TH1 and TH2 Responses Are Influenced by HLA Antigens in Healthy Neonates Vaccinated with Recombinant Hepatitis B Vaccine

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ABSTRACT

The immune response to hepatitis B surface antigen (HBsAg) is influenced by several factors, of which HLA antigens and balanced secretion of Th1/Th2 cytokines play important roles. The aim of this study was to evaluate the influence of HLA antigens on cytokine secretion by HBsAg-stimulated peripheral blood mononuclear cells (PBMC) from healthy neonates vaccinated with recombinant HBsAg.

PBMCs were isolated from 48 Iranian neonates vaccinated with a recombinant HBV vaccine. The cells were stimulated *in vitro* with rHBsAg and the concentration of IL-4, IL-10, IL-12 and IFN-γ were quantitated in culture supernatant by sandwich ELISA. HLA typing was performed by microlymphocytotoxicity method.

Significant diminished secretion of both Th1 (IFN-γ) and Th2 (IL-4, IL-10) cytokines was observed in HBsAg-stimulated PBMC from vaccinees expressing the HLA-DR7 compared to DR7 negative vaccinees. Similarly, lower production of these cytokines was also observed in vaccinees with DR7-DR53-DQ2, B7-DR7-DR53-DQ2 and A2-DR7-DR53-DQ2 haplotypes ($p<0.05$, $p <0.005$). While HBsAg-stimulated PBMC of DR13^{+} subjects produced lower levels of Th2-type cytokines (IL-4 and IL-10), those of HLA-B8[+] or HLA-A9[+] subjects produced higher levels of Th2-type cytokines. Cytokine secretion in response to PHA mitogen was not associated with a given HLA antigen or haplotype and was similarly represented in all groups of subjects irrespective of their HLA complex.

These results indicate that HLA antigens may differentially influence cytokine secretion by HBsAg-specific T-cells of healthy neonates vaccinated with recombinant HB vaccine. This phenomenon may have an important implication for control of the immune response to HBsAg vaccine.

Keywords: Cytokine; Hepatitis B vaccine; HLA antigens

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INTRODUCTION

Immunization of healthy individuals with the major surface antigen of hepatitis B virus (HBsAg) induces a

protective antibody response in the majority of vaccinated subjects, but 1-10% of neonates, children and adults remain non-responders and are at risk for infection.[1-3]

Production of anti-HBs antibody following vaccination or natural infection is helper T cell dependent. CD4^{+} T lymphocytes recognize different epitopes within the HBsAg molecule that can be presented by certain HLA class II antigens.[4] Despite the fact that the HLA system is highly polymorphic in human, many investigators have demonstrated significant associations between certain HLA alleles, antigens or haplotypes with hepatitis B vaccination outcomes. It has been demonstrated that the HLA- DR7, DR3 and DQ2 antigens and corresponding alleles, in particular, were more frequent in non-responders to hepatitis B vaccine.[5-7]

These observations suggest that HLA may play an important role in directing the antibody response to HBsAg. On the other hand, several studies have also been conducted to investigate the precise role of Th1 and Th2-derived cytokines in the immune response to hepatitis B vaccination. Analyses of *in vitro* HBsAg-induced cytokine production have revealed defects in Th1, Th2 or both Th1 and Th2 responses in adult non-responders to hepatitis B vaccine.[8-12]

Different patterns of cytokine production have also been observed in T-cell clones isolated from responder subjects, with either predominant Th0 and Th2 responses or Th1 and Th2 responses in high and low responders, respectively.[13-15]

We have previously demonstrated that both Th1 and Th2 responses are defective in our healthy neonates who failed to mount a protective antibody response to recombinant HBsAg.[16] Lack of antibody response to HB vaccine was also found to be associated with certain HLA antigens and haplotypes in a group of Iranian non-responder neonates and adults.[5,17]

Understanding the genetic factors that influence the variation of HBsAg-induced cytokine secretion following HB vaccination is important for developing strategies to increase HB vaccine efficacy. This study was undertaken to evaluate the influence of HLA antigens on cytokine secretion by HBsAg-stimulated PBMC from healthy neonates vaccinated with recombinant HB vaccine.

