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Hepatitis B Virus Vaccine–Induced Cell-Mediated Immunity Correlates with Humoral Immune Response following Primary Vaccination during Infancy

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ABSTRACT

Data on hepatitis B virus (HBV) vaccine–induced cell-mediated immunity (CMI) and humoral immune response during infancy is scarce. We assessed HBV vaccine–induced CMI among infants stratified as nonresponders (Ab against HBV surface Ag [anti-HBs] levels <10 IU/l), low-responders (anti-HBs levels 10–100 IU/l), and high-responders (anti-HBs levels ≥100 IU/l) following their primary vaccination against HBV. Moreover, we assessed the association between HBV vaccine–induced CMI and anti-HBs levels. Infants were immunized with HBV vaccine at ages 2, 4, and 6 mo. Hepatitis B surface Ag (HBsAg)-specific proliferation, intracellular cytokine production, and bulk cytokine secretion were assessed on PBMCs collected at 1 y and anti-HBs levels were measured at 1 and 2 y of age. Infants classified at 2 y of age as low-responders (n = 28) had lower median levels of secreted IFN-γ than high-responders (n = 29), 17.15 pg/ml versus 33.16 pg/ml, respectively, p = 0.009. Infants classified at 2 y of age as nonresponders (n = 15) had lower median levels of secreted TNF-α than high-responders (n = 29), 116.11 pg/ml versus 162.27 pg/ml, respectively, p = 0.032. There was a positive correlation between HBsAg-specific secreted IFN-γ levels at 1 y and anti-HBs levels at 1 and 2 y of age, rho = 0.269 and 0.302, respectively, (p = 0.019 and p = 0.01, respectively). There was a positive correlation between anti-HBs levels at age 1 y and the levels of secreted IL-10, rho = 0.297, p = 0.009. HBsAg-specific IFN-γ, IL-10, and TNF-α secretion correlated with HBV vaccine–induced humoral immune response. Assessment of CMI is a useful adjunct in demonstrating the persistence of HBV vaccine–induced memory immune response. ImmunoHorizons, 2017, 1: 42–52.

INTRODUCTION

Infection with hepatitis B virus (HBV) is a global public health concern, with a worldwide estimate of more than two billion individuals infected at some point in their lives (1). In some regions of the world, including sub-Saharan Africa, HBV is hyperendemic (≥8% hepatitis B surface Ag [HBsAg] prevalence) (2). The burden of HBV infection is chiefly driven by infections acquired in the first years of life (3). In southern and eastern sub-Saharan Africa, an increase in chronic hepatitis B infection occurred among young children from 1990 to 2005, reaching a prevalence of 8–9% in children 0–9 y old (3). In North America, although the prevalence...
of HBV infection has declined in the postvaccination era, children 0–4 y of age still have HBsAg positivity rates of nearly 2% in 2005, representing the highest age-specific positivity rate (3). Infections acquired during the first 5 y of life carry a high risk (nearly 30%) of progression to chronic disease (3, 4). Among immigrants to North America, the prevalence of chronic HBV infection is ~5% and 40% of them develop advanced liver disease (5). In Canada, nearly 300,000 individuals who were born abroad have chronic HBV infection. The socioeconomic burden of this condition among this population is significant (5).

Although a decline in the prevalence of HBV infection following expanded HBV vaccination has been established (3), breakthrough infections have been reported after primary vaccination against HBV (6–9).

Measurement of the humoral immune response (Ab against HBV surface Ag [anti-HBs] Ab levels) is currently the most commonly employed immune marker that correlates with protection from HBV infection. It is generally accepted that anti-HBs levels ≥10 IU/l are considered protective against HBV infection (10–12). In the vaccinated population, up to 10% of individuals only achieve anti-HBs levels of <10 IU/l despite full vaccination (termed “nonresponders”) (1). However, some data suggest that many individuals whose levels are <10 IU/l are still protected against HBV infection (13, 14). On the other hand, there is no absolute protection against HBV infection at any anti-HBs levels (11). Cases of HBV infection occur in healthy individuals with anti-HBs levels between 10 and 100 IU/l (termed “low-responders”) (11, 15, 16) and those with anti-HBs levels ≥100 IU/l (termed “high-responders”) (11).

The finding that protection from HBV infection in subjects with anti-HBs levels <10 IU/l (13, 14) and the potential for infection with HBV in persons whose anti-HBs levels are >10 IU/l (11, 15, 16) indicates that the full mechanisms of vaccine-induced protection are yet to be determined.

Cell-mediated immunity (CMI) may correlate with humoral immune response to HBV vaccination. To date, studies that have attempted to elucidate the biological mechanisms driving the humoral response to the HBV vaccine have been conducted mainly in adult populations and have thus far yielded inconsistent results. Sufficient data on these questions in infant cohorts is currently lacking.

