RESEARCH PAPER

Human Vaccines & Immunotherapeutics 10:8, 2336–2341; August 2014; © 2014 Landes Bioscience

Absence of occult hepatitis B virus infection in sera of diabetic children and adolescents following hepatitis B vaccination

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Keywords: Children, Egypt, HBV, IDDM, occult HBV infection, vaccination

Abbreviations: HB, hepatitis B; IDDM, insulin-dependent diabetes mellitus; anti-HBs, antibody to hepatitis B surface antigen; HBsAg, hepatitis B surface antigen; anti-HBc, antibody to hepatitis B core antigen; DM, diabetes mellitus; HbA1c, glycated hemoglobin; HBV, hepatitis B virus; OBI, occult hepatitis B infection

Background: The prevalence of occult hepatitis B (HB) infection (OBI) in HB-vaccinated diabetic children has not yet been tested. Here, we aimed to determine the prevalence of OBI among HB-vaccinated children and adolescents with insulin-dependent diabetes mellitus (IDDM).

Results: Eighty-seven (51.2%) children had a titer for antibodies to HB surface antigen (anti-HBs) of <10 IU/L. These included 44 (70%) IDDM children and 43 (40.2%) healthy children. Eighty-three (48.8%) children had an anti-HBs titer of ≥10 IU/L; they included 19 (30%) with IDDM and 64 (59.8%) healthy children. None of the enrolled children (n = 170) were reactive for total antibody to HB core antigen (anti-HBc) as determined by enzyme-linked immunosorbent assay. HB virus DNA was not detected in HB-vaccinated IDDM or healthy children and adolescents.

Method: An amount of 170 HBsAg-negative sera samples from HB-vaccinated children and adolescents was included. They were classified into the IDDM group (n = 63) and the healthy control group (n = 107). HBsAg, anti-HBc, and anti-HBs were tested by enzyme-linked immunosorbent assay, and HB virus DNA was tested by nested polymerase chain reaction using 3 pairs of surface, core, and X genes.

In Conclusion: Primary HB vaccination confers long-term protection against OBI in Egyptian diabetic children and adolescents. However, the number of cases tested in this study was relatively low, and further studies and long-term follow-up of large populations are needed to draw solid and convincing conclusions.

Introduction

Hepatitis B virus (HBV) is a global health problem that causes chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma.[1] Egypt is moderately endemic for HB infection, where the prevalence of HB surface antigen (HBsAg) is estimated to be 6.7% among the general population.[2] A significant reduction of HBsAg was recently reported in the Egyptian general population, which was largely attributed to a successful vaccination program.[3]

Occult HBV infection (OBI) is defined as the persistence of viral genomes in the liver tissue or serum of individuals who are HBsAg negative.[4] The occult status of infection in vaccinated children may be a result of host immunosuppression, genetic mutation of the S gene, transmission to children from occult-infected or HBsAg-positive mothers, or other host factors.[5-7] OBI may persist in individuals for years without obvious symptoms of HBV infection,[8] or it may progress to hepatitis, liver cirrhosis, and/or hepatocellular carcinoma.[7]

The incidence of OBI in HB-vaccinated children varies in groups with different risk factors and according to geographic location in concordance with the local incidence of HBV, irrespective of antibody to HBsAg (anti-HBs) serostatus. In Taiwan, the prevalence of OBI was 4.6% in anti-HBs–negative HB-vaccinated healthy children.[9] In addition, OBI was detected in 3.2% and 28% of anti-HBs–positive HB-vaccinated infants born to HBsAg-positive mothers in China and Iran, respectively.[10,11]

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Submitted: 04/24/2014; Revised: 05/30/2014; Accepted: 06/08/2014; Published Online: 06/19/2014
http://dx.doi.org/10.4161/hv.29521

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Table 1. Basic characteristics of children and adolescents with anti-HBs ≥ 10 IU/L

