Case Report

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HBV reactivation after fludarabine chemotherapy identified on investigation of suspected transfusion-transmitted Hepatitis B virus

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Background & Aims: Multi-transfused patients often receive treatments inducing various levels of immunodeficiency. Acute viral infections may then be attributed either to transfusion-transmitted infection (TTI) or reactivation of a past infection.
Methods: A patient with chronic lymphocytic leukemia (CLL) who had >250 blood donor exposures developed acute Hepatitis B virus (HBV) infection. Routine donor testing for HB core antibodies (anti-HBc) was in place in the relevant period and investigations undertaken on the blood donors were negative.
Results: Review of historical, molecular, and antigenic evidence demonstrated reactivation of a recovered HBV infection dating >30 years and the selection of a rare escape mutant that briefly replicated and caused acute liver disease. This mutant was unreactive with several HBsAg assays and poorly reactive with an HBV vaccine plasma. Correcting the C139Y substitution by site directed mutagenesis of recombinant surface proteins re-established assay reactivity.
Conclusions: Fludarabine, but not Chlorambucil, appeared sufficiently immunosuppressive to trigger reactivation despite low levels of neutralizing antibodies. Differentiating between TTI and reactivation of HBV becomes more challenging with the increasing frequency of immunocompromised blood recipients. Chemotherapy with Fludarabine alone should be considered as carrying high risk of viral reactivation. Pre-treatment testing and peripheral blood sample archiving may be indicated in HBsAg negative patients.

Introduction

In hematology, for patients receiving aggressive chemotherapy, reactivation of HBV is well described in populations with high endemicity and patients who are healthy HBV carriers [Hepatitis B Surface Antigen (HBsAg) positive] [1,2]. In other patients receiving chemotherapy for hematological malignancies who may be exposed to a large number of blood donors over the course of their treatment, acute HBV infection tends to be attributed to transfusion, notwithstanding the very low risk of transfusion-transmitted HBV (TT-HBV). An increasing body of evidence suggests that common viruses such as parvovirus B19 and HBV infections persist at very low levels after clinical recovery with the development of neutralizing antibodies in sanctuaries such as the bone marrow or the liver, respectively, but may be found in plasma with sensitive genomic amplification methods [3,4]. Reactivation of herpes viruses such as CMV has been described abundantly in severe immunodeficient patients receiving chemotherapy for cancer or organ and bone marrow transplantations. Common reactivation of viral infections, which generate neutralizing humoral immunity, is not always considered [5,6]. Since patients receiving chemotherapy often need transfusion, circumstances, in which TTI is suspected despite the high degree of safety of blood components, are not uncommon. The differential diagnosis between transfusion transmission and reactivation becomes critical for medical and legal reasons.

We describe a case where, reactivation of HBV occurred in an HBsAg negative patient treated with Fludarabine who had recovered from acute infection over 30 years earlier and had received HBV vaccine 18 months before this occurred.

Case report

In October 2004, the Munster Regional Transfusion Centre (MRTC), of the Irish Blood Transfusion Service (IBTS) received notification of a suspected TT-HBV in a transfusion-dependent hematology patient who had developed HBV serological markers. A look-back was initiated to trace and test implicated donors. An investigation into other possible causes was instigated concurrently.

This 54-year-old male, diagnosed with CLL in November 2001 was initially well maintained with intermittent alkylating cytotoxic chemotherapy (5 courses). In April 2003 he had a complete course of HBV vaccination, as a member of a voluntary defence force following which anti-HBs was detected. When his disease progressed in November 2003, Fludarabine was instituted. In March 2004 he was admitted with sepsis secondary to pancytopenia and bone marrow reserve was insufficient to tolerate further Fludarabine. He was first transfused on March 16, 2004

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and intermittently thereafter, with both red cell and platelet components becoming heavily transfusion dependant by July. His clinical course was stormy, requiring broad-spectrum antimicrobial therapy, (including treatment for CNS AFB). On admission in March 2004, ALT level was significantly elevated at 104 IU/L, and remained so to October when MRTC was notified (max 308 IU/l). Mild hyperbilirubinemia (29 μmol/L) persisted over the same time period. Serological markers for HBV were detected on the 29th October 2004, including HBsAg, anti-HBc, (IgM negative), and HBe antigen with anti-HBs detected at 10.4 IU/L (Table 1). HAV serological markers were consistent with past infection (anti-HAV total antibody positive/IgM negative). By April 2005, the patient was HBsAg and HBV DNA negative, and anti-HBc positive with undetectable anti-HBs.

Methods

All implicated components transfused to the patient were identified by the hospital blood bank and notified to MRTC. All donors were traced and deferred from the active donor panel. Donors were advised of their possible implication in a suspected TT-HBV and were invited to attend for HBV re-screening at an interval of >6 months after the implicated donation. Donors were re-tested for HBV by HBsAg (sensitivity <0.1 PEI units/ml) and anti-HBc by Abbott Prism Chemiluminescence at MRTC. In a few cases an archive sample of the index donation was tested for HBV DNA by PCR at University of Edinburgh.

The patient's medical and laboratory records were scrutinized and the patient and family were interviewed. Family contacts were tested for serological or virological evidence of current or past Hepatitis B virus infection by HBsAg, anti-HBc, HBe antigen (Abbott, Axym, Delkenheim, Germany), anti-HBs, and HBV DNA.