Adult studies, inherently limited by the necessity of including a heterogeneous population of postprimary and postbooster vaccinated subjects, reported a correlation between anti-HBs levels and T cell proliferation (17–19). Other studies reported a lack of T cell proliferative responses to HBsAg among nonresponders to HBV vaccine booster dose (19–21). Although one study reported that infant nonresponders to primary vaccination secreted less IFN-γ than responders (22), the correlation between anti-HBs levels and IFN-γ production remained inconclusive, as this finding was confirmed in some (23) but refuted in other studies (14, 24, 25). In one study in infants, nonresponders to primary vaccination against HBV secreted less IL-10 than responders (22). Similar results have been observed in adult populations (23, 26, 27). Last, TNF-α was found to be produced at significantly lower levels in adult nonresponders as compared with responders following in vitro stimulation with HBsAg (23).

To the best of our knowledge, systematic dissection of CMI measurements and the relationship between CMI and humoral immunity in infants after primary vaccination against HBV is lacking. Thus, the purpose of the current study was to assess HBV vaccine–induced CMI (HBsAg-specific proliferation, intracellular cytokine production, and bulk cytokine secretion) among healthy infants stratified as nonresponders, low-responders, and high-responders based on anti-HBs Ab levels achieved following their primary HBV vaccination. Moreover, we sought to ascertain if there is an association between the levels of different functional measures of HBV vaccine–and-induced CMI and the magnitude of HBV vaccine–induced humoral response (anti-HBs Ab levels).

**MATERIALS AND METHODS**

**Prospective birth cohort study design**

**Study settings, eligibility, and follow-up.** Healthy full-term newborns born at the British Columbia Women’s Hospital were assessed for eligibility during the years 2005–2008. The purpose of the cohort was to study the ontogeny of the immune system during the first 2 y of life (28). As such, study participants were recruited at birth and prospectively followed and enrolled further at 1 and 2 y of age.

Inclusion criteria were full-term healthy newborns born after elective caesarean sections without labor. It was previously shown that the mode of delivery (unlabored, elective caesarean section versus labored normal spontaneous vaginal) has significant impact on the immune status (29). Thus, the current study included only infants born after elective caesarean sections as detailed in the original study (28). All the participants in the study were considered healthy based on history-driven health assessments. Exclusion criteria have been described in a previous publication (28).

**Vaccination.** Participants were vaccinated with a pediatric formulation of the Recombivax HB vaccine (Merck) i.m. at 2, 4, and 6 mo of age. Each 0.5 ml dose contained 5 μg of recombinant HBsAg (adw subtype) with amorphous aluminum hydroxyphosphate sulfate (alum) adjuvant. Concomitantly with Recombivax HB vaccine, infants also received diphtheria, tetanus, acellular pertussis-inactivated polio-Haemophilus influenzae type b (DTaP-IPV-Hib) vaccine at 2, 4, and 6 mo of age, seven-valent pneumococcal conjugate vaccine at 2 and 4 mo of age, and meningococcal C conjugate at 2 mo of age, in accordance with the British Columbia childhood immunization schedule. The immunization history of each subject was recorded and updated at each visit.

**Timing of blood collection.** Peripheral venous blood was drawn prior to any vaccinations received on the same day at 1 and 2 y of age.

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Definition of response to primary HBV vaccination. The standard classification defines nonresponders, low-responders, and higher responders based on anti-HBs levels measured 1 or 2 mo after primary immunization. In this study, participants at year 1 and 2 with anti-HBs levels <10, 10–100 and ≥100 IU/l were considered as nonresponders, low-responders, and high-responders, respectively (10–12, 30–33).

Consent obtainment and ethics statement. During initial recruitment, parents were contacted prior to giving birth at the British Columbia Women’s Hospital and written informed consent was obtained from both parents. Prior to postnatal blood draws at year 1 and year 2, written informed consent was reobtained from the parent or legal guardian. The study was approved by the Institutional Ethics Review Board at the University of British Columbia (Certificate # H06-03339) and the University of Washington.

Blood sample processing

Seven to ten milliliters of venous peripheral blood was drawn into sodium–heparin tubes (Becton Dickinson, Franklin Lakes, NJ). Tubes were spun at 300 relative centrifugal force (rcf) for 10 min. Plasma was collected and frozen at −80°C until further analysis. The remaining blood products (WBCs, RBCs, and platelets) were diluted with Dulbecco’s PBS (Life Technologies, Burlington, ON) at a 1:1 ratio and layered on Ficoll-Paque Plus (GE Healthcare, Baie d’Urfé, QC). Cells were spun at 900 rcf for 25 min with no brake. PBMCs were isolated as per the manufacturer’s protocol. Cells were cryopreserved as previously described (29). PBMCs were stored in a liquid nitrogen storage unit (vapor phase) until further analysis.

Determination of HBsAg-specific Abs

HBsAg-specific IgG (anti-HBs IgG) was measured in plasma by commercial enzyme immunoassay (DiaSorin, Brussels, Belgium) as per the manufacturer’s instructions. The assay’s standard curve range is 1.25–1000 IU/l. All samples were tested at a 1:2 dilution. All samples with anti-HBs levels exceeding the upper limit of the standard curve were retested at a 1:10 dilution.