Parameters	Group I (n = 64)	Group II (n = 19)	X^2	*t*	*Mann–Whitney*	*P*
Age (Mean ± SD)	10.71 ± 1.36	9 ± 3.73		1.963		0.064
Sex Males Females	33 (51.60%) 31 (48.40%)	9 (47.40%) 10 (52.60%)	1.995			0.158
HBsAg	Negative	Negative				
Anti-HBc "Total"	Negative	Negative				
Anti-HBs Median (Minimum–Maximum)	52.4 (10.00–886.03)	47.8 (11.53–248.23)			523.5	0.36
HBV DNA by PCR	Negative	Negative				
Duration of IDDM	———–	2 ± 0.66 y				
HbA1c (Mean ± SD)	————	6.89 ± 1.78				

SD, standard deviation; HBsAg, hepatitis B surface antigen; Anti-HBc, antibody to hepatitis B core antigen; Anti-HBs, antibody to hepatitis B surface antigen; HBV, hepatitis B virus; DNA, deoxyribonucleic acid; PCR, polymerase chain reaction; IDDM, insulin-dependent diabetes mellitus; HbA1c, glycated hemoglobin.

OBI is more common in diabetic patients than in healthy control patients; it was detected in 11% of diabetic patients vs. 3% of healthy controls.[12] It is suggested that diabetic patients, due to their immune incompetence, respond poorly to various vaccines. The significant waning of anti-HBs titers in HB-vaccinated children and adolescents with insulin-dependent diabetes mellitus (IDDM) was demonstrated recently[13]; additionally, IDDM was associated with HB vaccine failure in children.[14] These data have fueled a debate regarding the efficacy and need for booster doses in HB-vaccinated IDDM children and adolescents. To our knowledge, OBI has not yet been tested in HB-vaccinated IDDM children. Here, we investigated the prevalence of OBI among Egyptian IDDM children with variable anti-HBs serostatus at 10 y after HB vaccination.

Results

Demographics

Among 170 HBsAg-negative children, the ages ranged from 4 to 17 y (mean 10.6 ± 2.1 y). Thirty-nine children (22.8%) and 131 children (77.2%) were <10-y-old and ≥10-y-old, respectively. Eighty-one were boys and 89 were girls. The healthy control group included 107 children with a mean age of 10.86 ± 1.21 y; 9 (8.4%) were <10-y-old and 98 (91.5%) were ≥10-y-old. Forty-nine (45.8%) were boys and 58 (54.2%) were girls. The IDDM group included 63 children with a mean age of 10.29 ± 3.04 y; among them, 4 (6.3%), 26 (41.2%), and 33 (52.5%) were <5-y-old, between 5- and 10-y-old, and ≥10-y-old, respectively. Thirty-two (50.8%) were boys and 31 (49.2%) were girls. There were no significant differences between the healthy and the diabetic children with respect to age or sex ($P > 0.05$).

The mean duration of IDDM in the diabetic children was 1.73 ± 1.96 y (range, 0.1–9 y). The mean glycated hemoglobin level (HbA1c) was 6.78% ± 1.37%.

Anti-HBs

Children with anti-HBs ≥ 10 IU/L

In the current study, 83 children (64 healthy and 19 IDDM) had an anti-HBs titer of ≥10 IU/L (**Table 1**). The mean age of the healthy children was 10.71 ± 1.36 y and the mean age of the IDDM children was 9 ± 3.73 y ($P > 0.05$). The healthy group included 33 (51.60%) males and 31 (48.40%) females, and the IDDM group included 9 (47.40%) males and 10 (52.60%) females. The mean anti-HBs titers were 104.86 ± 153.77 IU/L and 64.93 ± 66.65 IU/L among children in the healthy and IDDM groups, respectively. Seventeen (26.60%) HB-vaccinated healthy children had an anti-HBs titer of >100 IU/L; in contrast, only 3 (15.80%) HB-vaccinated IDDM children and adolescents had an anti-HBs titer of >100 IU/L.

Among those IDDM children with anti-HBs ≥ 10 IU/L, the mean duration of IDDM was 2 ± 0.66 y and the mean HbA1c was 6.89% ± 1.78%.

Children with anti-HBs < 10 IU/L

Eighty-seven children had an anti-HBs titer of <10 IU/L. These included 43 healthy children and 44 IDDM children (**Table 2**). The mean age of the healthy children was 10.84 ± 2.54 y and the mean age of the IDDM children was 11.07 ± 0.91 y ($P > 0.05$). The healthy group included 16 (37.21%) males and 27 (62.79%) females, and the IDDM group included 23 (52.27%) males and 21 (47.73%) females. The mean anti-HBs titers were 1.93 ± 3.17 IU/L and 2.46 ± 2.44 IU/L among healthy and IDDM children, respectively ($P = 0.38$).

