Failed Postnatal Immunoprophylaxis for Hepatitis B: Characteristics of Maternal Hepatitis B Virus as Risk Factors

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A retrospective case-control study was conducted to determine why some infants born full-term without obstetric intervention to hepatitis B e antigen (HBeAg)–seropositive mothers become infected by hepatitis B virus (HBV) despite having received passive-active immunoprophylaxis. Cases and controls comprised 12 hepatitis B surface antigen (HBsAg)–seropositive infants and 22 HBsAg-seronegative infants, respectively. Infants infected by putative vaccine-escape mutants were excluded. Risk factors, after adjustment for the level of maternal viremia, were the following allelic base changes in maternal HBV: C^{158}, A^{328}, G^{365}, and A^{479} ($P = .017$, .005, .003, and .005, respectively). High-level maternal viremia (i.e., $\geq 10^8$ genome equivalents/mL) was a significant factor only after adjustment for G^{365} ($P = .027$). HBV DNA sequences recovered from one of the cases, the case's mother, and three infected contacts all had the high-risk mutations. Specific allelic mutations in maternal HBV and level of maternal viremia are potential predictors of vertical breakthrough infection.

Hepatitis B virus (HBV) infection remains globally endemic. Transmission from mother to infant plays an important role in maintenance of endemicity, especially in regions where such infection is hyperendemic, e.g., East Asia [1]. Since a large fraction of vertical infections progress to chronicity [2], infected infants can in their lifetime be capable of initiating new cycles of both horizontal and vertical infection.

Infants born to hepatitis B e antigen (HBeAg)–positive mothers are at risk of acquiring infection maternally [3]. Prompt postnatal vaccination of at-risk infants effectively curtails perinatal infection, particularly when added protection is provided by hepatitis B immunoglobulin (HBIg) given shortly after birth. Nevertheless, a small proportion of those receiving complete passive-active prophylaxis become infected [4–6]. In these infected infants, the risks of developing sequelae of chronic infection [7] and transmitting HBV remain.

In the course of monitoring the outcome for at-risk infants in England and Wales, we observed some postvaccination failures among neonates born to HBeAg-positive mothers. In certain instances, failure could be attributed to factors that predispose to perinatal infection, such as prematurity [8], prolonged labor [9], delay or omission of HBIg administration, and failure to achieve full vaccination [10, 11]. However, HBV infection was also noted in full-term infants who were delivered without obstetric intervention and given full prophylaxis. Past studies have associated such ''breakthrough'' infections with intrauterine infection [6], high-level maternal viremia [12–14], and infection by vaccine-escape HBV mutants [15].

We report a case-control study of neonatal breakthrough HBV infection, showing that nucleotide changes at certain alleles in the genome of maternal HBV and high-level maternal viremia may be risk factors. In addition, to illustrate the public health impact of perinatal infection by an HBV variant with the high-risk characteristics identified in the case-control study, we provide a descriptive account of a molecular epidemiological investigation into a transmission chain traced to a child who became infected after birth despite having received full passive-active immunoprophylaxis.

Patients and Methods

Prevalence of Neonatal Breakthrough HBV Infection

The Public Health Laboratory Service administers a surveillance scheme in England and Wales for infants at risk of acquiring HBV infection. In this scheme, a database is kept of the age, ethnicity, parity, and HBeAg status of hepatitis B surface antigen (HBsAg)–positive mothers, and in relation to their infants, the obstetric history, vaccination schedule, dosage and batch numbers of HBIg and vaccine, and HBV serological status at age 1 year [16]. Data were extracted from records of infants born between 1 June 1988 and 30 May 1995 to assess the prevalence of HBV infection in neonates who were delivered to HBsAg/HBeAg-positive mothers at or near term without obstetric complications and received passive-active prophylaxis against HBV.

For this study, only records of infants who fulfilled the following criteria were reviewed. (1) They were delivered vagi-

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nally without assistance at or after 36 weeks' gestation; (2) they received 200 IU of HBIg (Bio Products Laboratory, Elstree, England) within 48 hours after birth; (3) they received three doses of hepatitis B vaccine (Engerix B; SmithKline Beecham, Welwyn Garden City, England) (0.5 mL each, containing 10 mg of HBsAg) at 0, 1, and 6 months of age; and (4) they underwent postvaccination serological testing for HBsAg.