Patient and control samples

Plasma samples studied included two samples from chronically HBV infected people (HBsAg positive, anti-HBc positive, negative for anti-HBs), one of whom (Patient 1) was the brother-in-law of the index patient, the second an unrelated donor (Patient 2), and both carried wild type genotype D strains. Plasma from two laboratory workers vaccinated with HBV recombinant S protein (genotype A2) were used as positive controls and samples from two blood donors negative for all HBV markers were used as negative controls.

Viral DNA isolation and amplification

Viral DNA was isolated and nested-PCR was used to amplify the S and BCP genes as previously described [7].

HBV DNA quantification

Viral DNA was quantified by QPCR using a TaqMan-based methodology as previously described [7]. The fluorogenic probe was 5′-labelled with Cy5 dye and 3′-labelled with Black Hole Quencher 2 (BHQ-2).

HBV sequencing and genotyping

Sequences of S and BCP/PC regions were obtained by direct sequencing of PCR products and phylogenetic analyses were performed as described previously [7]. In addition, full genome of the index patient was amplified as previously described, cloned, and two clones were sequenced using a series of seven primer pairs covering the complete HBV genome [7]. For comparison purposes, samples from Patients 1 and 2 were extracted for DNA, amplified and sequenced in the pre-S/S and S regions (1420 nucleotides). Phylogenetic analysis was performed by using PAUP* version 1.0 b10 and the neighbour-joining algorithm based on Kimura two-parameter distance estimation, ignoring all positions with gaps in pairwise comparisons. To confirm reliability of phylogenetic tree topologies, bootstrap reconstruction was carried out 1000 times and bootstrap values >75% were considered significant.

Preparation of recombinant S proteins

The S gene of controls and the index patient samples were amplified by PCR. The sense primer was designed to include a SnaB1 site and the ATG start codon in the context of a Kosac sequence: ACTCTACGTAACCATGGARAACAYMACATCAGGA. The antisense primer included the EcoRI site, a six histidine tag, flag tag, a strep tag II sequences, the stop codon, and the S gene: CCAGGGAATTCTCATTTTTCGAACTG CGGGTGGCTCCACTTGTCGTCATCGTCCTTGTAGTCATGGTGATGGTGATGATGGGCCG CAATGTATACCCAVAGACABAAGAA. Amplification was performed in a mix containing 1× Expand High Fidelity buffer, 2.5 mM $MgCl_2$, 0.20 mM dNTPs, 0.4 μM each primer, and 2.5 U of Expand High Fidelity Enzyme (Roche, Mannheim, Germany). Amplification was started at 94 °C for 5 min followed by 30 cycles of 94 °C for 40 s, 50 °C for 40 s, and 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR product of the S gene was inserted into pPIC3.5K (Invitrogen, Paisley, UK). The recombinant vector was then expressed in *Pichia pastoris* according to the manufacturer's instructions (Invitrogen). The selected colonies were grown in 1 L of buffered minimal glycerol (BMGY) medium [1% (w/w) yeast extract, 2% (w/w) peptone, 1.34% (w/w) yeast nitrogen base, 0.4 mg L^{-1} biotin, and 100 mM potassium phosphate (pH 6.0) 1% (v/v) glycerol] until optical density at 600 nm (OD_{600}) was >6. Cells were induced by 1% methanol by replacing the BMGY by buffered minimal methanol (BMMY) medium [1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 0.4 mg/ml biotin, and 100 mM potassium phosphate (pH 6.0) 1% methanol]. Methanol was added at a concentration of 1% every 24 h during the induction phase (72 h). Cultures were centrifuged and pelleted yeast cells were re-suspended in a 25 mM of Tris buffer (pH 7.6) and submitted to high pressure (30 kpsi) cell disruption in a cell disrupter system (Constant System, Daventry, UK). The lysates were further clarified by centrifugation. The supernatant was subjected to ultrafiltration through 300,000 nominal molecular weight cut-off (NMWC) hollow fibers (GE Healthcare, Slough, UK) post-clarification using 45-μm filter. HBsAg particles were further purified by immuno-affinity using an anti-Flag M2 affinity gel (Sigma, Gillingham, UK) eluted with 100 μg/ml of flag peptide (Sigma) in TBS. The eluted proteins were analyzed with 12% SDS–PAGE followed by staining with Coomassie brilliant blue and detection by Western blot with monoclonal anti-Flag® M2 antibody (Sigma). Concentration of HBsAg protein was determined by densitometry using known concentration of bovine serum albumin as standard (http://rsbweb.nih.gov/ij/). An additional, more specific, method was used to quantify and normalize the recombinant S protein based on StrepTactin®-coated plates (IBA, Gottingen, Germany).

Site directed mutagenesis by overlap extension PCR

Site directed mutagenesis (SDM) was carried out using two-step PCR procedure to restore the cysteine 139 mutation that was suspected to alter HBsAg conformation and assay recognition. Two reactions were performed with a primer pair [PIC3.5K-F/SDM-R(TCCGTCCGAAGGTTTGGTACAGCAACAGGA), PIC3.5K-R/SDM-F(562-TCCTGTTGCTGTACCAAACCTTCGGACGGA-591)] using 50 ng of the original mutated DNA. To obtain full-length mutated fragment, an equimolar concentration (100 ng) of the two PCR products was used as template in the second reaction with PIC3.5K forward and reverse primers as described above. The PCR product was cloned in PIC3.5 vector for expression in Pichia strain.

Table 1. HBV markers in index patient, index patient spouse, Patients 1 and 2 (unrelated chronic infection).