Cell culture and proliferation assay

PBMCs were thawed and washed twice with prewarmed R10 medium, RPMI 1640 medium, 10% FCS (Thermo Fisher Scientific, Logan, UT), penicillin (100 U/ml) (Life Technologies), streptomycin (100 µg/ml) (Life Technologies), and 50 µM β-mercaptoethanol (Sigma-Aldrich, Oakville, ON). Cells were then treated with DNase I (60 µg/ml) (Sigma-Aldrich) for 5 min at 37°C with 5% CO2. Following this, cells were then washed with RPMI 1640 medium, resuspended in 5 ml of R10 medium and transferred to a six-well polystyrene culture plate (Becton Dickinson) and incubated overnight at 37°C with 5% CO2.

After 24 h, EDTA (Sigma-Aldrich) was added to the cells at a final concentration of 2 mM and incubated for 10 min at 37°C with 5% CO2. Cells were then harvested, washed, and counted. Viability was assessed by trypan blue dye exclusion and found to be above 90% for all samples. Cells were resuspended in 500 µl of staining buffer (Dulbecco’s PBS with 5% FCS) and passed through a 70-µm cell strainer (Becton Dickinson). CellTrace Oregon Green 488 carboxylic acid diacetate, succinimidyl ester (OG488) (Life Technologies), a derivative of CFSE, was added to the cell suspension at a final concentration of 5 µM.

For unstained controls, an equal volume of staining buffer was added to cells and treated identically as stained samples. Cells were incubated with OG488 for 5 min, after which an equal volume of FCS was added to quench any residual OG488. To remove excess OG488, cells were washed three times with staining buffer. Cells were resuspended in R10 at a final concentration of 4 × 10⁶ cells/ml and plated at 0.5 × 10⁸ cells per well in a 96-well U-bottom polystyrene tissue culture plate (Becton Dickinson). The final volume of cultures in the 96-well plates is 200 µl. Samples of unstimulated controls were incubated with anti-CD28 (1 µg/ml) and anti-CD49d (1 µg/ml) monoclonal Abs (eBioscience, San Diego, CA) in R10 medium. Ag-specific samples were incubated with recombinant HBsAg (5 µg/ml) (Aldevron, Fargo, ND), anti-CD28 (1 µg/ml) and anti-CD49d (1 µg/ml) monoclonal Abs (eBioscience) in R10 medium. Positive controls were stimulated with anti-CD3 (30 ng/ml) and anti-CD28 (1 µg/ml) monoclonal Abs (eBioscience) in R10 medium.

Plates were incubated for 7 d at 37°C with 5% CO2. Plates were then spun at 300 rcf for 10 min to pellet cells and 50 µl per well of cell-free supernatant was transferred to a clean plate and frozen at −80°C until further analysis. Cells were then resuspended and restimulated with a mixture containing PMA (10 ng/ml) (Sigma-Aldrich), ionomycin (1 µM) (Sigma-Aldrich), and brefeldin A (2.5 µg/ml) (Sigma-Aldrich) for 6 h at 37°C and 5% CO2. After 6 h, EDTA (2 mM) was added to each well and incubated for an additional 10 min at 37°C with 5% CO2. The plates were then spun and supernatants were removed. Cells were fixed in a 1× FACS lysis solution (Becton Dickinson) and frozen at −80°C until further analysis.

Determination of bulk cytokine levels

The cell-free supernatant was thawed at room temperature (RT), and the levels of IL-10, IL-13, IL-17A, IL-21, IFN-γ, and TNF-α were measured by Luminex assay (Millipore, Billerica, MA) as per the manufacturer’s recommendations. Assays were read using the Luminex 200 Total System (Luminex, Austin, TX), and data were acquired and analyzed using MasterPlex software (MiraiBio Group, San Francisco, CA). The standard curves for the Luminex assay were as follows: 10–40,000 pg/ml for IFN-γ, 1–5000 pg/ml for IL-10, 7–30,000 pg/ml for IL-13, 12–50,000 pg/ml for IL-17A, 5–20,000 pg/ml for IL-21, and 2.5–10,000 pg/ml for TNF-α.

Immunophenotypic analyses by flow cytometry

Previously frozen plates containing cells fixed in FACS lysis solution were thawed in 37°C incubator for 10 min. Cells were washed once in PBSAN solution (PBS [Lonza, Basel, Switzerland], 0.5% BSA [Sigma-Aldrich], 0.1% sodium azide [Teknova, Hollister, CA]), resuspended in 1× FACS permeabilization solution (Becton Dickinson), and incubated for 10 min at RT.
Following one wash with PBSAN, cells were stained in a final volume of 100 μl per well of PBSAN for 30 min at RT. The following Abs were used: anti–CD3-APCeFluor780 (clone UCHT1; eBioscience), anti–CD4-eFluor450NC (clone RPA-T4; eBioscience), anti–CD8-PE-Cy7 (clone SKI; eBioscience), anti–IFN-γ-PE-CFS94 (clone B27; Becton Dickinson), anti–IL-2-PerCPeFluor710 (clone MAI-17H12; eBioscience), anti–IL-13-APC (clone JES50-5A2; Becton Dickinson), anti–IL-21-PE (clone eBio3A3-N2; eBioscience), and anti–TNF-α-AlexaFluor700 (clone MAb11; eBioscience). All Abs were used at a 1:100 dilution. OG488 was detected using the same filter as FITC. Following two washes with PBSAN, cells were resuspended in 200 μl per well of PBSAN and immediately analyzed using an LSRII Flow Cytometer (Becton Dickinson). FACSDiva (Version 9) (Becton Dickinson) was used as acquisition software. Compensation beads (Becton Dickinson) were used as single stain controls for all purchased Abs. For OG488, an equal number of unstained and stained PBMCs were combined and used as single stain control for compensation purposes.

