MEDICAL SCIENCE

Vaccine-induced escape mutant of hepatitis B virus

WILLIAM F. CARMAN ALESSANDRO R. ZANETTI
PETER KARAYIANNIS JENNY WATERS GIUSEPPE MANZILLO
ELISABETTA TANZI ARIE J. ZUCKERMAN HOWARD C. THOMAS

In southern Italy, 44 contacts of hepatitis B virus carriers, including infants of carrier mothers, became HBsAg positive despite passive and active immunisation according to standard protocols. In 32 of these vaccinees infection was confirmed by the presence of additional markers of viral replication. In 1 infant, serious disease occurred. The virus from this patient is an escape mutant with a different sequence from that of the isolate from the mother. A point mutation from guanosine to adenosine at nucleotide position 587 resulted in an aminoacid substitution from glycine to arginine in the highly antigenic *a* determinant of HBsAg. This mutation is stable: it is present in an isolate from the child 5 years later. In some of these patients, including this child, the *a* determinant, to which a large part of the vaccine-induced immunity is directed, has been partly lost. Binding to HBsAg of a monoclonal antibody, previously mapped to the region of the mutation, was reduced in the child relative to that of the mother.

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Introduction

During the past decade, several putative variants of hepatitis B virus (HBV) have been described, and some have caused illness. Patients infected with these variants have had no markers of HBV except for HBV-DNA in liver or serum,[1] or very low levels of hepatitis B surface antigen (HBsAg) detectable only by highly sensitive monoclonal antibody-based radioimmunometric assays (m-RIA).[2] There is a report of a patient in whom the only marker was HBV-DNA detectable by the polymerase chain reaction (PCR).[3] Sequencing of this isolate revealed a single aminoacid change in the conserved 3′ end of the surface protein, but the significance of this finding is unclear.

An HBV variant with one or two mutations in the pre-core region of the genome has been shown to be responsible for chronic hepatitis in Greek and Italian patients;[4,5] in these patients there has been evidence of continuing replication (positive HBV-DNA in serum measured by dot-blot hybridisation) without hepatitis B e antigenaemia (HBeAg). This variant has a base substitution that gives rise to a translational stop codon, which accounts for the failure of these patients to secrete HBeAg.

Two types of variant have been reported to replicate in the presence of vaccine-induced immunity. The first was found in a patient with HBsAg detectable only by m-RIA and low concentrations of HBV-DNA in the serum.[6] When serum from this patient was transfused into a vaccinated chimpanzee that had been challenged with wild-type HBV and found to be protected, it induced the same serological profile as that of the patient. The second was found in patients from Senegal, some of whom had vaccine-induced anti-HBs; despite their HBsAg positivity, in none of the patients did other markers of HBV infection (such as anti-HBc, HBeAg, or, in the unvaccinated cases, anti-HBs) develop, and the patients were thought to have been infected with a novel strain, termed HBV-2.[7] Neither of these putative variants was characterised at a molecular level.

Between 1982 and 1987, infants born to HBsAg-positive Italian carrier mothers and family contacts of carriers were vaccinated under trial conditions to assess the immunogenicity and efficacy of two licensed plasma-derived vaccines that met the WHO requirements for hepatitis B vaccine. While being monitored for an immune response, several subjects were found to have markers of HBV replication. This paper describes the clinical and serological profiles of some of these patients and characterises at molecular level the mutation in the isolate from a child.

Subjects and methods

Vaccine trial

Several HBV vaccine trials were begun in Italy in 1982. We followed up 1590 of the vaccines. They were mostly infants of HBsAg-positive carriers from two regions of southern Italy where

ADDRESSES: **Departments of Medicine, St Mary's Hospital Medical School, London** (W. F. Carman, M Med, P Karayiannis, PhD, J. Waters, PhD, Prof H. C. Thomas, FRCP); **Istituto di Virologia, University of Milan, Italy** (Prof A. R Zanetti, PhD); **4th Department of Infectious Diseases, Hospital Cotugno, Naples, Italy** (Prof G. Manzillo, MD); and **Department of Medical Microbiology, Royal Free Hospital School of Medicine, London** (Prof A. J. Zuckerman, FRCP). Correspondence to Dr W. F. Carman, Department of Medicine, St Mary's Hospital, London W2 1NY, UK.