Virological Studies

Specimens. To assess virological parameters that might account for the difference in outcomes of the infants' infections following immunoprophylaxis, antenatal booking sera (usually obtained at the end of the first trimester) from mothers of infants found to be HBsAg-positive at postvaccination testing were examined. Available antenatal sera from HBeAg-positive mothers of infants who were HBsAg-negative at postvaccination testing were used as controls. Sera from infected infants were additionally studied to compare the nucleotide sequences of HBV circulating in them and in their mothers. In the separate study of HBV transmission linked to an HBsAg-positive child, we studied sera obtained from contacts who developed acute HBV infection as well as sera and saliva from the child.

HBV strain differentiation. DNA in specimens was extracted by a silica/guanidium thiocyanate–based method [17]. Saliva was first subjected to ultracentrifugation before extraction. From each extract, a 476-bp segment from the surface antigen–coding region (hereafter the ''surface gene'') between nucleotide positions 82 and 558 was amplified by nested PCR. This segment was chosen because it encompasses sites in the surface gene that are regarded to confer antigenicity to HBsAg [18–20]. PCR primer sequences and cycling conditions used in this study have been specified elsewhere [21].

Strain differences in the surface gene were scanned by using a single-strand conformation polymorphism (SSCP) analytic procedure on the amplified products, performed essentially as described previously [21]. DNA in the amplified products was sequenced with use of the Taq DyeDeoxy Terminator Cycle Sequencing Kit and the ABI 373A DNA Sequencer (both from Perkin Elmer, Forest City, CA). HBV genotypic assignments were based on genotype-specific nucleotide motifs in the surface genes [22]. For viral load estimation and for the observational study, a 263-bp segment from the core antigen–coding region (hereafter the ''core gene''), from positions 1,891–2,154, was also amplified.

Viral load estimation. Following a series of 10-fold dilutions of each serum, DNA was extracted from each dilution and processed for PCR amplification of the core gene fragment. The end-point was compared to that of a similar dilution series made from 1 ng of the plasmid pHBV130.

Statistical Analyses

Variables examined were as follows: infant's birth weight; time between delivery and the administration of HBIg; mother's age, gravidity, and ethnicity; and maternal HBV titer, genotype, and base changes at non-genotype-specifying positions in the surface gene amplicons. The maternal HBV DNA titer was analyzed both as a continuous variable (expressed as genome copies/mL) and as a dichotomous variable (the number of mothers who carry 10^8 or $\geq 10^8$ genome copies per mL of HBV DNA). The value of 10^8 genome copies/mL was chosen because it divided the number of mothers into two roughly equal sets. Lot differences in HBIg and Engerix B were examined as potential variables, but the diversity in batch numbers of the two products was too wide for inclusion in the analysis.

Data were analyzed with use of statistical packages. Exact logistic regression was also used in multivariable analyses to investigate the joint effects of various pairs of variables. It was not possible to include >2 variables in any given model, owing to the small sample size and the high degree of correlation between many of the genetic variables (particularly allelic base substitutions that specify genotype). Logistic regression also enabled calculation of odds ratios adjusted for the effects of maternal HBV DNA titer and variation in the genotype or allele.

Analyses were based on the assumption that each mother-infant transmission was an independent event. Hence, variables for mothers with sequential pregnancies were considered separately. No multiple pregnancies were encountered.

Descriptive Account of HBV Transmissions Linked to a Child with Breakthrough Infection

A male infant, X, was born at 40 weeks' gestation in 1990 to a 22-year-old white woman, M, who had been discovered at antenatal evaluation to have detectable HBsAg and HBeAg. X was delivered vaginally and was given HBIg and the first dose of hepatitis B vaccine at different gluteal sites 1 hour after birth. The postnatal course was uneventful, and he received the second and third doses of the vaccine at 1 and 6 months of age. During postvaccination testing at age 1 year, his blood was found to be positive for HBsAg and HBeAg.

In 1992, a 52-year-old woman, A, who was providing daycare for X developed acute hepatitis B; she had no risk factors for HBV infection except contact with X. In 1994, a 6-year-old boy, B, who was a playmate of X in a nursery school facility, developed acute hepatitis B. In 1995, a 3-year-old girl, C, who attended the same nursery school, developed jaundice. Serological investigations confirmed acute hepatitis B. Her family members were screened and found to be seronegative for HBsAg.