Patient	HBsAg	HBeAg	HBV DNA load (IU/ml)	HBV genotype	Anti-HBc	Anti-HBe	Anti-HBs (IU/L)	ALT (IU/L)
Index 4-2004	Negative		ND		Positive		ND	104
10-2004	Positive	Positive	10^3	D	Positive	Negative	10.4	308
4-2005	Negative		Negative		Positive		Negative	
Index spouse[1]	Negative	Negative			Positive	Positive	200	ND
Patient 1, 2004	Positive		6.3×10^5	D	Positive		Negative	
Patient 2, 2004	Positive		2.4×10^3	D	Positive		ND	ND

[1] From an archived sample collected in 2000.

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Capture enzyme-linked immunosorbent assay (ELISA) of recombinant S proteins

StrepTactin®-coated plates (96-well) were incubated with recombinant S protein diluted with TBS buffer (25 mM Tris–HCl, 150 mM NaCl, 2 mM EDTA; pH 7.6) at 0.1 μg/wells (the saturation concentration for the StrepTactin® plates) for 1 h at 37 °C. The plates were washed five times with PBS/0.1% Tween 20 at room temperature. Diluted monoclonal anti-Flag® M2 antibody (1:5000) or plasma diluted 1:100 with TBS were incubated for 1 h at 37 °C and tested in duplicate. After washing with PBS/0.5% Tween 20, 100 μl of diluted horseradish peroxidase-conjugated anti-mouse (1:10,000) or anti-human Fc IgG monoclonal antibody (Sigma, 1:50,000) were added per well and incubated at 37 °C for 1 h. Plates were washed with 0.3 ml of PBS/0.5% Tween 20 and 100 μl 3,39,5,59-tetramethylbenzidine (TMB) substrate (Pierce, Cramlington, UK) per well were added, followed by incubation at room temperature in darkness for 20 min. The reaction was stopped by the addition of 100 μl of 1 M sulfuric acid and the absorbance of each well was read at 450 nm. The cut-off was determined as the mean absorbance of four anti-HBV negative control samples plus six standard deviations. Sample/cut-off ratios were calculated to express the reactivity of the samples: ratios above 1 were considered positive. An HBsAg standard curve was generated from the recombinant HBsAg positive control (wild type strain) following detection with flag antibody to normalize the protein concentration between samples.

Antibody-capture ELISA with recombinant S proteins

The recombinant S proteins produced in addition to a commercially available recombinant HBsAg (Aldevron, Fargo, USA) were used at 0.1 μg/wells to coat 96-well microplates overnight at 4 °C. Plates were washed five times with 0.3 ml/well of PBS/0.1% Tween 20 and then blocked with 250 μl PBS/0.05% Tween 20/4% BSA at 37 °C for 1 h, followed by five washes with 300 μl PBS/0.1% Tween 20 per well. Three monoclonal antibodies to HBsAg (Diagnostics for the Real World Ltd., Sunnyvale, CA, USA) and one animal polyclonal antibody (Abcam, Cambridge, UK) were serially diluted 1:1000, 1:5000, 1:10,000, 1:50,000 in PBS/0.05% Tween 20/1% BSA and 100 μl of each dilution were added to the wells and incubated for 1 h. One hundred microliters of 1:10,000 diluted secondary antibody labelled with horse radish peroxidase (HRP) were added and revealed by TMB as described above.

Commercial HBsAg screening assays

Semi-purified recombinant HBsAg were tested at a concentration of 1 μg/ml with four different commercial microplate EIAs and two lateral flow rapid tests: MonoLisa AgHBs Plus (Bio-Rad, Marnes Ia Coquette, France), Hepanostika® HBsAg Ultra (bioMerieux, Marcy-l'Etoile, France) Abbott/Murex (Abbott, Delkenheim, Germany), Bioelisa HBsAg Colour (Biokit, Barcelona, Spain), Determine HBsAg (Abbott, Delkenheim, Germany) and DRW-HBsAg (DRW, Sunnyvale, USA), respectively.

Results

The presence of anti-HBc prior to the acute episode of Hepatitis B during Fludarabine chemotherapy suggested prior exposure to HBV. As a result, investigation of the past history of the index patient was conducted. It uncovered evidence consistent with past HBV infection. Serological testing of 09 and 13 April 2004 samples revealed anti-HBc positive, HBsAg negative (anti-HBs not tested). The patient had had an episode of icteric hepatitis in 1975 and his spouse had an institutionalized sibling with established chronic HBV carriage (Patient 1). She had donated blood in 2000, prior to the introduction of anti-HBc blood donation screening and was found to be HBsAg negative. However, current testing results were consistent with past HBV infection and effective immunity (HBsAg neg, anti-HBs 200 IU/L, anti-HBc pos, and anti-HBe positive). In addition the couple's first-born child (DOB 1980) showed serological evidence of past infection (HBsAg negative, anti-HBs, and anti-HBc positive) while their younger child was seronegative. This historical data strongly suggested that, in addition to his brother-in-law who had chronic HBV infection (Patient 1), the index patient's spouse also had chronic HBV infection and transmitted HBV to both her husband and first-born child. She subsequently seroconverted to anti-HBs and recovered from the infection, apparently spontaneously [4].

The index patient having been infected as an adult recovered from the infection and 30 years later carried anti-HBc as the only indication of the previous infection until vaccinated to HBV.

Although this patient's history was highly suggestive of reactivation, community-acquired infection was not completely excluded. As a result, molecular evidence of relatedness between the HBV strains infecting the index patient and the only remaining family member with chronic infection, i.e. Patient 1, were studied. Samples from both individuals as well as a sample from an unrelated HBsAg-carrying blood donor from MRTC were sent under code to the Cambridge Laboratory, and examined for viral load, genotype and sequencing.