Among the IDDM children with anti-HBs titer of <10 IU/L, the mean duration of IDDM was 1.87 ± 2.2 y and the mean HbA1c was 6.73% ± 1.17%.

Antibody to hepatitis B core antigen status

None of the enrolled children (n = 170) was reactive for total antibody to hepatitis B core antigen (anti-HBc), as determined by enzyme-linked immunosorbent assay.

Serum HBV DNA by nested polymerase chain reaction

HBV DNA was not detected in any children of both healthy and IDDM groups (n = 170) by 3 different polymerase chain reaction (PCR) assays for the surface, core, and X genes.

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Table 2. Basic characteristics of children and adolescents with anti-HBs < 10 IU/L

Parameters	Group I (n = 43)	Group II (n = 44)	X^2	*t*	*Mann–Whitney*	*P*
Age (Mean ± SD)	10.84 ± 2.54	11.07 ± 0.91		0.561		0.577
Sex Males Females	16 (37.21%) 27 (62.79%)	23 (52.27%) 21 (47.73%)	1.995			0.158
HBsAg	Negative	Negative				
Anti-HBc "Total"	Negative	Negative				
Anti-HBs Median (Minimum–Maximum)	2 (0–9.75)	0 (0–9.25)			705.0	0.03*
HBV DNA by PCR	Negative	Negative				
Duration of IDDM	———–	1.87 ± 2.2 y				
HbA1c (Mean ± SD)	————	6.73 ± 1.17				

SD, standard deviation; HBsAg, hepatitis B surface antigen; Anti-HBc, antibody to hepatitis B core antigen; Anti-HBs, antibody to hepatitis B surface antigen; HBV, hepatitis B virus; DNA, deoxyribonucleic acid; PCR, polymerase chain reaction; IDDM, insulin-dependent diabetes mellitus; HbA1c, glycated hemoglobin. *Significant difference.

Discussion

The incidence of OBI breakthrough in HB-vaccinated children and adolescents varies in groups with different risk factors from different countries, irrespective of anti-HBs serostatus. OBI was reported in 4.6% of HB-vaccinated anti-HBs–negative healthy children in Taiwan.[9] It was also reported in HB-vaccinated anti-HBs–positive children born to HBsAg-positive mothers in China (3.2%)[10] and Iran (28%).[11] In the current study, OBI was not detected in IDDM children and adolescents, irrespective of anti-HBs serostatus. These discrepancies in the incidence of OBI may reflect differences in endemicity of HBV, maternal HBV serostatus, and use of measures to prevent vertical transmission; technical variations in immunization use and efficacy; variations in adherence to immunization protocols; and different time points of evaluation.

The current results demonstrated that OBI was not detected in HB-vaccinated IDDM children with either anti-HBs–positive or –negative serostatus. It is generally believed that an anti-HBs titer ≥ 10 IU/L is protective against HBV infection. However, in another study, OBI was detected in 6 (3.2%) infants with anti-HBs of ≥10 IU/L[10]; all of them had anti-HBs titers of ≤100 IU/L, suggesting that the neutralizing capacity of low anti-HBs titers is limited. In fact, these infants were born to HBsAg-positive mothers and in an area with high HBV endemicity.[10] Although HB vaccines induce protective anti-HBs after a primary series of immunizations, this is followed by a gradual decline in antibody titer.[15] Vaccinated children who had low or undetectable anti-HBs titers were reported to exhibit a robust anamnestic response up to 10 y, and not one of them had become a carrier 5 to 10 y after immunization.[16,17] This is further supported by the absence of OBI detected in vaccinated children with anti-HBs of <10 IU/L in our study. Notably, the children included (n = 170) in the current study had no history of exposure to risk factors of HBV infection, including previous surgery, blood transfusion, or household exposure. This was further confirmed by the undetectable anti-HBc in all children. Taken together, these data suggest that the occurrence of OBI in HB-vaccinated children may be largely related to HBV endemicity and the mother's HB serostatus, rather than the presence of IDDM or HB serostatus.

