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VIRAL SEQUENCE ANALYSIS OF OCCULT HBV INFECTION AND ITS REACTIVATION IN IMMUNOSUPPRESSED PATIENTS

D. FERRARO[1], P. PIZZILLO[1], N. URONE[1], E. IANNITTO[2], A. CRAXÌ[3] and R. DI STEFANO[1]

[1]Sezione di Microbiologia, Dipartimento di Scienze per la Promozione della Salute "G. D'Alessandro", University of Palermo, Palermo, Italy; [2]Unità Operativa Complessa di Ematologia con Trapianto, University of Palermo, Palermo, Italy; [3]Sezione di Gastroenterologia, Dipartimento Biomedico di Medicina Interna e Specialistica (Di.Bi.M.I.S.), University of Palermo, Palermo, Italy

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The first two authors contributed equally to this study

Mechanisms associated with reactivation of hepatitis B virus (HBV) in patients with occult HBV infection (OBI) remain unclear. In some cases immunosuppression is an enhancer of viral replication. However, not all patients with OBI who undergo immunosuppression experience reactivation. This study explores the role of viral heterogeneity as a determinant of occult HBV reactivation. HBV genotype, mutation patterns and quasispecies were assessed by sequencing the PreS/S region of 16 patients with OBI undergoing chemotherapy, 3 of whom experienced a OBI reactivation. The latter were also assessed at the time of reactivation. Phylogenetic analysis identified low nucleotide and amino acid diversity rates. There were no differences in the viral quasispecies, or common mutation patterns, detected between patients who underwent reactivation of OBI, and those who did not. Furthermore, upon reactivation, the quasispecies evolved towards a loss of most of the variants present during the initial OBI stage, probably representing the fittest version of the virus. The genetic variability of HBV alone did not account for the transition from occult to overt infection, which appears to be governed principally by the host's immune response.

Hepatitis B virus (HBV) can persist in a host as an occult infection (OBI), where HBV DNA can be detected in the liver, and sometimes in the serum, of patients who are seronegative for HBV surface antigen (HBsAg) (1).

In patients undergoing strong immunosuppression, OBI has been shown to reactivate overt HBV replication (2-5), resulting in an ensuing, and often severe, hepatitic flare.

When HBV infection persists, the viral genome is maintained in the nucleus of hepatocytes as a covalently closed, circular DNA (cccDNA) bound to histones and non-histone proteins and regulated by their acetylation status (6). It is estimated only a few molecules are typically detected in the liver of OBI patients (7). This condition may also explain the inability of HBsAg to be detected in serum by available diagnostic assays (8).

A number of studies have investigated the role of viral heterogeneity as a primary determinant of OBI.

Key words: occult HBV, HBV reactivation, phylogenetic analysis, molecular epidemiology

Mailing address: Dr Donatella Ferraro,
Dipartimento di Scienze
per la Promozione della Salute "G. D'Alessandro"
Via del Vespro, 133
90138 Palermo, Sicily, Italy
Tel.: +39 091 6553680 Fax: +39 091 655 3676
e-mail: dferraro@unipa.it

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For example, well-characterized epitope mutations have been detected in the preS/S region, especially in the major hydrophilic region (MHR) of the S gene (e.g., amino acids 100-160), which contains an "a" determinant. With this region being a main target for monoclonal antibodies used in diagnostic tests, these mutations can affect the recognition of conformational epitopes (9,10,11,12,13). Point mutations and deletions within the preS and S genes can also influence HBsAg secretion and expression, leading to low levels of HBsAg (14,15). However, the degree of variability associated with HBV quasispecies is not as well characterized (16,17). It is also not known how immune modulation and reconstitution affect the suppression and re-expansion of individual HBV clades within the HBV quasispecies at different stages of OBI. Therefore, the mechanisms underlying HBV reactivation in OBI patients remain unclear. In a previous study, we reported that 27.7% of patients with OBI that experienced an oncohematological disease requiring immunosuppression developed viral reactivation and a hepatitic flare (18). In the present study, we investigate the HBV genomic variability of a cohort of patients with OBI that undergo chemotherapy, with the goal of investigating possible differences between viral variants present in patients with OBI reactivation, versus those without reactivation. Correspondingly, HBV isolates from these patients were analyzed during the occult infection stage for genotype, subtype, and mutation patterns and then cloned to study the genetic variability of viral quasispecies. For patients in whom OBI reactivation occurred, the same studies were performed again at the time of reactivation.