Saliva specimens obtained from the rest of X's nursery school contacts and sera drawn from the nursery school supervisors tested negative for antibody to the hepatitis B core antigen [23]. A, B, and C all cleared HBsAg within 6 months of infection. M remained an HBeAg-positive carrier and subsequently gave birth to two other children. They were delivered vaginally at full term, received passive-active immunoprophy-

Table 1. Characteristics of 12 HBsAg-seropositive infants (cases) and 22 HBsAg-seronegative infants (controls).

Characteristic	Study group: Cases (n = 12)	Study group: Controls (n = 22)	P value
Infant			
Mean birth weight, kg (SD)	3.19 (0.46)	3.31 (0.51)	.500*
Median time in hours from delivery to HBIg administration (interquartile range)	4 (1–10)	4.5 (2–12)	.441†
Mother			
Ethnicity (no. of mothers)			
Asian	4	12	
Non-Asian	8	10	.297‡
Mean age, y (SD)	28.3 (5.2)	27.7 (4.7)	.810*
Primigravid? (no. of mothers)			
Yes	3	9	
No	7	10	
Not known	2	3	.449‡
Median titer (genome copies/mL) of circulating HBV (interquartile range)	10^8 (10^6–10^8)	10^7 (10^6–$10^{8.5}$)	.969†
No. of mothers with titer $\geq 10^8$/mL	7	10	.710‡

NOTE. HBIg = hepatitis B immunoglobulin; HBsAg = hepatitis B surface antigen; HBV = hepatitis B virus.
* Student's t-test.
† Mann-Whitney two-sample test.
‡ Fisher's exact test.

laxis, and were HBsAg-negative when tested at age 1 year. Both were included as controls in the case-control study. X is one of the cases in the case-control study.

Results

Case-Control Study

Of 1,128 infants born to HBsAg-positive mothers during the study period, 786 were born to mothers who were also HBeAg-positive. Of these, 568 were delivered vaginally at or after 36 weeks' gestation, 552 of whom were given HBIg within 48 hours after birth; 354 of the 552 received all 3 doses of vaccine. Postvaccination serological test records were available for 232 infants at 1 year of age and showed 16 (6.9%) to be HBsAg-positive.

Antenatal sera were available from mothers of 14 of the 16 HBsAg-positive infants. DNA sequencing of the segment amplified from the surface gene of 14 mother-infant serum pairs revealed sequence mismatches in two pairs. Data derived from these two latter pairs, whom we identified to be possibly infected by vaccine-escape HBV mutants [24], were excluded from further analysis. Only infants (n = 12) whose sera yielded HBV DNA sequences matching those of the corresponding mothers were included as cases in this study.

Differences between cases and controls with regard to the characteristics of infants and mothers, levels of maternal circulating HBV, and number of mothers with an HBV titer (genome copies) $\geq 10^8$/mL were not significant (table 1). The difference between the two groups in the distribution of maternal HBV genotypes was of interest only when type C was compared with other types. Table 2 shows that infants whose mothers were infected by non-C (A, B, D, and E) genotypes were at

Table 2. Univariate analysis: genotypic and allelic changes in maternal hepatitis B virus (HBV) as risk factors for HBV infection in infants.

Characteristic	No. of infants with characteristic: Cases	Controls	P value*	OR (95% CI)
Genotype				
Non-C†	10	11		
C	2	11	.075	4.77 (0.75–54.8)
Allelic substitutions				
C^{158}	12	14		
T^{158}	0	8	.030	8.49 (1.14–∞)
A^{328} or C^{479}	11	11		
C^{328} or G^{479}	1	11	.024	10.32 (1.14–515)
G^{365}	9	8		
A^{365}	3	14	.071	4.91 (0.9–37.2)
C^{377}	11	13		
T^{377}	1	9	.061	7.23 (0.78–362)

* Fisher's exact test.
† Cases: genotypes B (n = 3), D (n = 6), and E (n = 1). Controls: genotypes A (n = 1), B (n = 2), and D (n = 8).

higher risk of infection than those whose mothers carried genotype C, although this did not reach the 5% significance level.

Comparison of DNA sequences in the surface gene amplicons derived from mothers of cases and controls did not reveal base changes that were unique to mothers of the cases. Base substitutions at allelic sites were then examined. Substitutions at the following nonneutral allelic positions were compared: 136, 139, 146, 158, 167, 170, 176, 191, 203, 254, 328, 337, 365, 377, 401, 427, 476, 479, 482, and 503. Those at the following neutral allelic sites were also compared: 140, 192, 213, 246, 300, 354, 366, 420, 438, 444, and 513.