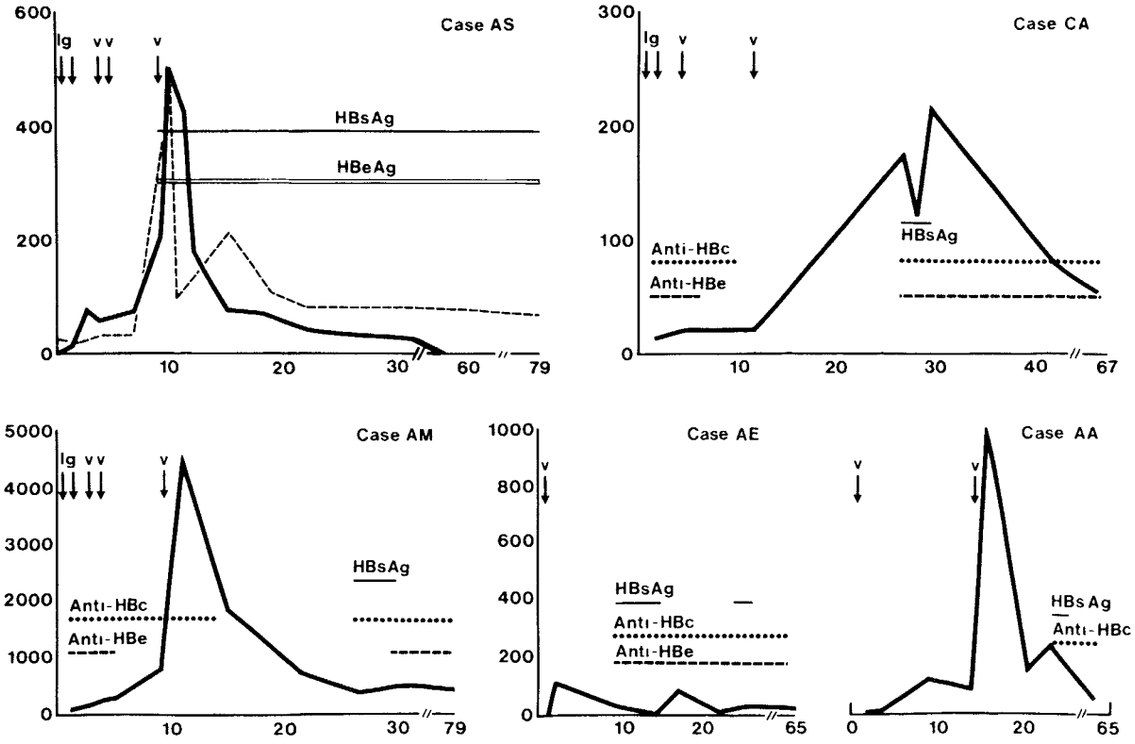

Fig 1—Serological changes in 5 patients with viral replication after vaccination.

X axes in months, Y axes in mIU/ml of anti-HBs Unlabelled solid lines represent anti-HBs concentrations, and unlabelled broken line in graph for patient AS represents alamine aminotransferase (ALT) concentrations. V = vaccine and Ig = hyperimmune globulin.

the HBsAg prevalence was greater than 5%.[8] The vaccines were either 'HB-VAX' (Merck Sharp and Dohme) or 'HEVAC-B' (Pasteur), both plasma derived. The dose was 20 μg of HB-VAX for adults (10 μg for infants) at 3, 4, and 9 months in the early phase of the trial, and at 0, 1, and 6 months later on in the trial, or 5 μg of HEVAC-B at 0, 1, 2, and 14 months. At birth and at age 1 month babies also received 0·5 ml hepatitis B hyperimmune globulin (HBIg) (Biagini), prepared by Cohn ethanol fractionation. Several family contacts of the carriers, both adults and children, were also vaccinated.