MATERIALS AND METHODS

Patients

Sixteen OBI (e.g., p1 - p16) patients with onco-hematological diseases were selected retrospectively from a cohort of 75 HBsAg negative patients previously studied for OBI (18). The selected subjects were positive in serum for the four different HBV genomic regions (e.g., preS/S, preC/C, Pol, and X) by nested PCR assays (limits of sensitivity between 0.1 and 0.05 IU/ml), while HBV DNA was undetectable using a bDNA assay (bDNA, Siemens, detection limit = 357 IU/ml) and a *home made* nested-PCR (sensitivity 60 UI/ml). All patients were treated with cytotoxic chemotherapy without Rituximab, and 6 patients also received corticosteroids. Virological analyses were performed for all patients prior to initiation of chemotherapy (baseline), and during the reactivation stage for patients positive for HBsAg (Table I). During chemotherapy, 3/16 patients (p1, p2, p3) experienced a virological and clinical HBV reactivation after a mean of 8 months. These patients were found to be HBsAg- and HBeAg-positive, with HBV DNA loads of 1.6×10^6, 7.6×10^6, and 1×10^4 IU/ml for p1, p2, and p3, respectively. These patients were subsequently treated with lamivudine (100 mg/day) and remained positive for HBsAg.

HBV DNA amplification and sequencing

Sequencing was performed for a fragment of 1059 bp that spanned the entire preS1 and preS2 regions and 537 nucleotides of the S gene. Viral DNA was at first amplified using O_1 (5'-GGGTCACCATATTCTTGGGAA-3') and O_2 primers (5'-CTCAGTTTACTAGTGCCATT-3'). An aliquot of PCR product was then reamplified in three separate reactions to obtain partially overlapping genome fragments of 479, 710, and 537 bases, respectively using the following primers: O_1 and N_2 (5'-CTGTTCTGACTACTGCCTCTC-3'), N_3 (5'-AATCCGGATTGGGACTTCAA-3') and N_4 (5'-GTTCTTCTGGACTATCAAGG-3'), N_5 (5'-CATGGAGAACATCACATCAGG-3') and O_2. The amplifications were performed with 35 cycles of: 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min, followed by a final incubation at 72°C for 10 min. Amplicons were sequenced with the same primers used for DNA amplification and a Big Dye Terminator v 1.1 Cycle Sequencing Kit (Applied Biosystem, Foster City, CA, USA) in an automatic DNA sequencer (ABI Prism 310 Genetic Analyzer, Applied Biosystem).

Determination of genotype, subgenotype, and mutation patterns

The three overlapping sequences, obtained by nested PCR, were assembled, edited, and aligned using BioEdit version 4.0, using the Clustal W multi-alignment system. Genotypes and subgenotypes were inferred by aligning each sequence with reference

sequences of HBV/D1 – HBV/D5 subgenotypes. Phylogenetic trees were subsequently constructed using Mega 4.1 software (19). Mutational patterns of the sequences analyzed was determined by comparison with consensus wild-type sequences specific for subtype D1 and D3, generated using sequences available in Gen-Bank database (e.g., D1: AF280817, Y07587, AF151735, L27106, X80924, M32138, AF121242, EU594396, EU594397, FJ386590, FJ904432, FJ904443, FJ904445, FJ904446, GQ167302, AB104709, AB104710, AB104711, AB104712, AB126581, AF121239, AF121240, AF121241, AY721605, AY721606, AY721607, AJ721612, AY741798, AY796030, AY796032, X02496; D3: X85254, AJ233291, AJ233292, AJ233293, AJ233294, AJ233295, AJ233296, U95551).

Cloning and quasispecies analysis

Viral quasispecies was investigated using clonal analysis, with the pGEM-T easy vector system according to the manufacturer's protocol (Promega, Madison, WI). For the occult HBV stage, where amplification was positive only after a second PCR step, the major amplicon of 710 bp (which spanned the preS1 region from nts 77-306 of the S region) was selected for cloning and quasispecies analysis. At the time of reactivation the entire 1059-bp sequence obtained at the first amplification step was cloned, because it was present in all patients. For each patient, DNA extracted from 4-10 recombinant colonies was directly sequenced. Neighbour-joining trees were then generated using MEGA 4.1 software (using 1000 bootstrap replicates) by applying

Table I. *Demographic and HBV Serological profiles of 16 patients prior to receiving immunosuppressive therapy.*