Substitutions occurring at five of these 31 sites correlated with neonatal HBV infection. Table 2 shows that the following allelic substitutions in the surface gene had the greatest association with neonatal infection: C^{158}, A^{328}, and A^{479}. In addition, G^{365} and C^{377} also showed some association with infection, although not at the 5% significance level. The allelic base changes were highly correlated, particularly those at positions 328 and 479.

The results of multivariable analyses are given in table 3. A high titer of maternal HBV DNA (i.e., $\geqslant 10^8$ genome equivalents/mL) was significantly associated with neonatal infection independently of G^{365} but not independently of the non-C genotype or of C^{158}, A^{328}, C^{377}, or A^{479}. However, four of the five allelic changes (C^{158}, A^{328}, G^{365}, and A^{479}) were significantly associated with infection independently of titer.

Molecular Epidemiology of HBV Transmission Linked to Breakthrough Infection

Identical banding patterns (figure 1) were produced in the SSCP assay procedure by amplicons derived from the surface and core genes of HBV in serum from X's mother (M) at the time of pregnancy, serum from X at age 1 year, and serum from the three contacts (A, B, and C) during acute hepatitis, as well as saliva from X collected during the investigation into C's hepatitis. Note that the study specimens had been collected over 6 years. Figure 1 also shows that sera from two unrelated cases of acute hepatitis B and the control plasmid yielded different banding patterns. DNA sequencing confirmed the sequence identity of HBV in all the serum samples and in the saliva of X. Sequencing also revealed that the HBV variant common to these individuals was distinct from HBV infecting the other 10 cases (data not shown).

The HBV variant carried by M, X, A, B, and C has the following base changes: C^{158}, A^{328}, G^{365}, and A^{479}. The circulating HBV titer in M when pregnant with X was 10^8 genome equivalents/mL. The titers during her pregnancy with the two other children were 10^6 and 10^2 genome equivalents/mL. There were no changes in the sequence of HBV characterized during the later pregnancies.

Table 3. Multivariate analysis: maternal hepatitis B virus (HBV) titer and genotypic and allelic changes as risk factors for HBV infection in infants.

Covariates	*P* value	OR (95% CI)
Titer plus genotype		
Titer of $\geqslant 10^8$ genome equivalents/mL	.115	4.72 (0.62–63.2)
Non-C	.041	9.98 (1.1–173)
Titer plus base change at position 365		
Titer of $\geqslant 10^8$ genome equivalents/mL	.027	8.33 (1.05–∞)
G^{365}	.003	15.8 (2–∞)
Titer plus base change at position 328 or 479		
Titer of $\geqslant 10^8$ genome equivalents/mL	.103	5.25 (0.67–70.6)
A^{328} or A^{479}	.005	20.6 (1.71–1,296)
Titer plus base change at position 158		
Titer of $\geqslant 10^8$ genome equivalents/mL	.257	3.32 (0.53–24.4)
C^{158}	.017	12 (1.49–∞)
Titer plus base change at position 377		
Titer of $\geqslant 10^8$ genome equivalents/mL	.345	2.83 (0.47–20.6)
C^{377}	.059	9.78 (0.94–542)

Discussion

The failure rate of passive-active immunoprophylaxis in infants of HBeAg-positive mothers, as measured by the HBsAg carriage rate at ~1 year of age, is low, ranging from zero to 14% [16, 25–27]. This variation reflects several factors: ethnic background of the study subjects, total number of HBIg injections given (ranging from 1 to 7), HBIg dosage (as fixed doses or calculated according to body weight), anti-HBsAg content of HBIg (varying according to the antibody levels prevailing in plasma donors), and vaccine type (plasma- or yeast-derived), dose (ranging from 2 mg to 30 mg of HBsAg), and schedule (3- or 4-dose, with varying intervals between each dose).