Our findings infer that the absence of detectable protective anti-HBs in vaccinated IDDM children and adolescents does not necessarily denote the absence of protection against OBI. Indeed, T-cell memory of HBsAg can be demonstrated by lymphocyte proliferation many years after HB vaccination, even in the majority of persons with anti-HBs titers of <10 IU/L.[18] Although it was shown that impairment of polymorphonuclear phagocytosis and reduction in granulocyte phagocytic capacity in IDDM persons with inadequate metabolic control lead to an increase in susceptibility to infection, these abnormalities are reversible after insulin therapy.[19-24] In previous studies, increased proliferation capacity of IDDM T-lymphocytes[25] and a tendency toward increased tumor necrosis factor-α and interleukin-6,[26] as well as increased levels of interferon-γ levels,[27] after metabolic control were reported. Ten years after HB vaccination, 70% of our IDDM patients had anti-HBs levels of <10 IU/L. While some IDDM patients may never develop protective antibodies after vaccination, some individuals with a history of vaccination who do not show detectable antibody may have lost their antibody titer over time. However, given the limitations of the available data, we were unable to determine who had lost antibody titer over time and those who had never developed protective antibodies. Despite this limitation, our data show that OBI was not detected in any of these patients. Given the good metabolic control (mean HbA1c, 6.78% ± 1.37%) in those children, these data suggest that diabetic control improves the capacity for activation and maintenance of the immune response, reducing susceptibility to infection, and support that the absence of OBI in our patients may be related to adequate metabolic control and a continuing ability to protect against HB in response to previous HB vaccination. The fact that all IDDM children and adolescents were vaccinated before discovery of DM in the current study allows us to speculate that anti-HBs immunity and OBI susceptibility may differ in children and adolescents vaccinated after the discovery of DM. One study showed that the anti-HBs seropositivity in Egyptian

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Table 3. Sequences of primer pairs used for PCR to detect HBV genome

Gene and primer [a]	Sequence (5′ 3′)	Nucleotide position
Surface gene		
P1s	TCACCATATT CTTGGGAACA AGA	2823-2845
S1–2a	CGAACCACTG AACAAATGGC	685-704
Core gene		
C1s	CTGGGAGGAG TTGGGGGA	1730-1747
C2a	GTAGAAGAAT AAAGCCC	2503-2487
C3s	GGTCTTTGTA CTCGGAGGCT G	1763-1783
C4a	ATACTAACAT TGACATTCCC	2455-2436
X gene		
X1s	CTAGCCGCTT GTTTTGCTCG	1282-1301
X2a	TTATGCCTAC AGCCTCCTAG	1666-1647
X3s	GGTCTTACAT AAGAGGACTC	1518-1537
X4a	GTTCACGGTG GTCTCCAT	1625-1608

[a]s, sense; a, antisense

diabetic children had declined to 30% 10 y after HB vaccination.[13] Clinical observation up to 20 y after neonatal vaccination showed that benign breakthrough of HBV infection occurred without leading to chronicity, as indicated by isolated anti-HBc positivity.[28,29] Vaccine-induced immunity appears to last for at least 15 y in immunocompetent individuals who respond adequately to primary immunization. Therefore, booster doses are not recommended for immunocompetent subjects 15 y after vaccination.[30] Recently we showed that HB-vaccinated Egyptian IDDM children and adolescents were negative to HBsAg at 10 y after vaccination.[13] Furthermore, anti-HBc and HBV DNA were not detected in the same cohort of diabetic children and adolescents in the current study. Taken together, one can hypothesize that the long-term efficacy of HB vaccination in diabetic adolescents will continue to be associated with a low risk of contracting HBV and a low serum level of neutralizing antibody, even at 10 y after vaccination, and booster vaccinations are not recommended for diabetic adolescents with good metabolic control up to 10 y after vaccination.

The sample size was limited in this study, which constitutes a shortcoming. Studies of large numbers of patients with long-term follow-up are needed for greater clarification of OBI occurrence in HB-vaccinated IDDM children and adolescents.

Conclusion

Primary HB vaccination confers long-term protection against OBI in Egyptian diabetic children and adolescents. However, the number of cases surveyed in this study was relatively small, and further studies and long-term follow-up of large patient populations are needed to make solid and convincing conclusions.