As shown in Fig. 1, the phylogenetic tree constructed with 1420 nucleotide pre-S/S sequences from the three patients and selected references indicated that all three Irish patients carried genotype D strains (sequence accession numbers for Index GU570523, for Patient 1 GU570524, and for Patient 2 GU570526). It also showed that the index patient and Patient 1 carried HBV strains that were closer to each other than to other strains although it was clustered with four other strains with a bootstrap value of 81% (Fig. 1). In particular Patient 1 carried a 24-nucleotide deletion in two out of four sequenced clones in the pre-S2 region (nt 31–54 from EcoR1 restriction site) that was not present in the index patient strain. The two other clones do not carry this deletion but have a mutation at the initiation codon of pre-S2.

The fact that the index patient had developed acute HBV infection despite having received HBV vaccine approximately 18 months prior to the acute episode and while carrying low levels of anti-HBs generally considered protective of HBV infection remained to be elucidated. This situation was compatible with an escape mutant involved in the acute infection. To examine this hypothesis, the S region sequences containing the major hydrophilic region (MHR) from the index patient, Patients 1 and 2 strains were obtained and translated to compare amino acid sequences. As shown in Fig. 2, the three strains substantially differed from each other. In particular, the index patient had a C139Y substitution that was assumed to disrupt considerably the presentation of the MHR loop 4 epitope. In addition, two substitutions were seen in loop 3: G130R and Y134N compared to the Patient 2 reference strain and one in loop 1: P120S. These substitutions were compatible with an escape mutant being responsible for the acute HBV infection.

The escape mutant hypothesis was further explored by preparing purified recombinant S protein from the index patient and the genotype D control (Patient 2). The reactivity of these two proteins was tested against the pre-acute HBV infection plasma of the index patient and of two other vaccinated healthy individuals, six commercial HBsAg assays and four monoclonal or polyclonal antibodies to HBsAg. Results are summarized in Table 2. The index patient recombinant S protein did not react with the index patient plasma, with one of four HBsAg commercial EIA, with two HBsAg rapid tests and was poorly reactive with two of three monoclonal antibodies to MHR. However, this heavily mutated protein remained strongly reactive with 2/4 commercial HBsAg EIA, one monoclonal and one polyclonal antibodies. In contrast to the index sample, both controls