Patients	*Anti-HBc* [a]	*Anti-HBs* [b]	*M/F*	*Mean age (range)*	*Treatment (steroid/no steroid)* [c]
PATIENTS WITH REACTIVATION					
p1	Neg [d]	Neg	3/0	78 (73-81)	1/2
p2	Neg	Neg			
p3	**Pos** [e]	**Pos**			
PATIENTS WITHOUT REACTIVATION					
p4	**Pos**	**Pos**	6/7	63.4 (41-81)	5/8
p5	**Pos**	Neg			
p6	Neg	Neg			
p7	**Pos**	**Pos**			
p8	Neg	Neg			
p9	Neg	neg			
p10	Neg	Neg			
p11	Neg	Neg			
p12	Neg	Neg			
p13	Neg	**Pos**			
p14	Neg	Neg			
p15	Neg	**Pos**			
p16	**Pos**	**Pos**			

[b] *HBs: surface antigen*
[c] *None of these patients were treated with Rituximab*
[d] *Neg: negative*
[e] *Pos: positive*

pairwise deletion and the Kimura-2 parameter model. This model was also used to calculate nucleotide and amino acid substitution rates.

Statistical analysis

Statistical analyses were performed using Student's t-test and the χ^2 test, with Fisher's exact test used to compare categorical data. *P* values less than 0.05 were considered statistically significant. Calculations were performed using GRAPHPAD PRISM version 5.00 (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

HBV genotype and subtype

All 16 patients included in this retrospective study were infected with a genotype D HBV strain (HBV/D), with 6/16 (37.5%) having the subtype HBV/D1, and 10/16 (62.5%) having subtype HBV/D3. There were no gender- and mean age-related differences in the incidence of subtype ($p = 0.15$, $p = 0.43$). One reactivation event was reported in the HBV/D1 group and 2 were reported in the HBV/D3 group ($p = 0.87$).

S-gene sequences at baseline

When isolates from 16 OBI patients (p1–p16) were compared with consensus wild-type sequences specific for HBV subtypes, D1 and D3, between 3 and 37 nucleotide substitutions in the 1059 bp region of the preS/S gene were detected. The nucleotide substitutions were evenly distributed throughout the region analyzed, with only a few substitutions occurring at identical positions in all patients. Furthermore, the number of non-synonymous substitutions ranged from 2 to 20. Overall, the highest number of mutations detected in the preS1

Table II. *Complete list of amino acid changes detected in HBV isolates obtained from a patients (n=16).*

	PreS1	PreS2	S
p1[a]	A28E, A84V	P41H, P52H	S167L
p2[a]		G30E	E164G
p3[a]	W66R, Q89K	S5Y, H9L, Q10K, deletion of amino acid 22, P23H, P41H ,L54R	E2G, G10E, S31N, T115N, T123P,Q129R,T131N, P135L, C147Y, I150T,E164V
p4	A79V	T31A	P120S
p5		P36L	L89P
p6	G48R	R18K, A39V,P41H,L42I	T126S
p7	S90P, G91R, G92R, frame shift from position 96	I42L, S43L	P120S, P127L, G145A, S154P
p8	L77V		P120S, Y134H, W165S
p9	L77V,N103T		
p10	A79V		P120S
p11	L77V		P120S, Y134H, E164G, W165S
p12	L77V		Stop codon at position 30
p13	A79V		P120S
p14	L77V	G50A	L109R, P120S, Y134H, E164G, W165S
p15	L77V		Stop codon at position 30
p16	D16G,S90P, G91R	P52H	Wild-type/Stop codon at position 30, P127L, T140I, E164G

[a] *Patients with OBI reactivation under immunosuppression*

region was associated with one isolate from p7, who did not experience a reactivation of OBI. In this case, insertion of a thymidine base between nucleotides G289 and C290 caused a shift from amino acid 96 in the reading frame. L77V mutation in preS1 region was identified in six viral isolates and A79V mutation