Subjects

Of the 1590 vaccinees, 44 (2·8%) became HBsAg positive; of these 12 showed weak reactivity and no other HBV markers whereas the other 32 showed further evidence of HBV infection. Some data are available on 18 of these patients (the term patients is used to refer to vaccinees who became HBsAg positive after vaccination, and carriers refer to those who remained HBsAg positive for more than 6 months)—5 infants born to carrier mothers, 5 child family contacts, and 8 adult family contacts. All the 44 vaccinees had protective titres of anti-HBs before emergence of HBsAg positivity. Serological findings on the children are given below.

Changes for AS, an infant born to an HBe antigen-positive carrier mother (and chosen for detailed study), and for CA and AM, infants born to anti-HBe positive carrier mothers, are shown in fig 2. Serological changes for patients AA (7 years old) and AE (4 years old), brothers living with an HBsAg and anti-HBe positive mother, are also shown in fig 2. Both brothers were vaccinated at the same time.

PG, a boy born to an HBsAg positive, anti-HBe positive carrier, received HBIg at birth and vaccine (HB-VAX) at 1, 2, and 7 months of age. 27 months after the last immunisation, he became HBsAg positive with anti-HBs at 55 mIU/ml. ALT remained normal and HBsAg was positive for 2 weeks. He is now anti-HBe and anti-HBc positive.

AC, a girl aged 4 years living with an HBsAg positive sister, showed, at the time of the third injection of vaccine, an anti-HBs titre of 2850 mIU/ml and a positive HBsAg test, which remained positive for only 3 weeks. Anti-HBc and anti-HBe were still positive 40 months later, when anti-HBs concentration was 160 mIU/ml.

RV, an infant born to an anti-HBe positive carrier mother, was vaccinated at age 3 and 4 months, after having received HBIg at 0 and 1 months. At age 4 months anti-HBs was 70 mIU/ml and HBsAg was positive. It became negative at 5 months. The child is now anti-HBc and anti-HBe positive. CV, a 1-year-old boy, and PE, a 10-year-old girl, had similar histories. Both were found to be HBsAg positive at time of the third vaccination. CV had an anti-HBs concentration of 90 mIU/ml at this stage and PE 12 mIU/ml.

Hepatitis markers

Blood samples were tested for HBsAg, HBeAg, anti-HBe, anti-HBs, and anti-HBc with commercial kits (Abbott Laboratories). HBsAg positivity was confirmed both by retesting and by the HBsAg confirmatory neutralisation assay (Abbott Laboratories). Serum containing almost exclusively anti-*a* was used for the neutralisation assay. Other markers of HBV replication were confirmed in an independent laboratory.

HBsAg subtyping and monoclonal binding studies

HBsAg from the following were subtyped: patients AS, AA, and AE, 2 of the adult patients the carrier contacts of the 5 infected babies and the 5 infected children, and 6 of 8 of the family contacts of infected adults. For subtyping, beads coated with anti-*a*, anti-*d*, or anti-*y* (Sorin Biomedica) were incubated with serum, then washed, and the bound HBsAg was picked out with ^{125}I-anti-HBs. A sample was considered *ay* when the sample to negative ratio with anti-*a* or anti-*y* on the solid phase exceeded 2·1 and that obtained with the anti-*d* beads was lower than the cut-off value. Samples were considered to have only *y* reactivity if the counts were higher than the cut-off value with anti-*y* on the solid phase, but lower with anti-*a* or anti-*d* solid phases. Specificity of reactivity was confirmed by neutralisation with monoclonal antibodies (Sorin Biomedica). To our knowledge, these antibodies have not been mapped exactly. Briefly, HBsAg positive sera were mixed with monospecific anti-*a*,

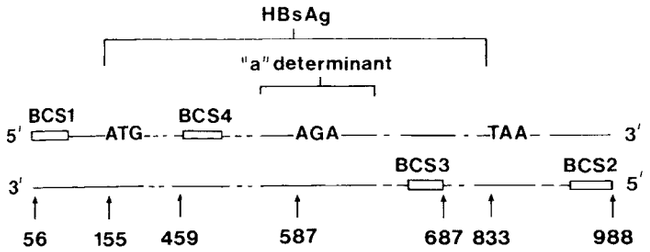

Fig 2—Target sequence of the mutant strain of HBV for PCR-direct sequencing, showing the translational start (ATG) and stop (TAA) codons of HBsAg and the site of the *a* determinant.