Few studies have considered, in addition, variation due to obstetric factors. We excluded this consideration by restricting the study to infants who were delivered full-term and without obstetric intervention: those born to mothers who underwent gestational complications that could have led to transplacental materno-fetal hemorrhage (e.g., threatened abortion and preterm and prolonged labor [28]) were excluded. The 6.9% HBsAg carriage rate we observed consequently represents an infection rate in which the effect of variation in peripartum materno-fetal hemorrhage is minimized. Since the denominator (232) for calculating the carriage rate at 12 months of age comprises infants who received high-dose HBIg and the full course of vaccine, the rate reflects infection acquired in the absence of antenatal and peripartum complications and despite optimal immunoprophylaxis. Under such favorable circumstances, the observed rate of breakthrough infection is 5.8%, disregarding the two cases of infection by putative vaccine-escape mutants.

In the case-control part of this study, univariable analysis did not show high-level viremia to be a significant risk factor, but multivariable analysis with certain allelic changes, particularly G^{365}, revealed that high-level viremia was associated with

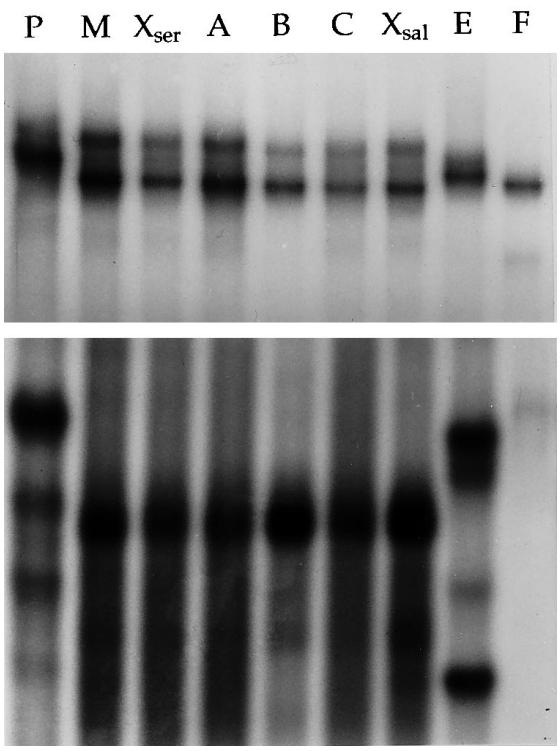

Figure 1. Evidence of horizontal hepatitis B virus (HBV) infection subsequent to vertical breakthrough infection. Single-strand conformation polymorphism (SSCP) banding patterns of HBV DNA fragments amplified from the surface gene (*top panel*) and the core gene (*bottom panel*) are shown. *P* = pHBV130; *M* = serum of mother, M, taken when pregnant in 1990 with X; X_{ser} = serum of index case, X, at 1 year of age (1991); *A*, *B*, and *C* = serum of infected contacts A, B, and C, taken in 1992, 1994, and 1995, respectively; X_{sal} = saliva of X, obtained in 1995; and *E* and *F* = serum from unrelated patients with acute hepatitis B.

an independent increase in risk. In this respect, the finding agrees with data from previous studies [12–14]. The independent effect of viremia is further exemplified in the case of the mother (M) of the source (X) of the HBV transmission series studied in detail in this report: while the sequence of HBV carried by M remained identical throughout the three pregnancies, it was only during her pregnancy with X, when the level of viremia was high, that breakthrough infection occurred.

However, our study identifies allelic substitutions at positions 158, 328, 365, 377, 479, and 2,064 as additional risk factors. Thus, when viremia was included as a predictor in multivariable analysis, the base changes at positions 158, 328, 365, and 479 were seen to increase in significance and gave higher estimated odds ratios. Owing to the small size of this study and the many variables examined, it was not possible to show which of the identified risk factors would be the best predictor of breakthrough infection or whether a combination of factors would lead to an increased risk.

There are several limitations to this case-control study. One is selection bias, because only 40% (232) of the 568 full-term infants born without obstetric intervention to HBeAg-positive mothers during the study period served as the denominator for estimating the HBsAg carriage rate. In the United Kingdom, the failure to vaccinate at-risk infants against HBV completely and to fully achieve postvaccination testing is a recognized problem [10]. Another limitation arises from the restricted availability of mother-infant serum pairs for use as controls. Several reporting laboratories were not able to provide archived HBsAg-positive sera for our study. The small size of the control group, as well as that of the case group, accounts for the relatively wide confidence intervals observed in the odds ratios.