Prior to each acquisition, Cytometer Setup and Tracking beads (Becton Dickinson) were run on the LSRII and previously standardized and saved cytometer settings were applied. Cells and beads were acquired uncompensated using a high-throughput sampler. The maximum number of cells were acquired from each well and ranged from 144,000 (116,000–179,000), median (interquartile range [IQR]), for unstimulated and HBsAg-stimulated samples. Compensation matrices were calculated and applied using FlowJo (Version 9.4) (TreeStar, Ashland, OR). All samples were analyzed compensated. Biological controls were used to set appropriate gates for analyses.

**Determination of cellular proliferation**

Proliferation data were analyzed using the proliferation platform on FlowJo. Positive controls were used to set the gates identifying successive rounds of cell division (generation gates) and sub-sequently copied to unstimulated and HBsAg-stimulated samples on a per subject basis. Number of cells in each generation gate were determined and used to calculate the percentage of the starting population that divided at least once. The gating strategy employed for the flow cytometric data are represented (Supplemental Fig. 1).

**Statistics**

All demographic data were compared between study groups by the use of the Pearson Chi-Square test. Anti-HBs Ab levels below the lower limit of detection were assigned the lower limit of detection of the assay. Ab levels were log transformed prior to analysis. Geometric mean concentration and 95% confidence interval for infants’ sera at the age of 1 and 2 y were calculated. The Mann–Whitney U test was used to assess differences in log transformed Ab levels between low-responders (anti-HBs levels 10–100 IU/l) and high-responders (anti-HBs levels ≥100 IU/l) at year 1 and the Kruskal–Wallis test was used to test for differences in log transformed Ab levels between nonresponders (anti-HBs levels <10 IU/l), low-responders, and high-responders.

For CMI parameters, technical replicates were averaged and unstimulated values were subtracted from stimulated values. Wilcoxon matched pairs signed rank test was used to test for differences between unstimulated and stimulated values from the same subject.

Nonparametrical tests were used to compare the proliferation, bulk cytokine production, and secretion between study groups stratified according to anti-HBs levels. Cytokines expressed below the lower limit of detection were assigned the value of 0. The Mann–Whitney U test was used to test for differences in CMI responses (proliferation, bulk cytokine production, and secretion) between low-responders and high-responders. The Kruskal–Wallis test was used to test for differences in CMI responses (proliferation, bulk cytokine production, and secretion) between nonresponders, low-responders, and high-responders. Correlations between anti-HBs levels and CMI responses were studied by use of Spearman rank analysis.

Data was analyzed using SPSS software version 24.0 (SPSS, Chicago, IL). Significance was set at $p < 0.05$.

**RESULTS**

**Baseline characteristics and humoral immune response**

Blood for anti-HBs Abs and CMI measurement was available from 76 and 72 participants at age 1 and 2 y, respectively (Fig. 1).

The demographic, clinical characteristics and anti-HBs levels of the study population are presented (Table I). At 1 y of age, there were no statistical differences between the low-responders ($n = 16$) and high-responders ($n = 57$) in sex or ethnicity. Immunization records confirmed that by 1 y of age, all participants received three doses of HBV vaccine. At 2 y of age, there were no statistical differences between the nonresponders, low-responders, and high-responders in sex or ethnicity (Table I). A correlation analysis between anti-HBs levels measured at the age of 1 and 2 y showed a significant positive correlation between anti-HBs levels measured at the age of 1 and 2 y (Spearman correlation coefficient $\rho = 0.588, p = 0.000$). This indicates that those with higher anti-HBs levels at 1 y of age were likely to have higher anti-HBs levels at 2 y of age.

**HBsAg-specific cellular proliferation**

The median percentage of proliferating CD4 T cells was higher as compared with the percentage of proliferating CD8 T cells, 0.575 ($n = 76$, IQR = 0.635) versus 0.168 ($n = 76$, IQR = 0.309), respectively ($p = 0.000$, Wilcoxon signed rank test). There was a significant positive correlation between the percentage of proliferating CD4 and proliferating CD8 T cells in response to HBsAg (Spearman $\rho = 0.346, p = 0.002$ [two-tailed]) indicating that those with a greater percentage of proliferating CD4 T cells were likely to have a greater percentage of proliferating CD8 T cells as well.

