB033559

Epitope	Amino acid position	Wild type	Mutation(s) (No)		
			Before therapy	After therapy	
Non	4	I	-	V (1)	
	20	F	S (1)	-	
	21	L	-	S/C (1)	
	26	L	R (1)	-	
	36	W	R (1)	-	
	T helper	40	N	-	S (1)
		45	T	-	P (1)
		49	L	-	-
		54	Q	-	R (1)
	T h	78	R	Q (1)	-
87		L	P (1)	-	
101		Q	R (1)	-	
B cell	109	L	Q (1)	-	
	143	S	L (1)	-	
	186	L	P (1)	-	
	187	S	F (1)	-	
	188	P	L (1)	-	
T helper	189	T	-	I (1)	
	190	V	A (1)	-	
	193	S	L (1)	-	
	196	W	-	L (1)	
Non	200	Y	F (1) C (1)	F (1)	
	204	S	N (1)	K (1) N (1) R (1)	
	206	Y	N (1) C (2)	F (1) C (2)	
CTL	207	S	I (1) N (2) R (1)	T (2) N (3) R (1)	
	208	I	T (2)	T (1)	
	209	L	V (2) W (1)	V (1)	
T h	216	L	S/C (1)	-	

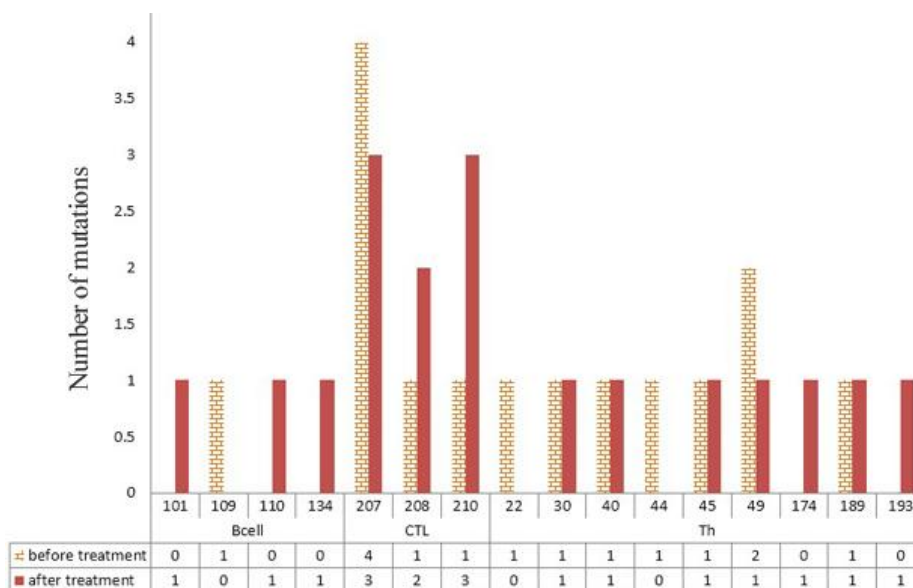
In non-responders, of the total of 92 amino acid changes, 51 (55.4%) occurred in immune epitopes: 5 were in B cell epitopes, 21 in T helper cell recognized epitopes, and 25 in CTL epitopes. Mutational patterns before and after vaccine therapy

showed an increase in 3 patients, a decrease in 5 patients and there were no differences in 8 patients. In the control group, of a total 76 amino acid changes, 32 (42%) substitutions occurred in immune epitopes: 14 (43.7%), 14 (43.7%) and 4 (12.5%) occurred in CTL, Th and B cell epitopes, respectively.

There has been a considerable interest in the investigation and characterization of HBV variants. Recent studies have shown that HBsAg is more variable than initially thought, and amino acid exchanges are scattered over the whole molecule (21). The hepatitis B surface protein is an important target for immune mediated virus elimination and several B, Th and CTL immune epitopes within the surface protein have been described (22-25). Appropriate reactivity of T-helper cells is a prerequisite for adequate anti-HBs production after infection with HBV, as well as after hepatitis B vaccination. Thus, the T-cell epitopes of HBsAg as targets for recognition by T cells should also be affected (26). Some mutations are able to impair the binding of neutralizing antibodies to the viral surface; viruses carrying such mutated T-cell epitopes that cannot be recognized by specific T-cells of a vaccinated individual, will not enhance anti-HBs production (24). Naturally occurring HBV with surface mutations have been reported in different groups and isolated cases of chronic infection who did not receive any vaccine or HBIG, regardless of whether they were within (18-20) or outside the "a" determinant (27-31).