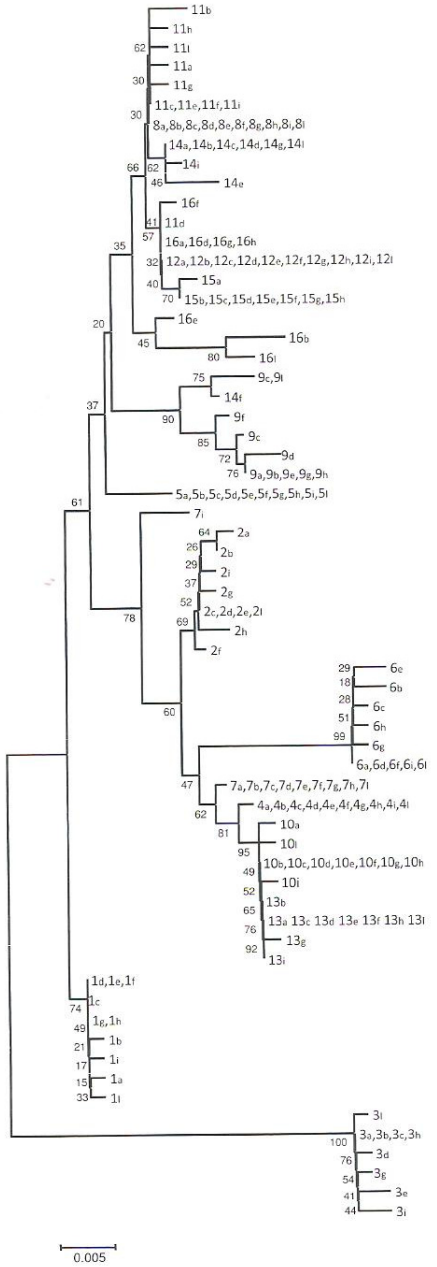

Fig. 1. *Neighbour-joining tree of the HBV preS/S region (710 bp) sequenced from 16 OBI patients prior to the start of chemotherapy. Bootstrap values from 1000 replicates were used.*

in three other viral strains isolated from the thirteen patients that did not undergo OBI reactivation

An analysis of the preS2 region identified an in-frame deletion present in a strain isolated from p3, who later experienced a reactivation of OBI with administration of chemotherapy. In the S gene of the same isolate was found an amino acid change in the putative human leukocyte antigen class I-restricted cytotoxic T lymphocyte epitope (aa 28–51) of HBsAg. For three other patients (p12, p15, p16) that did that not experience a reactivation of OBI, the same region contained a point mutation, C87T, that formed a stop codon (CAG → TAG) at position 30. Between 1 and 4 substitutions were detected within the MHR of the S gene (amino acids 100–160) in 11/13 patients without OBI reactivation. Moreover, eight viral isolates had at least one amino acid change in the "a" determinant region (aa 124–147). Of these substitutions, only the P120S mutation has previously been described in studies of vaccine escape mutants (20,21). Furthermore, of three patients who later had experienced a reactivation of OBI, only an isolate from p3 showed a peculiar mutation pattern in S region, which included 11 amino acid changes, 7 of which were present in the MHR. Furthermore, of these mutations, only the Q129R (22) and C147Y (12) mutations have been shown to correlate with a possible immune escape phenomenon. The complete list of amino acid changes in HBV isolates from all patients is listed in Table II.

The substitutions detected in the preS/S region resulted in up to 17 amino acid changes in the overlapping polymerase region, and between 1 and 4 changes in the RT domain. Furthermore, any of these mutations could potentially result in replication-defective HBV mutants, except for an isolate from p7 which was associated with a stop codon at amino acid position 281.

Phylogenetic analysis of HBV at baseline and during OBI reactivation

The genomic variability prior to chemotherapy (baseline) was studied by cloning 710 bp of HBV preS/S gene. The phylogenetic analysis of 8-10 clones for each isolate found a low nucleotide (0–1.6%) and amino acid (0–3.4%) diversity rates for all patients, with only a few mutations in individual clones not detected previously by direct sequencing.