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Age 1 y: comparing HBsAg-specific CMI and humoral immune response

Age 1 y: HBsAg-specific CMI of low-responders versus high-responders. At 1 y of age, three infants had anti-HBs levels <10 IU/l. This small number of subjects precluded the analysis of a non-responder group at the 1-y time point. Thus, the comparison between groups at 1 y reported below is between low-responders and high-responders.

HBsAg-specific cellular proliferation and HBsAg-specific intracellular cytokine production. The median percentage of starting CD4 T cells, starting CD8 T cells, IFN-γ+ CD4 T cells, IL-13+ CD4 T cells, and IL-21+ CD4 T cells that proliferated were not significantly different among infants classified as low-responders versus high-responders (Fig. 2).

HBsAg-specific bulk cytokine secretion. Several differences were observed in the bulk cytokine levels when comparing different responder groups (Table II). The median levels of IL-10 were lower in low-responders as compared with high-responders (p = 0.05). Lower median levels of TNF-α were observed in low-responders as compared with high-responders (p = 0.06).

Age 1 y: correlation between HBsAg-specific CMI and anti-HBs levels. To determine if there is a relationship between anti-HBs levels and HBsAg-specific CMI, a correlation analysis was performed at the 1-y time point. There was no significant correlation between anti-HBs levels at age 1y and HBsAg-specific proliferation or intracellular cytokine production (Table III). Interestingly, there was a significant positive correlation between anti-HBs levels at age 1 y and the levels of secreted IFN-γ and IL-10 with rho of 0.269 and 0.297, respectively, (p = 0.019 and 0.009, respectively) (Table III).

Age 2 y: comparing HBsAg-specific CMI and humoral immune response

Age 2 y: HBsAg-specific CMI of nonresponders, low-responders, and high-responders. HBsAg-specific cellular proliferation and HBsAg-specific intracellular cytokine production. The median percentage of starting CD4 T cells and CD8 T cells that proliferated were not significantly different among infants classified as non-responders, low-responders, and high-responders (Fig. 3).

The median percentage of IFN-γ+ CD4 T cells that proliferated was 8.02 (n = 12, IQR = 10.47), 12.25 (n = 25, IQR = 16.67), and
TABLE I. Clinical characteristics and anti-HBs levels of subjects stratified by humoral immune response to primary series of HBV vaccine at age 1 and 2 y

<table>
<thead>
<tr>
<th>Clinical Characteristics</th>
<th>High-Responders at 1 y (n = 57)</th>
<th>Low-Responders at 1 y (n = 16)</th>
<th>p Value</th>
<th>High-Responders at 2 y (n = 29)</th>
<th>Low-Responders at 2 y (n = 28)</th>
<th>Nonresponders at 2 y (n = 15)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, n (%)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Male</td>
<td>29 (50.8)</td>
<td>7 (43.8)</td>
<td>0.614a</td>
<td>14 (48.3)</td>
<td>16 (57.1)</td>
<td>5 (33.3)</td>
<td>0.330a</td>
</tr>
<tr>
<td>Female</td>
<td>28 (49.2)</td>
<td>9 (56.2)</td>
<td></td>
<td>15 (51.7)</td>
<td>12 (42.9)</td>
<td>10 (66.4)</td>
<td></td>
</tr>
<tr>
<td>Ethnicity, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>23 (40.3)</td>
<td>9 (56.2)</td>
<td>0.649a</td>
<td>11 (40)</td>
<td>16 (57.1)</td>
<td>8 (53.3)</td>
<td>0.376a</td>
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<tr>
<td>Asian</td>
<td>6 (10.5)</td>
<td>3 (18.7)</td>
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<td>5 (17.2)</td>
<td>1 (3.4)</td>
<td>3 (20)</td>
<td></td>
</tr>
<tr>
<td>South Asian</td>
<td>8 (14)</td>
<td>1 (6.2)</td>
<td></td>
<td>4 (13.8)</td>
<td>4 (14.3)</td>
<td>2 (13.3)</td>
<td></td>
</tr>
<tr>
<td>Asian-Caucasian</td>
<td>5 (8.8)</td>
<td>1 (6.6)</td>
<td></td>
<td>4 (13.8)</td>
<td>2 (7.1)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Anti-HBs IgG (IU/l)</td>
<td>340.24</td>
<td>57.37</td>
<td>0.000c</td>
<td>231.64</td>
<td>36.03</td>
<td>1.73 (1.17–2.51)</td>
<td>0.000d</td>
</tr>
<tr>
<td>(GMC, 95% CI)</td>
<td>(275.42–407.38)</td>
<td>(44.66–72.44)</td>
<td></td>
<td>(186.20–281.83)</td>
<td>(27.52–45.70)</td>
<td></td>
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</tr>
</tbody>
</table>

Nonresponders: anti-HBs levels <10 IU/l; low-responders: anti-HBs levels 10–100 IU/l; high-responders: anti-HBs levels ≤100 IU/l. Categorical data are presented as a number (%).