The DNA sequence of the maternal viral genome was not examined in its entirety. Consequently, the possibility that residues other than the four identified here, particularly in non-C genotypes, are associated with increased risk could not be excluded. However, that the four changes in the surface gene are all missense mutations may be significant. In early studies, investigators using synthetic peptides located the immunodominant *a* antigen, against which neutralizing antibodies are raised, as residing between codons 138 to 149 of HBsAg [19, 20]. Later studies, with use of mutagenesis [29] and amino acid replacement assays of immunizing oligopeptides [30], identified key antigenic residues at a disulphide loop stretching from codons 140 to 146.

The missense mutations at positions 158, 328, 377, and 479 affect codons 53, 110, 122, and 160, respectively, and would appear to lie outside the *a* determinant. However, one study located *a*-specifying epitopes within a wider domain of the HBsAg polypeptide, extending back to codon 110 [18]. In more recent studies, phage display technology revealed the presence of continuous HBsAg epitopes between codons 115 and 129 [31], 117 and 122 [32], and 121 and 124 [33], as well as discontinuous epitopes between codons 101 and 207 [33]. Hence three of the four missense mutations (at positions 328, 377, and 479) lie within regions that specify HBsAg epitopes.

The extent to which the newly characterized epitopes contribute to the antigenicity of the *a* determinant is not clear; they may play accessory roles in eliciting antibodies with specificities and affinities different from those elicited by the more immunodominant epitopes of the *a* determinant. The base changes in codon 122 are particularly significant. In this codon, G^{365} specifies arginine while A^{365} specifies lysine, and these are the key determinants of the *y* and *d* subtype, respectively [22].

It is interesting to note that Lelie et al. [34] observed subtype *y* occurring more commonly than *d* in their series of infants with breakthrough infection. It is possible that the mutation affecting this codon, and perhaps those involving codons 110 and 160, interferes with the suppression of perinatally transmitted HBV by HBIg [35, 36] and by vaccine-induced antibody [37]. The chances of failure to clear the virus may be enhanced when the exposure dose is high. The significance of the mutation at position 158, affecting codon 53, is unknown.

We did not attempt to detect HBsAg serially in infants during their first year of life. Other groups that had done so reported

HBsAg positivity at or within 3 months of birth in some of their cases [4–6, 13], pointing to intrauterine infection. Infants found to be HBsAg-positive between 6 and 9 months of age have also been regarded to be infected in utero, because the vast majority of infants not receiving prophylaxis become positive in this period and not later [6, 35, 38]. The retrospective nature of our study does not allow intrauterine and perinatal infection to be distinguished. Thus, the possibility cannot be excluded that the high-risk mutations identified are associated with the propensity for HBV variants bearing these mutations (or other base changes that co-segregate with these mutations) to be transmitted in utero.

A factor not considered in this study is nonresponse or poor response of the infant to vaccination, thereby predisposing to postnatal infection. However, positive HBsAg detection at 1 year of age seldom reflects postnatal infection [38]. Furthermore, a Dutch study examining the role of nonresponse to hepatitis B vaccine in infants born to HBeAg-positive mothers found that the HLA haplotype regarded to be strongly associated with nonresponse in adults, HLA-B8-DR3, was not represented in any of the infants in the infected nonresponder group [14].

The findings in our study of serial HBV transmission exemplify how, in a community where the level of herd immunity is low, unimmunized contacts continue to be susceptible to infection by HBV carriers. The public health impact of HBV transmission from individuals who become carriers as a result of failed immunoprophylaxis should be much reduced if universal vaccination policies are carried out [39, 40], as substantial boosting of the level of herd immunity is then achieved. The implementation of wide vaccination would have to be accompanied by continued efforts to identify carrier mothers and to provide follow-up testing of their offspring.

The problem of preventing chronic HBV infection in the infants remains. It is therefore important to determine whether HBeAg-positive pregnant women who carry the virus at a high titer and have the high-risk genetic characteristics identified here are more likely to give birth to infected infants who may not eliminate the virus despite immunoprophylaxis. To effect this, procedures for quantifying viremia and for the identification of specific high-risk nucleotide changes in the HBV genome in maternal sera could be developed and applied. Different management strategies could then be implemented to lessen the risk that the infant would become a carrier. Possible measures are preconceptual antiviral chemotherapy to suppress maternal viremia, administration of HBIg into the fetus by cordocentesis to prevent intrauterine infection [41], and cesarean section to prevent perinatal transmission [42].

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