MATERIALS AND METHODS

Subjects and Vaccination Scheme

Triple 10 microgram doses of a recombinant hepatitis B vaccine (Heberbiovac Co., Cuba) were administered i.m to a large group of healthy Iranian neonates at 0, 1.5 and 9 months intervals. Gestational age, birth weight and sex of the neonates were registered and only physically healthy neonates with a minimum weight of 2500 g were enrolled in the study. The first dose was given 24-48 h after delivery and subsequent doses were administered in selected local health centers of Kerman, a city that is located in south-east of Iran. Two to four weeks after completion of the vaccination course, peripheral blood was taken from 48 randomly selected vaccinees. The samples were selected from a study that originally was approved by the ethical committee of Research Chancellor of the Minister of Health and Medical Education. Moreover, a consent letter was taken from parents of the vaccinated neonates.

In vitro Stimulation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMC) were separated from heparinized blood by Ficoll (Pharmacia, Uppsala, Sweden) gradient centrifugation. After washing in RPMI-1640 medium (Gibco Life Techologies Ltd, Paisley, UK), PBMC were resuspended in complete culture medium containing RPMI-1640 supplemented with 10% heat inactivated fetal calf serum (Seromed, Berlin, Germany) and antibiotics, including penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were then seeded at 10^6 cells/ml in a 24-well sterile tissue culture plate (Nagle Nunc International, Roskilde, Denmark) in presence of 10 μg/ml of purified rHBsAg without vaccine additive (Heberbiovac, Cuba) or 10 μg/ml phytohemagglutinin mitogen (PHA) (Sigma-Aldrich Corporation, St Louis, MO, USA). Following 72 h incubation at 37° C in a humidified $CO2$ (5%) incubator, culture supernatants were collected and stored in –7° C until use.

Quantification of Cytokines

All cytokines including IL-4, IL-10, IL-12 and IFN-γ were measured by sandwich ELISA using commercial kits (Biosource International, Camarillo, CA, USA).

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Table 1. Cytokine secretion by HBsAg-stimulated PBMC from HB vaccinated neonates with different HLA profiles

HLA antigens	No. of subjects	HLA status	IL-4 (pg/ml)	IL-10 (pg/ml)	IFN-γ (pg/ml)	IL-12 (pg/ml)	Serum anti-HBs levels (IU/L)	*P.* value
A9	48	A9$^+$(n=8)	85.6 (16.01)	53.3 (13.96)	192.8 (109.6)	103.8 (23.9)	895.7 (198.3)	**0.01**, 0.1, 0.49, 0.33, 0.19
		A9$^-$ (n=40)	48.3 (4.66)	36 (8.36)	117.5 (46.82)	85 (11.95)	583.2 (97.8)	
B8	48	B8$^+$ (n=7)	79.5 (18.26)	57.4 (15.84)	165.7 (123.06)	89.2 (23.4)	1042.7 (177.1)	**0.02**, **0.03**, 0.46, 0.66, 0.057
		B8$^-$ (n=41)	50.2 (4.85)	35.7 (8.14)	124 (46.12)	88 (11.95)	565.7 (95.9)	
DR7	48	DR7$^+$(n=14)	36.7 (5.34)	26.2 (16.04)	52.1 (28.79)	69.8 (20.96)	282.5 (146.6)	**0.01**, **0.02**, **0.02**, 0.1, **0.009**
		DR7$^-$(n=34)	62 (6.43)	44.1 (7.89)	162 (58.6)	95.7 (12.33)	780.5 (100.8)	
DR13	48	DR13$^+$(n=12)	41.4 (7.83)	27.5 (13.09)	40.5 (15.95)	69.3 (21.73)	326.2 (166.5)	**0.04**, **0.03**, 0.42, 0.22, **0.04**
		DR13$^-$(n=36)	58.9 (6.01)	42.7 (8.78)	160 (56)	94.5 (12.25)	738.3 (99.9)	
DR7-DR53-DQ2	48	Hap.$^+$(n=9)	34.8 (8.23)	14.6 (8.76)	59.6 (44.46)	56 (26.26)	305.8 (197.8)	**0.02**, 0.07, 0.03, **0.04**, 0.07
		Hap$^-$ (n=39)	59.1 (5.73)	44.5 (8.62)	146.3 (51.55)	95.6 (11.68)	711.3 (96.3)	
B7-DR7-DR53-DQ2	48	Hap.$^+$(n=5)	27 (5.15)	2 (1.03)	1.8 (0.3)	35.8 (30.17)	257.8 (250.5)	**0.02**, **0.008**, **0.002**, **0.05**, 0.14
		Hap$^-$ (n=43)	57.7 (5.41)	43.2 (8.01)	145 (47.35)	94.3 (11.11)	679.2 (93.3)	
A2-DR7-DR53-DQ2	48	Hap.$^+$(n=7)	30.2 (5.79)	8.3 (3.07)	12.5 (16.59)	71.2 (29.43)	365.4 (234.2)	**0.01**, 0.09, **0.04**, 0.33, 0.20
		Hap$^-$ (n=41)	58.7 (5.6)	44.1 (8.14)	148.4 (49.56)	91.1 (11.54)	681.4 (95.2)	

†The results represent mean (SEM). The *p* values were obtained for IL-4, IL-10, IFN-γ, IL-12 and anti-HBs antibody levels, respectively. All significant values are made bold.