*Pearson Chi-Square test.
*aPearson Chi-Square test.
*bMann–Whitney U test.
*cKruskal–Wallis test.
Cl, confidence interval; GMC, geometric mean concentration.

21.05 (n = 23, IQR = 18.60) among infants classified as nonresponders, low-responders, and high-responders, respectively, p = 0.013 (Fig. 3C). Moreover, the median percentage of IFN-γ CD4 T cells was significantly lower in infants classified as nonresponders compared with high-responders, (p = 0.006); and infants classified as low-responders as compared with high-responders (p = 0.033).

The median percentage of IL-13 CD4 T cells that proliferated was 7.88 (n = 12, IQR = 8.11), 14.21 (n = 25, IQR = 8.24), and 17.12 (n = 23, IQR = 16.39) among infants classified as nonresponders, low-responders, and high-responders, respectively, p = 0.089 (Fig. 3D). Moreover, the median percentage of IL-13 CD4 T cells was significantly lower in infants classified as nonresponders as compared with high-responders (p = 0.032) and a trend was noted in infants classified as nonresponders when compared with low-responders (p = 0.065).

The median percentage of IL-21 CD4 T cells that proliferated was 7.18 (n = 12, IQR = 10.23), 13.09 (n = 25, IQR = 14.93), and 19.12 (n = 23, IQR = 18.21) among infants classified as nonresponders, low-responders, and high-responders, respectively, p = 0.075 (Fig. 3E). In addition, the median percentage of IL-21 CD4 T cells was significantly lower in infants classified as nonresponders versus high-responders (p = 0.034). It should be noted that despite being lower than responder groups in several parameters, nonresponders do demonstrate evidence of HBsAg-specific CMI as they had measurable levels of secreted cytokines.

**HBsAg-specific bulk cytokine secretion.** Several differences were observed in the bulk cytokine levels when comparing different responder groups (Table II). Specifically, nonresponders had lower median levels of IL-10 than low-responders (p = 0.05). As well, the median levels of TNF-α were significantly lower in nonresponders than high-responders (p = 0.032). The median levels of IFN-γ were significantly different in infants stratified by humoral immune response (p = 0.025). Specifically, low-responders had significantly lower median levels of IFN-γ secretion than high-responders (p = 0.009). Moreover, it should be noted that nonresponders demonstrated evidence of HBsAg-specific CMI as they had measurable levels of secreted cytokines.

**Age 1 y: correlation between HBsAg-specific CMI.** There was a positive correlation between anti-HBs levels at 2 y of age and the percentage of IFN-γ CD4 T cells, rho = 0.350, p = 0.006 (Table III). In addition, there was a significant positive correlation between anti-HBs levels at 2 y of age and the levels of secreted IFN-γ, rho = 0.3, p = 0.01 (Table III).

**DISCUSSION**

In the current study, we interrogated the HBsAg-specific CMI after primary HBV vaccination during infancy as measured by CD4 and CD8 T cell proliferation, CD4 T cell intracellular cytokine production, and bulk cytokine secretion at the age of 1 y as compared with humoral immune response at 1 and 2 y of age.

Our study provides evidence on a significant association between humoral immune response at the age of 1 and 2 y and IFN-γ secretion. IFN-γ is produced by CD4 and CD8 T cells, supports the differentiation of CD4 T cells to Th1 cells, and suppresses the development of Th2 cells (34). It also supports T cell proliferation (34). Moreover, IFN-γ enhances HBV-specific humoral immune response in vitro as the addition of IFN-γ resulted in a dose-dependent increase of anti-HBsAg secreting B cells (35). IFN-γ produced by HBV-specific CD8 T cells is necessary for controlling HBV infection (36–38). Our results correlate with previous literature on adult cohorts, which showed that low- or nonresponder adults had low or undetectable expression or secretion of IFN-γ (18, 20, 21, 23, 39). However, the cohort included in these studies was a mixed adult population of those having their primary and booster vaccination against HBV. In the pediatric population, only one study reported that infant
nonresponders to primary vaccination against HBV secreted less IFN-γ than responders (22). The correlation between anti-HBs levels and IFN-γ production is controversial, as some studies showed a correlation (23), whereas others have not (14, 24, 25).

Our data showed that there was a significant positive correlation between anti-HBs Ab levels at age 1 y and the levels of secreted IL-10. IL-10 is a potent growth and differentiation factor for activated B cells inducing secretion of Igs (40). Our findings are in agreement with evidence from adult studies, which included a heterogeneous population following primary and booster vaccination and showed that individuals who did not mount a protective humoral immune response following vaccination against HBV secreted significantly lower levels of IL-10 in response to HBsAg stimulation (23, 26, 27).

We also found that the median levels of TNF-α were significantly lower in nonresponders than high-responders to primary vaccination against HBV secreting less IFN-γ than responders (22).