Numbering of nucleotides is from the unique *Eco*R1 site. The adenosine at position 587 is the mutation. PCR primers were BCS2 (5'CCGGATCCATACTTTCCAATCAATAGG; underlined is a *Bam*HI site and a terminal redundancy) and BCS4 (5'GTATGTTGCCCGTTTGTCCTC) The sequencing primer is BCS3 (5'GGCACTAGTAAACTGAGCCA).

anti-*d*, or anti-*y* antibody before subtyping. A reference HBsAg-containing serum was used as a control.

These studies were then extended to define more specifically the loss of *a* determinant antigenicity in serum from a mother-child pair. A previously mapped set of anti-HBs monoclonal antibodies known to bind to the *a* determinant, were used. Beads were coated with antibodies RFHBs-1, RFHBs-2, and RFHBs-7, then incubated with a dilution series of serum. Bound HBsAg was detected with ^{125}I-anti-HBs from an 'Ausria II' kit (Abbott Diagnostics). An identical dilution series was assessed under standard kit conditions. RFHBs-1 and RFHBs-2 bind to a cyclical peptide made from aminoacids 124–137, and RFHBs-7 to a cyclical peptide from aa 139–147 of HBs antigen.

Patients for sequencing studies

A boy who was HBeAg positive and his mother were the patient pair chosen for sequencing studies. The mother was an HBsAg, HBeAg, and anti-HBc positive carrier (patient IE) with an ALT of 105 IU/l. Her son (AS), was born on March 3, 1983. The father was anti-HBc and anti-HBs positive. At birth and 1 month later, the infant was given 1·5 ml of HBIg, and he was vaccinated with HB-VAX at 3 months, 4 months, and 9 months of age. The serological profile of the child is shown in fig 1. The following samples were used for sequencing studies: serum from the mother at delivery and serum from the child at age 11 months (HBsAg, HBeAg, and anti-HBc positive; anti-HBs 420 mIU/ml, ALT 120 IU/l) and 5 years later (HBsAg and HBeAg positive, anti-HBs negative, ALT 36). Serum from 4 randomly chosen Italian HBeAg-positive carriers not taking part in the vaccine trial and serum from 3 British HBsAg-positive patients were also sequenced.

PCR and direct sequencing

50 µl of serum was digested in 25 mmol/l sodium acetate, 2·5 mmol/l edetic acid, 0·5% sodium dodecyl sulphate, and 1 mg/ml proteinase K (Boehringer Mannheim), in a volume of 200 µl overnight at 37°C. After two phenol/chloroform and two chloroform extractions, the DNA was precipitated with enthanol and the pellet washed with 70% ethanol. The pellet was resuspended in 20 µl of water. 10 µl of the DNA and 300 pmol each of primers BCS and BCS4 were used for the polymerase chain reaction[4] (fig 2). Sequencing[4] was done with the 'Sequenase' kit (United States Biochemicals) and end-labelled primer BCS3. This primer was not column-purified after labelling.

EPITOPE MAPPING OF HBsAg FROM MOTHER (IE) AND CHILD (AS) PAIR

Subject	RFHBs-1	RFHBs-7	RFHBs-2	AUSRIA
AS				
11 months	6·5	2·7	1·7	27·3
5 years	13	12	7 9	56
IE	129·5	140·2	24·8	137 2

Data are expressed as positive to negative ratio at maximum antigen binding AS was anti-HBs positive at 11 months, negative at 5 years Sample of IE was taken at birth of AS

Hydrophobicity plots

Aminoacids 139 to 147 of the *a* determinant from the normal and the mutant HBsAg were analysed with Prosis (Pharmacia) software.