The assay for IL-4, IL-10 and IL-12 was optimized by titration of the paired capture and detection antibodies as suggested by the manufacturer to determine the optimum concentration of both antibodies. Accordingly, the capture antibody was coated in polystyrene ELISA plates (Maxisorp, Nunc, Denmark) at 1 μg/ml and the biotinylated detection antibody was employed at 0.4 μg/ml for all 3 cytokines. The assay detection limit was found to be 62.5 pg/ml for IL-4, 19 pg/ml for IFN-γ, 62.5 pg/ml for IL-10 and 8 pg/ml for IL-12.

HLA Typing

HLA class I and II antigens were determined by the standard microlymphocytotoxicity technique. HLA class II was determined on B lymphocytes enriched by nylon wool. HLA typing was performed using commercial polyclonal antisera (Blood Transfusion Center of Iran, Tehran, Iran). Antisera recognizing the HLA antigens including HLA-A: A1-A3, A9, A11; HLA-B: B5, B7, B8, B13-B18, B21, B22, B27, B35, B38, B39, B40, B44, B45, B53, B55, B56; HLA-C: CW1-CW6; HLA-DR: DR1-DR4, DR7, DR9, DR11, DR13, DR14, DR15; DR52, DR53; HLA-DQ: DQ1-DQ3 were employed in this study.

Statistical Analysis

Differences of the cytokine levels were compared between vaccinated subjects positive or negative for the specified HLA antigens or haplotypes using two tailed Mann-Whitney U-test. *P* values of less than 0.05 were considered significant.

Table 2. Cytokine secretion by PHA-stimulated PBMCs from HB vaccinated neonates with different HLA profiles

HLA antigen	No. of subjects	HLA status	IL-4 (pg/ml)	IL-10 (pg/ml)	IFN-γ (pg/ml)	IL-12 (pg/ml)	P. value
A9	48	A9$^+$(n=8)	66.6 (16.7)	84.1 (33.4)	805 (405.5)	155.6 (25.4)	0.61, 0.45, 0.77, 0.33
		A9$^-$ (n=40)	77.9 (9.3)	112.2 (15.4)	924.4 (171.5)	191 (15.2)	
B8	48	B8$^+$ (n=7)	96.1 (16.3)	102.8 (28.7)	1148.9 (434.2)	222.1 (41.2)	0.31, 0.89, 0.92, 0.25
		B8$^-$ (n=41)	72.6 (9.1)	108.3 (15.4)	1087.2 (169.7)	178.7 (14.0)	
DR7	48	DR7$^+$(n=14)	65.0 (8.3)	91.1 (24.8)	1060.7 (330.7)	215.0 (19.3)	0.39, 0.45, 0.52, 0.13
		DR7$^-$(n=34)	80.6 (11.0)	114.2 (16.5)	840.2 (176.1)	171.5 (17.0)	
DR13	48	DR13$^+$(n=12)	69.9 (18.4)	138.7 (30.0)	563.3 (242.9)	171.25 (23.5)	0.66, 0.19, 0.21, 0.55
		DR13$^-$(n=36)	78.1 (9.1)	97.1 (15.1)	1140.9 (190.1)	189.7 (16.1)	
DR7-DR53-DQ2	48	Hap.$^+$(n=9)	69.4 (4.9)	91.7 (23.1)	1421.1 (44.46)	230.5 (26.9)	0.70, 0.58, 0.20, 0.10
		Hap$^-$ (n=39)	77.6 (10.0)	111.1 (16.1)	785.3 (156.3)	174.6 (14.9)	
B7-DR7-DR53-DQ2	48	Hap.$^+$(n=5)	75.2 (6.9)	74.2 (25.6)	1316.0 (700.6)	225.0 (42.8)	0.97, 0.41, 0.37, 0.31
		Hap$^-$ (n=43)	76.1 (9.1)	111.3 (14.9)	856.7 (156.8)	180.4 (14.1)	
A2-DR7-DR53-DQ2	48	Hap.$^+$(n=7)	68.3 (5.6)	96.6 (29.4)	1205.0 (563.6)	217.5 (35.5)	0.72, 0.76, 0.47, 0.36
		Hap$^-$ (n=41)	77.1 (9.3)	109.0 (15.2)	861.6 (161.7)	180.4 (14.4)	

†The results represent mean (SEM). *The p* values were obtained for IL-4, IL-10, IFN-γ and IL-12 levels, respectively. No significant *p* values were obtained.