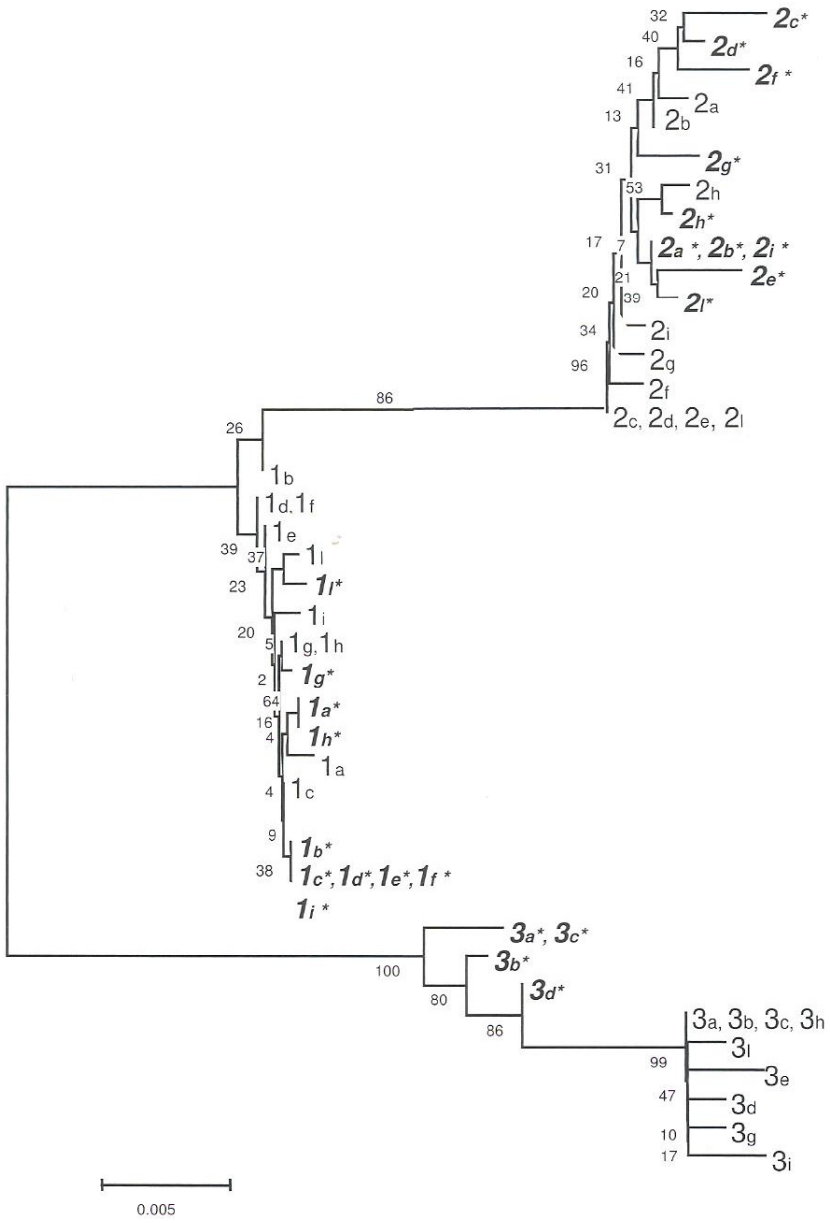

Fig. 2. *Neighbour-joining tree of the HBV preS/S region (710 bp) from 3 OBI patients prior to the start of chemotherapy and following of reactivation. Bootstrap values from 1000 replicates were used. Asterisks indicate the clones that were detected during the reactivation stage.*

The phylogenetic tree also showed that most clones for each patient were clustered, with between 4 and 10 clones being identical (Fig. 1).

For the three patients with OBI reactivation (p1, p2, p3), phylogenetic analysis was performed on isolates obtained at baseline (9-10 clones) and at the time of viral reactivation. At this time, 10 clones were obtained from p1 and p2, while only four clones were obtained from p3 due to reduced cloning efficiency attributed to insufficient DNA. Phylogenetic analysis was performed for each isolate and rates of nucleotide and amino acid diversity was found to

range from 0–2.6%, and 0–4.8%, respectively (Fig. 2). The HBV quasispecies, which was initially found to be relatively diverse, evolved during the stage of reactivation towards a loss of most of the variants present during the initial OBI stage.

DISCUSSION

It has previously been shown that the full-length HBV genome persists in an OBI patient despite suppression of its replicative activity and gene expression (1). When the immune system is induced to be unresponsive, HBV can undergo reactivation and overcome the control systems of the host. However, it remains unclear why this occurs in only some OBI patients.

The heterogeneity of HBV in 16 patients experiencing an occult infection prior to the start of strongly immunosuppressive chemotherapeutic regimens was assessed in the present study. In 3/16 patients, despite comparable degrees of immunosuppression, reactivation of OBI occurred. However, only one case involved the administration of corticosteroids.