In our study, mutations occurred outside the "a" determinant. The responder and control group showed no mutations before or during therapy in the "a" determinant. As a whole, comparison of variations between patient and control groups showed no role for the vaccine in inducing immune escape mutations; as the number of substitutions and their distribution showed no significant differences in both groups. We believe that, in chronic carriers who had already contained HBsAg variants selected by the host-immune response, administration of the vaccine to provoke an active immune response, had no effect in these patients. The absence of vaccine escape mutations argues against the production of an anti-HBs response.

The presence of amino acid mutations distributed in different surface protein immune epitopes, indicated that these proteins were under a significant selection pressure which had already been applied by both arms of cytotoxic and humoral host immune system. The occurrence of Th and CTL epitope mutations indicates an ineffective T cell response, and as already shown these responses are weak and sometimes undetectable during the chronic state of the infection (32, 33). On the other hand, even in chronic hepatitis B infection, B and T-cell escape mutants are not common, which is consistent with a weak HBV-



**Figure 3.** Position of amino acid and number of mutations at immuno epitopes in control group before and after therapy

**Table 2.** Amino acid mutations (described by single-letter code) within HBsAg of control groups before and after therapy. Only positions at which changes occurred are shown

Epitope	Amino acid position	Wild type	Mutation(s) (No)	
			Before therapy	After therapy
T helper	22	L	M (1)	-
	44	G	E (1)	-
	49	L	P (2)	P (1)
	101	Q	-	X (1)
B cell	109	L	Q (1)	-
	110	I	-	L (1)
	134	Y	-	F (1)
T helper	174	S	-	N (1)
	193	S	-	L (1)
Non	198	M	-	I (1)
	199	W	-	S (1)
CTL	207	S	R (3)	R (2)
	208	I	T (1)	T (2)
	210	S	R (1)	R (3)

specific T-cell response. In the few chronic hepatitis B cases in which T-cell escape mutants have been observed, the T-cell response was unusually strong and narrowly focused and thereby might have exerted stronger selective pressure (30). The results obtained by the present study showed that the vaccine was not able to enhance either such an immune response or such selective pressure,

emphasizing that in the spectrum of HBV chronicity, the occurrence of genomic variation (especially in immune epitopes) is a reflection of virus-host adaptation.

Compared to other immune epitope mutations, the occurrence of 25 (52.9%) CTL epitope changes in only 5 amino acid residues suggested a focused immune selection pressure at a hotspot position. Considering no correlation between vaccine administration and mutational pattern in non-responders, these mutations could be natural immune escape mutations regardless of non-respondiveness to vaccine therapy.

However, this hypothesis is in disagreement with the findings obtained by some other authors. In several studies on chronic HBV-infected patients, investigators found that in anti-HBe positive patients, who went into remission, putative escape mutations appeared in the T helper epitopes of the core protein. Conversely, in those with ongoing disease, they occurred in B cell epitopes (18, 34). They suggested a significant role for core protein humoral immune response and they hypothesized that chronic exposure of hepatocytes to HBcAg could lead to a T cell-independent B cell immunogenicity (35-38).

**Conclusion**

Vaccine therapy has inspired optimism as a new therapeutic approach; however, the efficacy of current vaccines in chronic patients is disappointing. Vaccine administration has no particular effect on the evolution of new mutations in the genome which had already been under host immune pressure. Hepatitis B virus genomes containing mutated immune epitopes were no longer recognized by

specific T-cells of a vaccinated individual and did not lead to anti-HBs production. Finally, strategies for vaccination programs and post-transplantation prophylaxis of recurrent hepatitis need to be developed to prevent immune escape mutant HBV from spreading and to prevent these strains from becoming dominant in the coming decades.

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