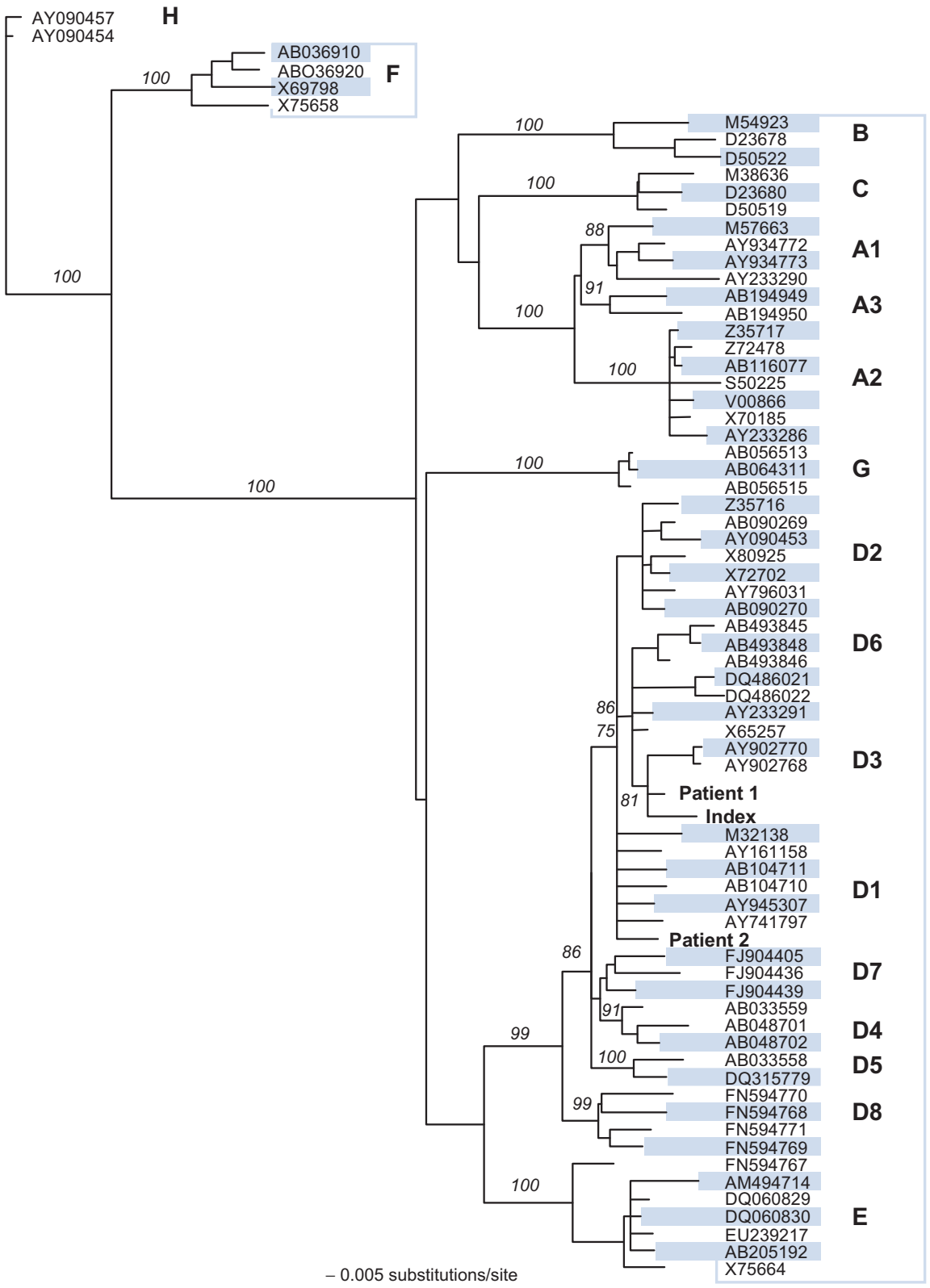

Fig. 1. Phylogenetic tree of the pre-S/S region of Index, Patients 1 and 2 HBV strain compared to genotype D and other genotype strains. The phylogenetic tree was constructed with a 1420 nucleotide sequences in the pre-S/S region of HBV. Phylogenetic analysis was performed with the neighbour-joining algorithm based on Kimura two-parameter distance estimation method. Only bootstrap values ⩾75% are shown (1000 replicates). The GenBank accession numbers of Pro and Patients 1 and 2 are GU570523, GU570524, and GU570526, respectively.

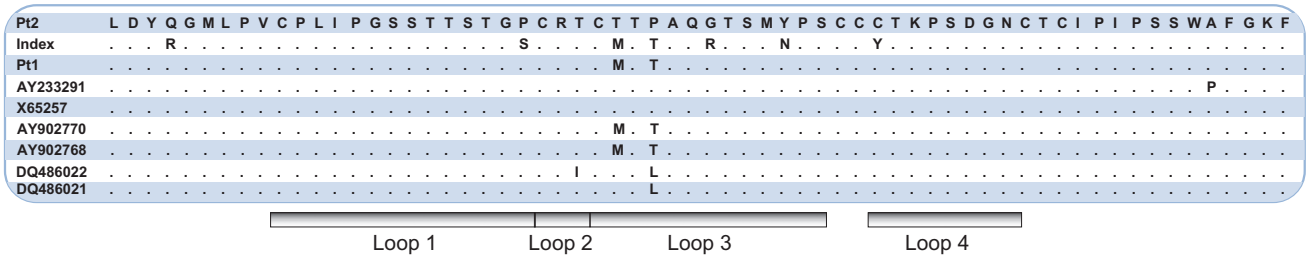

Fig. 2. Amino acid sequence of the central part of MHR of three Irish genotype D patients and subgenotype D3 references. Index patient amino acid sequence is aligned against Patient 2 sequence used as wild type subgenotype D1 reference. Patient 1 appears possibly related to the index strain and shows similarities with but also considerable divergence from the index strain. Other subgenotype D3 reference strains are indicated by their GenBank accession number. Grey bars indicate antigenic loops 1–4 between cysteines 107 and 121, 121 and 124, 124 and 137, and 139 and 147, respectively.

(Patient 2 and Aldevron) reacted with all HBsAg commercial EIAs and rapid tests as well as with all monoclonal or polyclonal anti-HBs. The index patient protein was not as well recognized as the genotype D control recombinant protein from Patient 2 and the genotype A2 commercial recombinant HBsAg by plasmas from vaccinated people (Table 2). However, plasma from the index patient did not carry sufficient level of anti-HBs to react with any of these three recombinant antigens. This is not particularly surprising since the level of anti-HBs detected at the time of sample collection was very close to the cut-off (10 IU/L) and when re-tested in 2009 with two commercial anti-HBs assays were non-reactive. The absence of recognition of this mutant antigen by three HBsAg screening assays, low level of reactivity with one EIA and two monoclonal antibodies (Ab1 and Ab2) and strong reactivity with only two commercial HBsAg screening assay clearly indicated considerably modified MHR epitopes.

When cysteine 139 mutation was corrected by SDM, the reactivity of one commercial EIA and two rapid tests unable to detect the mutated S protein (EIA1 and EIA4) was restored (Table 2). The S/CO of all other assays moderately or highly reactive with the index recombinant protein was enhanced. All monoclonal antibodies reacted strongly.

The accumulation of historical, phylogenetic, and S protein studies concurred to support reactivation of an escape mutant from recovered HBV infection 30 years earlier. However, the look-back study of potentially implicated blood donors had been put in motion. This onerous exercise identified and traced 268 donors to the 74 red cell and 46 pooled platelet components transfused. One donor met re-instatement criteria at the time of notification. Subsequently a further 114 donors re-tested HBV negative $\geqslant$6 months after their index donation while 29 additional donors re-tested HBV negative $\geqslant$3 months after theirs. In four cases where the donor never re-attended, the archive donation samples tested negative for anti-HBc and HBV DNA. Negative look-back information was available from 148/268 implicated donors when clear evidence of reactivation was obtained and the look-back was closed.

Table 2. Detection of recombinant HBsAg with different plasmas, assays or antibodies.