**TABLE II.** HBsAg-specific bulk cytokine secretion stratified by humoral immune response to primary immunization against HBV at age 1 and 2 y

<table>
<thead>
<tr>
<th>CMI Parameter at 1 y</th>
<th>Low-Responders at 1 y (n = 16)</th>
<th>High-Responders at 1 y (n = 57)</th>
<th>p Value</th>
<th>Nonresponders at 2 y (n = 15)</th>
<th>Low-Responders at 2 y (n = 28)</th>
<th>High-Responders at 2 y (n = 29)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ secretion (pg/ml), median (IQR)</td>
<td>18.64 (32.18)</td>
<td>24.23 (30.98)</td>
<td>0.119</td>
<td>22 (37.84)</td>
<td>17.15 (34.26)</td>
<td>33.16 (44.87)</td>
<td>0.025 (0.009b)</td>
</tr>
<tr>
<td>IL-10 secretion (pg/ml), median (IQR)</td>
<td>32.11 (87.17)</td>
<td>82.32 (163.16)</td>
<td><strong>0.05</strong></td>
<td>35.09 (114.69)</td>
<td>79.87 (162.62)</td>
<td>79.06 (119.45)</td>
<td>0.112 (0.05b, 0.069c)</td>
</tr>
<tr>
<td>IL-13 secretion (pg/ml), median (IQR)</td>
<td>529.61 (1219.63)</td>
<td>978.30 (957.53)</td>
<td>0.125</td>
<td>796.25 (1104.05)</td>
<td>1157.21 (1313.74)</td>
<td>860.19 (988.54)</td>
<td>0.262</td>
</tr>
<tr>
<td>TNF-α secretion (pg/ml), median (IQR)</td>
<td>103.50 (131.24)</td>
<td>171.24 (209.45)</td>
<td>0.06</td>
<td>116.11 (161)</td>
<td>178.65 (231.64)</td>
<td>162.27 (175.13)</td>
<td><strong>0.015 (0.032c)</strong></td>
</tr>
</tbody>
</table>

Nonresponders: anti-HBs levels <10 IU/l; low-responders: anti-HBs levels 10–100 IU/l; high-responders: anti-HBs levels ≥100 IU/l. Categorical data are presented as a number (%). The bold text indicates statistical significance.

*Low-responders versus high-responders.
*Nonresponders versus low-responders.
*Nonresponders versus high-responders.
at the age of 2 y. TNF-α is a proinflammatory cytokine (41) and studies have shown that TNF-α produced by HBV-specific CD8 T cells is necessary for controlling HBV infection (36–38). In our study, the source of TNF-α was not demonstrated. Despite the importance of TNF-α in controlling HBV infection, there is limited data on its role in the response to vaccination against HBV, particularly in infants. In line with our results, adult nonresponders produced significantly less TNF-α than strong responders following stimulation with HBsAg (23).

<table>
<thead>
<tr>
<th>CMI Parameter at age 1 y</th>
<th>Correlation Coefficient* (Rho) to Anti-HBs Levels at 1 y (n)</th>
<th>p Value (Two-Tailed)</th>
<th>Correlation Coefficient* (Rho) to Anti-HBs Levels at 2 y (n)</th>
<th>p Value (Two-Tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of starting CD4 T cells that proliferated</td>
<td>0.115 (n = 76)</td>
<td>0.321</td>
<td>-0.001 (n = 72)</td>
<td>0.994</td>
</tr>
<tr>
<td>Percentage of starting CD8 T cells that proliferated</td>
<td>0.176 (n = 76)</td>
<td>0.129</td>
<td>0.179 (n = 72)</td>
<td>0.132</td>
</tr>
<tr>
<td>Percentage of IFN-γ+ proliferating CD4 T cells</td>
<td>0.170 (n = 63)</td>
<td>0.182</td>
<td>0.350 (n = 60)</td>
<td><strong>0.006</strong></td>
</tr>
<tr>
<td>Percentage of IL-13+ proliferating CD4 T cells</td>
<td>0.097 (n = 63)</td>
<td>0.447</td>
<td>0.106 (n = 60)</td>
<td>0.420</td>
</tr>
<tr>
<td>Percentage of IL-21+ proliferating CD4 T cells</td>
<td>0.068 (n = 63)</td>
<td>0.598</td>
<td>0.180 (n = 60)</td>
<td>0.169</td>
</tr>
<tr>
<td>IFN-γ secretion (pg/ml)</td>
<td>0.269 (n = 76)</td>
<td><strong>0.019</strong></td>
<td>0.302 (n = 72)</td>
<td><strong>0.01</strong></td>
</tr>
<tr>
<td>IL-10 secretion (pg/ml)</td>
<td>0.297 (n = 76)</td>
<td><strong>0.009</strong></td>
<td>0.088 (n = 72)</td>
<td>0.461</td>
</tr>
<tr>
<td>IL-13 secretion (pg/ml)</td>
<td>0.114 (n = 76)</td>
<td>0.325</td>
<td>-0.029 (n = 72)</td>
<td>0.812</td>
</tr>
<tr>
<td>TNF-α secretion (pg/ml)</td>
<td>0.091 (n = 76)</td>
<td>0.433</td>
<td>0.122 (n = 72)</td>
<td>0.308</td>
</tr>
</tbody>
</table>

The bold text indicates statistical significance.