Results

Subtyping and monoclonal antibody binding studies

HBV was of subtype *ay* in all the contacts of the infected infants and children and in all the 6 contacts of adult cases who were tested. Patient AS was found to be weakly positive for subtype *y* at 12 and 18 months of age and *ay* positive at 46 months, by which time the anti-HBs became undetectable. Patient AA, AE, and the 2 adults tested (2 years, 9 months, 26 months, and 28 months after the start of vaccination respectively) were *y* positive only. These results were confirmed in three independent laboratories and by neutralisation (ie, the serum samples were neutralised with anti-*y* but not with anti-*a*).

Binding studies in patient AS showed that the HBs antigen in the serum at 5 years of age, when anti-HBs was no longer present, bound significantly less to RFHBs-1, RFHBs-2, and RFHBs-7 monoclonal antibodies than to the polyvalent anti-HBs (table). In contrast, the HBs antigen in the mother's serum showed similar binding to RFHBs-1 and RFHBs-7 monoclonal antibodies as to polyvalent anti-HBs, though binding to RFHBs-2 was reduced.

DNA sequencing of the a determinant

HBV-DNA from patient AS revealed a single mutation from guanosine to adenosine at position 587 (numbered from the unique *Eco*R1 site of the HBV genome) that resulted in an aminoacid substitution from glycine to

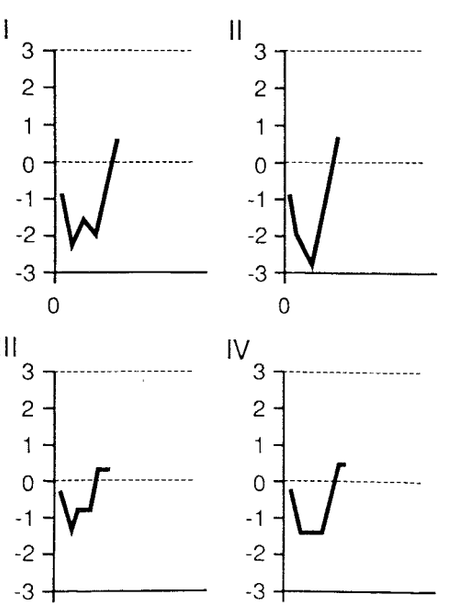

Fig 3—Hydrophobicity plots of the second loop of *a* determinant (amino)acids 139–147) from normal (I, III) and mutant (II, IV) isolates.

Numbers I and II are generated by the method of Kyte and Doolittle[9] and numbers III and IV by that of Hopp and Woods.[10] Prosis software (Pharmacia) was used to generate the plots, which were then reproduced.

arginine at aminoacid 145 of HBsAg. This change was stable, since it was present at the age of 11 months and at 5 years of age. The viral DNA from the mother, IE, and from the 7 control patients encoded for glycine at this point in the epitope, as in all sequences published to date. The DNA sequence of the region encoding the *a* determinant was otherwise the same in patients AS and IE and the same as those previously published.

Hydrophobicity plots

According to the method of Kyte and Doolittle,[9] the mean hydrophobicity index for the second loop of the *a* determinant was −1·3 in a normal isolate and to −1·9 in the isolate from patient AS; the corresponding values obtained by the method of Hopp and Woods[10] were −0·5 and −0·9 (fig 3). Thus the mutant isolate was more hydrophilic than the normal isolate.

Discussion

The lipid envelope of HBV contains molecules of the large, middle, and major surface gene-encoded proteins, which share the same caboxyl terminus—ie, HBsAg. Antigenic subtypes of HBV are defined serologically and have been shown to be due to single base changes in the region of the genome encoding HBsAg.[11] Thus, major antigenic variation can result from minor changes to the genome. These subtypes occur naturally and have particular geographical distributions.