Samples (dilution)	Reactivity of recombinant S protein expressed as sample/cut-off (S/CO)			
	Index HBsAg	C139 corrected	Patient 2 HBsAg	Aldevron HBsAg[1]
Index plasma (1/100)	Negative	Negative	Negative	Negative
Patient 1[2] (1/100)	Negative	Negative	Negative	Negative
Patient 2[2] (1/100)	Negative	Negative	Negative	Negative
Donor 1[3] (1/100)	Negative	Negative	Negative	Negative
Donor 2[3] (1/100)	Negative	Negative	Negative	Negative
Vaccine 1[4] (1/100)	7.9	8.9	9.9	18
Vaccine 2[4] (1/100)	4.6	7.8	9.9	10
Microtiter EIA 1	Negative	7.3	36.4	30
Microtiter EIA 2	32.1	>50	36.9	>50
Microtiter EIA 3	26.5	>50	43.6	>50
Microtiter EIA 4	3.7	13.2	42.2	20
Rapid test 1	Negative	Positive	Positive	Positive
Rapid test 2	Negative	Positive	Positive	Positive
Monoclonal Ab1 (1/1000)	5.6	8.6	26.8	18.1
Monoclonal Ab2 (1/1000)	4.0	7.3	25.1	52.6
Monoclonal Ab3 (1/1000)	18.6	16.7	27.1	70.7
Polyclonal Ab (1/1000)	11.5	14.2	18.7	9

[1] Since the commercial HBsAg did not carry strep tag II, HBsAg was directly coated at 1 μg/ml overnight at 4 °C as described in Methods. This direct coating compared to Strep tag captured HBsAg explains the difference in S/CO levels.
[2] Patients 1 and 2 were HBV chronic carriers with wild type genotype D strains.
[3] Donors 1 and 2 were healthy and non-vaccinated to HBV.
[4] Vaccines 1 and 2 were healthy laboratory workers vaccinated against HBV and carrying 68 IU/L and 18 IU/L anti-HBs, respectively.

Discussion

Estimates of the residual risk of TT-HBV range from 1 in 50,000–426,000 (UK) [8], 1 in 60–150,000 (US) [9], 1 in 400,000 [10] (France) and 1 in >1 million (Australia) [11], without HBV Nucleic Acid Testing (NAT). In the 5 years to end of 2004, testing of 219,677 donations at MRTC identified a single donor with serological evidence of HBV (<0.5/100,000 donations). All donations to the IBTS since January 2002 were screened prospectively for anti-HBc although less than 1% anti-HBc positive donations may have detectable DNA, a proportion of which are potentially infectious [12,13].

The residual risk therefore reflects either incident primary infection window period (WP) donations or very rare cases of 'occult' Hepatitis B without anti-HBc with only viral DNA as a marker of the infection [14]. The Serious Hazards of Transfusion (SHOT)/Communicable Diseases Surveillance Centre (CDSC) reported WP donations resulting in 8/10 cases of TT-HBV in the 9 years to the end of 2004, from more than 25 million blood components (unscreened for anti-HBc) transfused [15]. The Irish Haemovigilance System (National Haemovigilance Office) had not confirmed any TT-HBV since its inception. However, a single implicated case could not be excluded (donor untraced, patient with other risk factors) [13]. In 1999, the prevalence of anti-HBc in the Republic of Ireland was estimated at 0.51% in the general population [12]. When anti-HBc screening was initiated in Irish blood donors, the prevalence in the first year of testing was 0.17% [16]. In 60 anti-HBc reactive donations identified in the first 120,000 donations tested none contained detectable HBV DNA (personal communication Dr. Joan O' Riordan). The blood components received by the index patient had been screened with the most sensitive HBsAg assay and anti-HBc and the likelihood of having transmitted HBV to the recipient was extremely low.

The alternative explanation of reactivation is well documented by concordant epidemiological and molecular evidence. The index patient had experienced acute hepatitis in 1975. The probability of having acquired HBV infection through heterosexual contact is high since his spouse was likely to have been a then carrier of HBV as not only the index patient but also their first-born child were infected. The spouse apparently seroconverted to anti-HBs after years of carriage (Table 1) so that strong but only indirect evidence of infectivity was obtained. However, the proven chronic carrier status of the brother-in-law (Patient 1, Table 1) and the phylogenetic link between Patient 1 and the index patient seem to implicate the index patient's spouse in his initial infection (Fig. 1). The sharing of the same genotype D and subgenotype D3 by the index patient and Patient 1 (In Ireland genotype A2 is dominant) is not particularly informative since the blinded unrelated individual examined (Patient 2) also carried genotype D although subgenotype D1 and there is evidence that this genotype is increasing in proportion (32%) in the southern part of the country [17]. Fig. 1 indicates some relatedness of nucleotide sequences covering half of the viral genome between the index patient and Patient 1, more so than with other genotype D available sequences including Patient 2. However, these two sequences have substantial divergence and unrelated sequences of HBV subgenotype 3 available in GenBank appear in the same cluster with 83% bootstrap value (Fig. 1). Such genetic distance is not particularly surprising since Patient 1 and his sister's strains have evolved with high-level replication (viral load >10E5 in 2004) for 20 years until the index patient was infected and Patient 1 strain further evolved for 30 years after the index patient infection. However, although the homology between the two sequences is 98.2%, the phylogenetic analysis is compatible with but does not formally proved genetic relatedness. In addition, the reactivated strain replicating in the index patient was submitted to considerable immune pressure related to the presence of anti-HBs probably generated by the vaccination 1.5 years prior to the acute episode and by the likely stimulation of the patient's own memory B-cells triggered by the vaccine antigen (Fig. 2).