* Spearman rank analysis.

FIGURE 3. HBsAg-specific proliferation and intracellular cytokine production stratified by humoral immune response to primary immunization against HBV at age 2 y.

(A) Percentage of starting CD4 T cells that proliferated. (B) Percentage of starting CD8 T cells that proliferated. (C) Percentage of IFN-γ+ proliferating CD4 T cells. (D) Percentage of IL-13+ proliferating CD4 T cells. (E) Percentage of IL-21+ proliferating CD4 T cells. Data are presented in boxplots as median (lines) and IQR (boxes). Small circles indicate outliers and stars indicate extreme values. p < 0.05 indicates significance. 

1nonresponders versus low-responders versus high-responders, 2nonresponders versus high-responders, 3low-responders versus high-responders.

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In our study, we found that the median percentage of IL-13+ CD4 T cells was significantly lower in infants classified as nonresponders as compared with high-responders. Limited knowledge is available on the role of IL-13 in the response to vaccination against HBV. Following primary or booster vaccination of adults, nonresponders had a lower percentage of IL-13+ CD4 T cells than responders (20). Although immunization of naive adults against HBV was associated with increased levels of secreted IL-13, infants showed no difference in IL-13 following full vaccination series against HBV (24). The correlation between IL-13 genotype and response to immunization against HBV is controversial. Certain genotypes in the IL-13 gene were associated with nonresponse to vaccination against HBV in an adult population (42). However, no associations between anti-HBs levels and genetic variation in IL-13 genes was reported in a cohort of patients undergoing hemodialysis (43).

We found that the median percentage of IL-21+ CD4 T cells was significantly lower in infants classified at the age of 2 y as nonresponders when compared with high-responders. IL-21 is an effector cytokine of follicular helper T cells, a subset of T helper cells that are critical in the formation and maintenance of germinal centers as well the differentiation of germinal center B cells into plasma and memory B cells (44, 45). The role of IL-21 in the immune response to HBV infection remains relatively undetermined and there is limited data in humans for the role of IL-21 in the immune response to vaccination against HBV.

In our study, we did not find a correlation between the proliferative response of CD4 and CD8 T cells in infants categorized according to their humoral immune response to immunization against HBV. Previous literature on this topic is controversial. Although some adult studies reported on a correlation between anti-HBs levels and T cell proliferation (17–19), others showed a lack of T cell proliferative response to HBsAg among nonresponders to HBV vaccine booster (19–21).

It should be noted that we demonstrated that infants who were defined as nonresponders have evidence of HBsAg-specific CMI as they had some degree of HBsAg-specific CD4 and CD8 T cell proliferation and measurable levels of secreted cytokines following stimulation with HBsAg. Our findings and the evidence that there is clinical protection from HBV infection in subjects with anti-HBs levels <10 IU/l (13, 14), clearly indicates that an alternative or additional immunological mechanism might also correlate with HBV vaccine–induced protection. Further support for the role of CMI generated after vaccination in the prevention of clinical infection was reported for influenza. CMI to influenza correlated with protection from clinical infection, whereas serum Ab response did not (46).

Our study has several limitations. First, we did not assess peak anti-HBsAg levels (1 mo following primary vaccination against HBV), thus limiting our ability to classify the cohort into the standard groupings of nonresponders, low-responders, and high-responders. It should be noted that there is increased anti-HBs response to the third dose of primary immunization against HBV as compared with the first two doses (47), with the vaccine-induced anti-HBs levels achieving a peak usually 4 wk after immunization (48). This is then followed by a rapid decline during the next few years after immunization (especially the first year) and more slowly thereafter (48, 49). Second, the small sample size of our cohort limited our comparison at age 1 y to low-responders and high-responders, which might have limited our ability to detect significant correlation between CMI and humoral immune response. It should be emphasized that the vast majority of studies on HBV vaccine responses have concentrated on older children, adolescents, or adults. Thus, the strength of our study is that we add significant data on CMI at age 1 y and following primary vaccination against HBV.

In conclusion, in the current study we demonstrate HBsAg-specific CMI in infants following primary immunization against HBV. We found that HBsAg-specific IFN-γ and IL-10 secretion correlated with HBV vaccine–induced humoral immune response indicating that there is a balanced Th1/Th2 cytokine profile in response to vaccination against HBV. This is consistent with previous literature (18, 24). Although our results were less robust with TNF-α, this cytokine should also be targeted by future research as another CMI candidate that correlates with an HBsAg-specific humoral immune response.

Furthermore, the demonstration in our study of evidence of HBsAg CMI response among nonresponders and the fact that individuals whose levels are <10 IU/l are protected against HBV infection clearly argue that an alternative or additional immunological mechanism might also correlate with vaccine-induced protection.

Assessment of T cell responses could be a useful adjunct in demonstrating the persistence of HBsAg-specific immune response.

DISCLOSURES

The authors have no financial conflicts of interest.

REFERENCES


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