RESULTS

Levels of cytokines were measured in supernatant of HBsAg-stimulated cultures of all vaccinated neonates and compared between different groups of subjects positive or negative for a given HLA antigen or haplotype, including all HLA class I and II antigens and haplotypes tested. Significant differences are depicted in table 1.

Significantly decreased levels of IL-4 (p=0.01), IL-10 (p=0.015), IFN-γ (p =0.015), but not IL-12 (p=0.1) were found in DR7$^+$ as compared to DR7$^-$ subjects. Moreover, lower production of these cytokines was also observed in vaccinees expressing DR7-DR53-DQ2, B7-DR7-DR53-DQ2 or A2-DR7-DR53-DQ2 haplotype. HBsAg-stimulated PBMC from neonates with DR7-DR53-DQ2 and B7-DR7-DR53-DQ2 haplotypes produced lower levels of IL-12 (p =0.04 and p =0.05, respectively) (Table 1).

Secretion of IL-4 and IL-10 was significantly lower in DR13$^+$ vs DR13$^-$ subjects, however, a reverse profile (significantly higher levels of IL-4 and IL-10) was evident in HLA-B8$^+$ compared to HLA-B8$^-$vaccinees. Vaccinated neonates positive for HLA-A9 tended to produce higher levels of IL-4 (p =0.01), but not the other cytokines when compared with HLA-A9 negative subjects.

Comparison of cytokine levels produced following PHA mitogen stimulation between vaccinees positive or negative for all combinations of HLA antigens or haplotypes tested revealed no significant differences for any of the cytokines (Table 2).

DISCUSSION

We have previously demonstrated that the antibody response to HB vaccine is associated with the HLA complex and certain HLA antigens and alleles or haplotypes are linked to vaccination failure in healthy adults and neonates.[5,6,17] In parallel to these findings, we and others observed a diminished Th1 and/or Th2 cytokine response to HBsAg in HB vaccinated non-responder healthy adults and neonates.[8-12,16] Down-regulation of Th1 and Th2 cytokines in non-responder neonates was associated to suppression of IL-12 production suggesting involvement of dysregulated antigen presentation in HB vaccination failure.[18]

In the present study, we sought to determine the influence of HLA antigen on HBsAg-induced cytokine secretion in healthy neonates vaccinated with recombinant HB vaccine. Our results demonstrated that the DR7 antigen and some DR7$^+$ haplotypes including DR7-DR53-DQ2, B7-DR7-DR53-DQ2 and A2-DR7-DR53-DQ2 are associated with significantly lower

secretion of IL-4, IL-10 and IFN-γ by HBsAg-stimulated PBMCs.

It has previously been demonstrated that there is a positive association between DR7, DR3 and DQ2 antigens and related alleles with unresponsiveness to hepatitis B vaccine.[5-7,19,20] Furthermore, viral persistence is over-represented in HBV-infected DR7^{+} subjects.[21] HBsAg seems to encompass only a limited set of peptides that are immunogenic at the level of Th cells.[22, 23] It has been demonstrated that the antigen presenting cells (APC) from DR3^{+} and DR7^{+} non-responders to HB vaccine were able to present HBsAg on their surface and the expression of accessory molecules such as CD86 were not deficient after culturing the PBMC with HBsAg.[24] Thus it is conceivable that T cells recognizing the dominant epitope(s) of HBsAg which can associate with certain HLA class II molecules (e.g. DR7 or DQ2) may be absent, because these HBsAg-derived peptide-MHC class II complexes so closely resemble self peptide-MHC class II complexes (molecular mimicry) that may lead to elimination of specific T cells in the thymus or in the periphery. Alternatively, the inhibitory effects of these HLA antigens or haplotypes on cytokine secretion may be due to the influence of HLA-DR7 and HLA-DQ2 antigens at the level of antigen presentation. Although defective APC function has not been strongly confirmed.[25-27] Our previous findings of diminished IL-12 secretion by PBMC from non responder neonates to HB vaccine strongly suggest contribution of APC dysfunction in unresponsiveness to HBsAg.[18]