All known antigenic subtypes contain the *a* determinant, and antibody to this confers protection against all subtypes. An important region of this determinant lies between amino-acids 124 to 147 of HBsAg (fig 4). Antibodies that bind to peptides representing amino-acids 124 to 137 and 139 to 147 have been demonstrated in a variety of serum samples.[12] Cyclic synthetic peptides of these two regions have greater antigenicity than that of the linear peptides, and antigenicity of HBsAg is lost if the cyclic form is treated with detergents;[13] these findings imply that the epitopes are conformational. It has been shown by in-vitro mutagenesis, in which single aminoacid changes can be introduced into the HBsAg molecule, that the cysteine at position 147 and the proline at position 142 are very important for the display of full antigenicity of this region.[14]

The HBV vaccine, which consists of HBsAg either in the native or a recombinant form, is known to be highly effective. Although in about 5% of vaccinees an adequate anti-HBs response does not develop, those who do have such a response are considered protected. In persons with an adequate anti-HBs level HBV infection does not develop on subsequent exposure. It is therefore of interest that several vaccinees from southern Italy, all of whom were considered to have responded well to the vaccine, should subsequently have markers of HBV replication. In those who became HBsAg positive transiently a few months after the start of vaccination, the easiest interpretation is that the infection was incubating at the time of vaccination, although post-vaccination infection with the variant virus could also have accounted for this situation. In most cases, there was only a transient appearance of HBsAg in the patients' serum, and this was followed by the appearance of anti-HBc and anti-HBe, as would be expected in acute infection. However, in one case, acute hepatitis B, followed by a persistent rise in transaminases, occurred.

Viruses continually change during the course of infection:[15,16] patients with chronic HBV infection may have

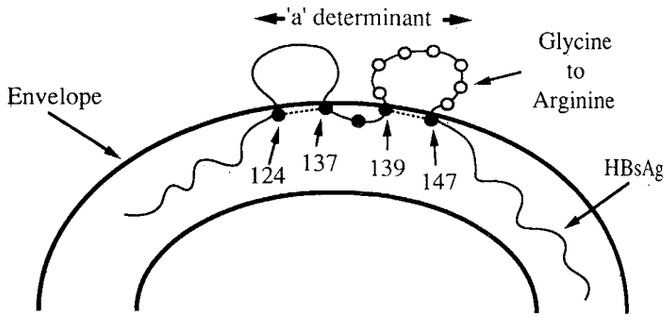

Fig 4—Diagrammatic representation of normal and mutant HBsAg in the envelope of HBV.

Both loops of the *a* determinant are shown. Numbering of the aminoacids is from the start of HBsAg and refers to the cysteine molecules (solid circles), joined by disulphide bridges (broken lines). Open circles are the aminoacids of the second loop. Glycine is found in the normal protein at position 145. Arginine replaces it in the mutant.

a variety of genotypes in their serum.[17] The fact that the immune response is directed against a number of viral epitopes (some of which are neutralising) leads to mutants being selected from the constantly changing viral population. Not all such mutants will be able to replicate, however. When an epitope is also vital to virus survival, such as when it is necessary for cell entry, a virus mutated at this position may not be viable. Replicating, infectious viruses that have mutated so that they are no longer susceptible to neutralising immunity are termed escape mutants. Such mutation might be responsible for the antigenic drift seen regularly in the epidemiology of influenza virus. Exposure of tissue culture cells infected with hepatitis A virus to monoclonal antibodies enables escape mutants to become dominant.[18] A remarkable parallel to HBV is to be found in the biology of herpes simplex virus. One of the envelope glycoproteins, gC, has two highly antigenic hydrophilic loops; mutant forms of these loops have enabled the virus to escape from monoclonal antibody pressure in vitro.[19] Such changes are found widely in viral biology.