Reactivation of HBV in the index patient is supported by the elevation of ALT levels prior to October 2004 and a peak of ALT concomitant with the occurrence of HBsAg. Although infection through another route cannot be formally excluded, the genetic data is compatible with a relatively mild acute hepatitis caused by a host-selected endogenous escape mutant. Reactivation of HBV in the presence of anti-HBs has been described in recipients of organ transplantation [18–21] hematology patients [2,6] including two cases with CLL treated with Fludarabine [22] and breakthrough infections in vaccinated individuals are not exceptional [23–25]. However, the combination of single chemotherapy-induced reactivation and breakthrough escape mutant has not been described. One case anti-HBs positive receiving multiple drug chemotherapy reactivated by selecting a mutant of cysteine 137 associated with G145R [26]. The mutations found in the index patient, in particular the substitution of the cysteine at codon 139 of the S gene (Fig. 2) was not observed post-vaccination or described in other circumstances except occult HBV infection (OBI). The associated mutations in loops 1 and 3 have been previously described. Loop 1 P120S was observed in at least eight cases of vaccinated or passively immunized patients [24,26–28]. Mutation G130R was identified in at least three cases, one of them in a genotype D strain [26,29,30]. Codon 134 is genotype specific, phenylalanine in genotypes A, B, and C and Tyrosine in genotype D strains [26].

The nearly undetectable anti-HBs only 18 months after vaccination in a previously exposed individual might be related to part of the anti-HBs complexed with viral antigen produced in small amounts but still recognized by the circulating anti-HBs. Only when the escape mutant emerged with high level of replication reactivation became clinically recognizable. The escape mutant hypothesis is further supported by the lack of detection of the recombinant mutant protein by three of six commercial HBsAg assays as well as its poor reactivity with two of three monoclonal antibodies specific of HBsAg (Table 2). This was further supported by the enhanced reactivity of the SDM recombinant protein with all assays and monoclonal antibodies despite the two substitutions left in the loop 3 region (Table 2). This data is strong evidence of the critical importance of cysteine 139 in the conformation and presentation of the 'a' determinant to monoclonal antibodies used in the commercial HBsAg assays. It also suggests that the selection of the mutated cysteine 139 by the patient anti-HBs played a significant role in the escape mechanism.

It can be speculated that the immunosuppressive effect of treatment with Chlorambucil was sufficiently mild not to disturb immunological control over the probably very few viral particles persistent in the index patient liver. Such mild effect has been demonstrated in a large randomized trial comparing the reactivation of HSV and CMV in patients treated for lymphoma with

Chlorambucil alone, Fludarabine alone or both drugs [31]. The higher degree of immunosuppression induced by Fludarabine triggered reactivation but, probably in relation with the presence of neutralizing anti-HBs, took approximately 1 year of treatment-induced viral replication to select a variant able to escape these antibodies. In a study of Fludarabine-induced HBV reactivation without anti-HBs, HBsAg became detectable after a few months of treatment [32]. The pathogenic impact of this mutant was probably limited (relatively low level of ALT and short duration of detectable HBsAg) by the ability of the stimulated immune system to adapt to this new antigen.

This case strongly suggests that patients should be screened for serological hepatitis markers in advance of instituting Fludarabine therapy and an archive sample should be retained. Such screening could lead to prevention or control by early antiviral therapy of reactivation in the patient and reduction of the need for onerous look-back investigations of suspected TT-HBV, especially as this risk is extremely low and look-back traumatic for donors [33].

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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References