Our data demonstrated that the production of Th2-type cytokines (IL-4, IL-10) was lower in HLA-DR13^{+} vaccinees. It has been reported that DRB1*1301 and DRB1*1302 alleles were associated with responsiveness and nonresponsiveness to HBsAg, respectively.[28] DRB1*1301 and DRB1*1302 alleles were also associated with chronic hepatitis B cirrhosis.[29] Association between DRB1*13 alleles with the clearance of HBV and self-limited course of acute hepatitis B has also been reported.[30-32] It remains unknown whether this is due to the DR13 molecule itself or to a linkage disequilibrium of DR13 with another gene on the same chromosome. However, it has been demonstrated that patients with DR13 can mount a more vigorous CD4^{+} T cell response to HBV core antigen during acute HBV infection and the progression to chronic hepatitis B is rare in that group of patients.[32] Although, the effect of DR13 on HBcAg-induced cytokine production has not been reported, it seems that it differs from those observed for stimulation with HBsAg. In other words, both HLA and antigenic peptide may contribute in cytokine secretion profiles which subsequently determine outcome of responsiveness.

In our study higher production of Th2-type cytokine (IL-4 and IL-10) was observed in HLA-B8[+] neonates. Similar results were obtained for HLA-A9 vaccinees. In other studies B8 allele has been associated with viral persistence in HBV-infected subjects.[33] Moreover rapid progression of AIDS has been reported in HLA-B8-DR3 positive subjects.[34] Moreover, decreased natural killer cell activity and decreased production of type 1 cytokines, including IL-2, IFN-γ and IL-12 have also been noted in subjects with this HLA antigen.[35,36] Thus, the immune status of healthy individuals with B8 antigen seems to be characterized by an impairment in cellular immunity due to the effect of TH2-type cytokines.

We have shown that higher levels of Th2 cytokines (IL-4) were produced in A9[+] vaccinees (Table 1). There is no published report on the association of HBV and HLA-A9 antigen. Further studies are needed to confirm and extend this association.

In general, the present results indicate that HLA antigens, especially A9, B8, DR7, and DR13 and some HLA extended haplotypes could influence cytokine secretion from HBsAg-stimulated PBMCs of healthy vaccinated neonates. Basically, it should be noted that the differentiation of naïve CD4^{+} Th cells into Th1 and Th2 phenotype is influenced by several factors such as the affinity of the interaction between the MHC-peptide-TCR complex and the numbers of MHC-peptide complexes available on the surface of APC.[37] It has been reported that Th1 development is favored with high concentrations of peptides or increased affinity of the TCR-MHC-peptide interactions. Moreover, high ligand density favored IFN-γ producing cells, whereas lower densities largely resulted in differentiation of IL-4 response or mixed responses.[37] It has been demonstrated that there are different immunodominant T cell epitopes within the HBsAg molecule that can be presented by different HLA class II alleles and induce differentially Th0/Th2 responses.[13] It has been shown that the differences in HBsAg peptide binding affinity between the HLA-DR alleles do not explain the known HLA-DR association and HBsAg vaccination failure, so that differences in the T cell recognition of

peptide/MHC complexes is the critical event in T cell responsiveness to HBsAg.[38] Accordingly, the differences in the TCR affinity for binding to particular HLA-HBsAg-derived peptides may contribute to differential Th1 or Th2 response.

It should be also noted that $CD4^+CD25^+$ regulatory T cells are an important component of the immune regulation in the periphery. Recently, it has been demonstrated that regulatory T cells specific for particular peptide-MHC complexes can be generated from human $CD4^+CD25^-$ T cells *in vitro*.[39] Accordingly, induction of regulatory T cells via certain HLA-HBsAg-derived peptides may also be responsible for diminished Th1- and/or Th2-type cytokine profiles.

In addition to HLA molecules, single-nucleotide polymorphisms in either cytokine or cytokine receptor genes may also influence cytokine secretion after HBsAg stimulation or HB vaccination.[40, 41]

In conclusion, the results of the present study show that certain HLA molecules significantly influence the cytokine response to HBsAg stimulation. In turn, these cytokine responses may influence the outcome of both cellular and humoral immune responses to HBV vaccine or infection. Further studies are required to get insights into the complex interplay between HLA polymorphisms and cytokines in HBV immunity among populations with various HLA allelic distributions.

ACKNOWLEDGMENTS

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