The isolate from AS is an escape mutant with an aminoacid substitution of arginine for glycine at position 145 of HBsAg, and the binding of monoclonal antibodies specific to this region is much reduced. The reduction in binding of antibodies to the region of aminoacids 124-137 suggests that the mutation has resulted in a conformational change affecting several epitopes in the region of the *a* determinant. These antigenic changes are to be expected, since arginine is a much larger residue than glycine and is charged. The hydrophilicity of the prototype sequence and that of the escape mutant in this region are quite different. The hydrophilicity, which often correlates with antigenicity, is increased, and a new HBsAg specificity may be present in these patients.

It seems likely that the region in which the mutation occurs is an important epitope of HBV, to which vaccine-induced neutralising antibody binds; it also seems likely that the mutant virus lacks this epitope and is not therefore neutralised by antibody of this specificity. The ability of the mutant virus to replicate implies that this aminoacid substitution does not alter the attachment of the virus to the hepatocyte.

Another seven patients, infected after successful vaccination, have recently been noted from this area although they are, as yet, not fully characterised. A major unanswered question arising from this study is why these

mutants are localised to a particular geographical area. A parallel can be drawn between this variant and the pre-core variant found in Greece and Italy.[4] There is some evidence that the latter variant arises de novo in the patient in response to host-selection pressure, probably immune in nature (personal observation). For both these variants, therefore, it may be the host's immunogenetic make-up, which determines the nature of the immune pressure, that allows their appearance. Another explanation can be found in the virus itself. Several published sequences have thymidine rather than cytosine in the base preceding the mutation described here. In these isolates, the mutation would give rise to TAG, rather than CAG, in the polymerase gene reading frame. The former is a translational stop codon and therefore the polymerase would not be translated and the virus would not be able to replicate. The C in this position seems to be a random event and not related to serotype. Finally, vaccination was not given according to schedule in some cases, and this irregularity may have allowed the virus to become established in the liver and to mutate before the infected hepatocytes were destroyed by other components of the host response. In the case of the child from whom the viral sequence was obtained, the hyperimmune globulin was given at birth and 1 month later but, as was the practice then, the vaccine was not given until the 3rd, 4th, and 9th month of age.

There has been a recent report in which a patient with HBV-induced end-stage liver disease received a liver transplant and was given monoclonal anti-HBs to prevent reinfection of the homograft. After therapy, the patient again became serum HBV-DNA positive, and sequencing of the genome revealed the identical mutation to the one described here.[20] Thus there are two lines of evidence pointing to this region of the viral envelope being susceptible to mutation under "immune pressure". Consideration may have to be given to including the sequence of this mutant virus in future vaccines, to prevent the emergence of this escape mutant.

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From The Lancet

The Harveian Oration

The Harveian Oration was delivered on Tuesday last, by Dr Acland, and was in all respects worthy and able. For the first time a great innovation was practised: the oration was delivered in English. It would be affectation to deny that this change is in some respects to be regretted. It had unquestionably been well for the fellows and members of the College to have maintained that easy familiarity with good Latin which would have made the Latin oration easy to the orator and his audience. Still we heartily approve of the change. It is just one of those things that speak for themselves. It would not have happened if it had not been needed. And if the present generation of physicians be behind its predecessors in point of familiarity with Latin, let us be thankful that in other kinds of knowledge it more than makes up for that defect. It may not be too much to hope that men may retain Latin enough for pleasure and culture of thought; but it does seem hopeless to expect that the run of busy physicians now-a-days should retain enough of it to enable them, with anything like satisfaction to themselves, either to compose or appreciate a Latin oration. And a Latin oration should be done well, or not at all. There is one severe demand, however, which will be made upon the orators who have been relieved of the duty of Latin,—that is, that they give us English of the first order. In this respect the oration of Dr Acland was quite equal to the dignity of the occasion, whether we consider the audience or the subject. His Royal Highness the Prince of Wales, Mr Gladstone, Dr Watson (happily adverted to by the orator as the "Cicero of Medicine"), and a very unusual gathering of the chief physicians and surgeons of London, constituted the audience, and gave the most discriminating and close attention to the orator for decidedly more than "one short hour".

(July 1, 1865)