[1] Yeo W, Chan PK, Zhong S, Ho WM, Steinberg JL, Tam JS, et al. Frequency of hepatitis B viral reactivation in cancer patients undergoing cytotoxic chemotherapy: a prospective study of 626 patients with identification of risk factors. J Med Virol 2000;62:299–307.
[2] Lalazar G, Rund D, Shouval D. Screening, prevention and treatment of viral hepatitis B reactivation in patients with haematological malignancies. Br J Haematol 2007;136:699–712.
[3] Lefrère JJ, Servant-Delmas A, Candotti D, Mariotti M, Thomas I, Brossard Y, et al. Persistent B19 infection in immunocompetent individuals: implications for transfusion safety. Blood 2005;106:2890–2895.
[4] Rehermann B, Ferrari C, Pasquinelli C, Chisari FV. The hepatitis B virus persists for decades after patients' recovery from acute viral hepatitis despite active maintenance of a cytotoxic T-lymphocyte response. Nat Med 1996;2:1104–1108.
[5] Roche B, Samuel D, Gigou M, Feray C, Virot V, Schmets L, et al. De novo and apparent de novo hepatitis B virus infection after liver transplantation. J Hepatol 1997;26:517–526.
[6] Lalazar G, Rund D, Shouval D. Screening, prevention and treatment of viral hepatitis B reactivation in patients with haematological malignancies. Br J Haematol 2007;136:699–712.
[7] Zahn A, Li C, Danso K, Candotti D, Owusu-Ofori S, Temple J, et al. Molecular characterization of occult hepatitis B virus in genotype E-infected subjects. J Gen Virol 2008;89:409–418.
[8] Allain JP, Hewitt PE, Tedder RS, Williamson LM. Evidence that anti-HBc but not HBV DNA testing may prevent some HBV transmission by transfusion. Br J Haematol 1999;107:186–195.
[9] Kleinman SH, Bush MP. HBV: amplified and back in the safety spotlight. Transfusion 2001;41:1081–1085.
[10] Pillonel J, Laperche S. Le groupe Agents Transmissibles par Transfusion de la Societe francaise de transfusion sanguine. Trends in residual risk of transfusion transmitted viral infections (HIV, HCV, HBV) in France between 1992 and 2002 and impact of nucleic acid testing. Transfus Clin Biol 2004;11:81–86.
[11] Seed CR, Kiely P, Keller J. Residual risk of transfusion transmitted human immunodeficiency virus, hepatitis B virus, hepatitis C virus and human T lymphotrophic virus. Intern Med J 2005;35:592–598.
[12] O'Connel T, Thornton L, O'flanagan D, Staines A, O'connell J, Dooley S, et al. Prevalence of hepatitis B anti-core antibody in the republic of Ireland. Epidemiol Infect 2000;125:701–704.
[13] National Haemovigilance Office. Annual Report 2004, Dublin, Ireland; 2004.
[14] Raimondo G, Allain JP, Brunetto MR, Buendia MA, Chen DS, Colombo M, et al. Statements from the Taormina expert meeting on occult hepatitis B virus infection. J Hepatol 2008;49:652–657.
[15] Serious Hazards of Transfusion (SHOT). Annual Report 2004, Manchester, UK; 2004.
[16] Lundy D, Laspina S, Kaplan H, Rabin Fastman B, Lawlor E. Seven hundred and fifty-nine (759) chances to learn: a 3-year pilot project to analyse transfusion-related near-miss events in the Republic of Ireland. Vox Sang 2007;92:233–241.
[17] Horgan M, Brannigan E, Crowley B, Levis J, Fanning LJ. Hepatitis B genotype and YMDD profiles in an untreated Irish population. J Clin Virol 2006;35:203–204.
[18] Blanpain C, Knoop C, Delforge ML, Antoine M, Peny MO, Liesnard C, et al. Reactivation of hepatitis B after transplantation in patients with pre-existing anti-hepatitis B surface antigen antibodies: report on three cases and review of the literature. Transplantation 1998;66:883–886.
[19] Dhedin N, Douvin C, Kuentz M, Saint-Marc MF, Reman O, Rieux C, et al. Reverse seroconversion of hepatitis B after allogeneic bone marrow transplantation: a retrospective study of 37 patients with pretransplant anti-HBs and anti-HBc. Transplantation 1998;66:616–619.
[20] Roche B, Feray C, Gigou M, Roque-Afonso AM, Arulnaden JL, Delvart V, et al. HBV DNA persistence 10 years after liver transplantation despite successful anti-HBS passive immunoprophylaxis. Hepatology 2003;38:86–95.
[21] Berasain C, Betes M, Panizo A, Ruiz J, Herrero JI, Civeira MP, et al. Pathological and virological findings in patients with persistent hypertransaminasaemia of unknown aetiology. Gut 2000;47:429–435.
[22] Alexopoulou A, Theodorou M, Dourakis SP, Karayiannis P, Sagkana E, Papanikolopoulos K, et al. Hepatitis B virus reactivation in patients receiving chemotherapy for malignancies: role of precore stop-codon and basic core promoter mutations. J Viral Hep 2006;13:591–596.
[23] Huang X, Lu D, Ji G, Sun Y, Ma L, Chen Z, et al. Hepatitis B virus (HBV) vaccine-induced escape mutants of HBV S gene among children from Qidong area, China. Virus Res 2004;99:63–68.
[24] Hsu HY, Chang MH, Ni YH, Chen HL. Survey of hepatitis B surface variant infection in children 15 years after a nationwide vaccination programme in Taiwan. Gut 2004;53:1499–1503.
[25] Zheng X, Weinberger KM, Gehrke R, Isogawa M, Hilken G, Kemper T, et al. Mutant hepatitis B virus surface antigens (HBsAg) are immunogenic but may have changed specificity. Virology 2004;329:454–464.
[26] Awerkiew S, Daumer M, Reiser M, Wend UC, Pfister H, Kaiser R, et al. Reactivation of an occult hepatitis B virus escape mutant in an anti-HBs positive, anti-HBc negative lymphoma patient. J Clin Virol 2007;38:83.
[27] Grethe S, Monazahian M, Böhme I, Thomssen R. Characterization of unusual escape variants of hepatitis B virus isolated from a hepatitis B surface antigen-negative subject. J Virol 1998;72:7692–7696.
[28] Wallace LA, Echevarria JE, Echevarria JM, Carman WF. Molecular characterization of envelope antigenic variants of hepatitis B virus from Spain. J Infect Dis 1994;170:1300–1303.
[29] Candotti D, Grabarczyk P, Ghiazza P, Roig R, Casamitjana N, Iudicone P, et al. Characterization of occult hepatitis B virus from blood donors carrying genotype A2 or genotype D strains. J Hepatol 2008;49:537–547.

[30] Lu HY, Zeng Z, Xu XY, Zhang NL, Yu M, Gong WB. Mutations in surface and polymerase gene of chronic hepatitis B patients with coexisting HBsAg and anti-HBs. World J Gastroenterol 2006;12:4219–4223.
[31] Morrison VA, Rai KR, Peterson BL, Kolitz JE, Elias L, Appelbaum FR, et al. Impact of therapy with chlorambucil, fludarabine, or fludarabine plus chlorambucil on infections in patients with chronic lymphocytic leukemia: intergroup study cancer and leukemia group B 9011. J Clin Oncol 2001;19:3611–3621.
[32] Picardi M, Pane F, Quintarelli C, De Renzo A, Del Giudice A, De Divitiis B, et al. Hep B virus reactivation after fludarabine based regimens for indolent non-Hodgkin's lymphomas: high prevalence of acquired viral genomic mutations. Haematologica 2003;88:1296–1303.
[33] Miller R, Hewitt PE, Warwick R, Moore MC, Vincent B. Review of counselling in a transfusion service: the London (UK) experience. Vox Sang 1998;74:133–139.