Method

Setting

This study was conducted at Al-Azhar University Hospital, Cairo, Egypt, from April 2013 to January 2014. All procedures were performed in accordance with the ethical standards of Al-Azhar University's committee on human experiments. Informed consent was obtained from all children's parents.

All patients

A total of 170 children who had been routinely vaccinated against HBV were included in the study. The children enrolled in the study were divided into healthy (n = 107) and IDDM (n = 63) cohorts. Any history of previous surgery or blood transfusions, as well as maternal history of HBV infection, was denied for all children (n = 170).

IDDM children

IDDM was diagnosed on the basis of hyperglycemia and absolute insulin dependence. All diabetic children were receiving human insulin through 2, 3, or 4 injections daily. All patients were compliant with insulin therapy. Any diabetic patients with associated comorbidities or who were receiving immunosuppressive drugs were excluded from participation in the study.

HB vaccination

Three doses of recombinant vaccine had been administered to all participating children (n = 170) at 2, 4, and 6 mo of age between 1996 and 2009 according to the vaccine schedule of the Egyptian Ministry of Health and Population. All included children had completed their HB vaccination schedule at least 3 y before the study began. All diabetic children had been vaccinated before the discovery of diabetes. A vaccination history was obtained from all parents.

Blood sampling

Five milliliters of venous peripheral blood were obtained from all children. Blood samples were centrifuged, and the serum samples were immediately stored at –21 °C. For HbA1c testing, an additional 1.5 mL of whole blood was collected from the IDDM children and placed into tubes with EDTA.

Serology

HBsAg (Murex Biotech Limited, 9F80–01/05), total anti-HBc (DiaSorin, N0137), and anti-HBs (DiaSorin, Poo1603) were measured by enzyme-linked immunosorbent assay according to the manufacturers' instructions.

HBV DNA by nested PCR

Serum samples were assayed for HBV DNA through the use of 3 different PCR assays with primer pairs whose sequences were taken from the surface, core, and X genes of the viral genome (**Table 3**).

HBV DNA extraction

Briefly, 200 µL of serum from each sample were used to extract HBV DNA in viral particles using a *QIAamp MinElute Virus Spin kit* (QIAGEN, USA, 2010), according to the manufacturer's instructions.

Detection of HBV surface gene

The reaction mixture contained 5 µL of extracted DNA in 25 µL of 1 × PCR buffer containing 1.5 mM $MgCl_2$, 1 pmol of

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each primer, 200 μmol/L of each deoxynucleotide, and 1 U of *Taq* DNA polymerase (*Taq* PCR, QIAGEN, 2010); the solution was completed with the addition of sterile distilled water to 25 μL. The samples were incubated at 95 °C for 10 min, followed by 40 amplification cycles at 94 °C for 20 s (denaturation), 55 °C for 20 s (annealing), and 72 °C for 1 min (extension); this was followed by a further extension at 72 °C for 10 min. The sensitivity of the PCR assay reached 100 copies of HBV DNA per specimen.[31]

Detection of HBV core and X genes

For the first round of PCR, the DNA in 25 μL of a reaction mixture containing 5 μL of the DNA sample, 1 × *Taq* buffer, 1.5 mM $MgCl_2$, 200 μmol/L of each deoxynucleotide, 1 pmol of each outer primer pair, and 1 U of *Taq* DNA polymerase (*Taq* PCR, QIAGEN, 2010) was amplified in a thermal cycler for 30 cycles. Each cycle entailed denaturation at 95 °C for 60 s, primer annealing at 55 °C for 30 s, and extension at 72 °C for 60 s with a final extension step at 72 °C for 7 min. After the first amplification, 2 μL of the PCR products was reamplified for another 30 cycles with 100 ng of each inner primer pair. The second round of PCR was done in the same manner as the first round. The sensitivity of the PCR assay reached 10 copies of HBV DNA per specimen.[32]

The amplified products were separated by electrophoresis in a 1.5% agarose gel containing 1.5 μL ethidium bromide for staining and were visualized with an UV trans-illuminator. The sensitivities of the PCR assay reached 10 copies of HBV DNA per specimen.

Statistical analysis of the data

SPSS version 17 was used for analysis. Differences in frequency between groups were compared with the chi-square test or the Fisher exact test. A P value < 0.05 was considered significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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