At baseline in the OBI state, most HBV isolates did not display significant variants, although there were a few exceptions. For example, the isolate from p7, that did not undergo OBI reactivation, showed a frame shift from the amino acid 96 in the preS1 region, within the PreS2/S promoter. This mutation determines the change of a significant proportion of the *Large* protein, potentially altering its folding and function. The preS1 region has previously been shown to harbor the preS2/S promoter, the binding sites for the recognition of hepatocytes (23) and some epitopes recognized by neutralizing antibodies (11), and is involved in the incorporation of the capsid into the envelope (24). Correspondingly, a frameshift would induce the defective assembly of virions, inhibit the recognition of hepatocyte receptors, and alter antigenicity. However, an *in vitro* study of HBV variants, in which the preS2 start codon was abolished by an in-frame deletion or a point mutation, have shown that normal viral replication occurred. However, lower levels of HBsAg were associated with these variants compared with wild-type strains (16,17).

The mutations at positions 77 and 79 of pre-S1 region falls in the major cytosolic loop (residues 29-80) that interacts with core particles. These mutations could affect the formation and secretion of empty envelopes and the nucleocapsid envelopment.

A stop codon at amino acid position 30 of the S region in three other viral isolates from patients that did that not experience a reactivation of OBI, resulted in the production of a truncated protein devoid of the main antigenic HBV determinant, and of the second determinant (aa 119-128, coreceptor) required for infectivity, as described in an HBV/HDV model by Jaoudè G.A. and Sureau C.(25). Both these characteristics might help maintain the OBI state, and prevent the transition to overt infection.

In seven patients who did not undergo OBI reactivation, a P120S mutation was detected. This mutation was originally described in infants born to HBV-carrying mothers who underwent anti–HBV immunization at birth. However, more recently, an *in vitro* study found that P120S variants from isolates of OBI patients synthesize a fully functional S protein (Pollicino T., unpublished data).

Regarding the baseline status of the three patients who experienced an OBI reactivation following the administration of chemotherapy, only p3 harbored a HBV with a specific mutational pattern characterized by 20 substitutions in the *Large* protein. Moreover, a subset of these mutations have previously been reported (26) and associated with the production of a predicted novel HBsAg variant. This hypothesis is also supported by the presence of the relevant C147Y mutation. Indeed, cysteine substitutions within the "a" determinant have been shown in *in vitro* studies to alter tertiary structure (12), resulting in loss of antigenicity, consistent with a lack of detectable HBsAg reactivity. Remarkably, p3 was also found to be anti-HBs positive, suggesting that this variant may be an immune escape mutant.

Although it has been suggested that the selection of immune escape mutants is characteristic of OBI with HBV/D (27), both the findings of the present study and others (16, 7), show that are present in only a small proportion of OBI cases. In addition, taking into account the partial overlap of the open reading frames for the preS/S and P regions, none of the mutations identified in the present study have the potential to affect viral fitness by altering polymerase status, except for an isolate from p7 which bears a

stop codon within the spacer domain. However, the identification of this single isolate is not sufficient to assert that the virus infecting this patient is unable to replicate, since the variant may only represent a subset of the viral population present.

The phylogenetic analysis at baseline showed a low quasispecies diversity in all patients, with no differences found between patients with or without OBI reactivation. However, since the MHR was not analyzed, the observation of low rates of diversity must be recognized in this context.

For the three patients who underwent reactivation of OBI, the HBV quasispecies observed at the time of viral reactivation was found to contain less variants than those detected at baseline. However, the genetic diversity observed may also reflect the natural evolution of the HBV genome during an occult infection that is related to maintenance of viral replication, even if it is at low levels, prior to reactivation.

The pattern of quasispecies evolution observed in the present study may represent a "funnel effect". In the resting stage at baseline, the HBV population is hypothesized to be selected by mechanisms of immune pressure and viral senescence. This could potentially result in the generation of strains with low replicative fitness and reduced HBsAg expression. During a strong immunosuppression, a more fit virus could emerge and expand, ultimately maintaining its selective advantage over weaker competitors in the quasispecies present. Although it was not possible to investigate cellular compartments of HBV replication in this study, such as hepatocytes and peripheral blood mononuclear cells, the viruses present in serum samples would be expected to reflect the viral population maintained in liver cells.

These data, although obtained from a small number of cases, confirm that OBI is a complex phenomenon of virus-host interactions. Moreover, mutations altering the expression of preS/S and the production of new viruses have been observed in some cases, but there was not a pattern of mutations that characterizes the probability of reactivation. These considerations are in according to studies that examined HBV isolates from liver tissue occult HBV carriers (28,29) Therefore, the genetic variability of HBV alone does not explain the transition from an occult infection to an overt infection, which is principally governed by the host's immune response, and which subsequently influences the further evolution of HBV quasispecies.

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