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RAPID COMMUNICATION

Relationship between T-lymphocyte cytokine levels and sero-response to hepatitis B vaccines

Vijayakumar Velu, Shanmugam Saravanan, Subhadra Nandakumar, Esaki Muthu Shankar, Appasamy Vengatesan, Suresh Sakharam Jadhav, Prasad Suryakant Kulkarni, Sadras Panchatcharam Thyagarajan

Vijayakumar Velu, Shanmugam Saravanan, Subhadra Nandakumar, Esaki Muthu Shankar, Sadras Panchatcharam Thyagarajan, Department of Medical Microbiology, Dr ALM PGIBMS, University of Madras, Chennai 600113 and National Referral Centre for Viral Hepatitis, India
Vijayakumar Velu, Vaccine Research Centre, Department of Microbiology and Immunology, Emory University, Atlanta, Georgia, 30329, United States
Sadras Panchatcharam Thyagarajan, Sri Ramachandra University, Porur, Chennai 600113, India
Saravanan Shanmugam, Esaki Muthu Shankar, Sadras Panchatcharam Thyagarajan, the YRG Centre for AIDS Research and Education, VHS campus, Taramani, Chennai 600113, India
Suresh Sakharam Jadhav, Prasad Suryakant Kulkarni, Serum Institute of India Ltd., Pune, India
Appasamy Vengatesan, Clinical Epidemiology Unit, Stanley Medical College, Chennai 600001, Tamilnadu, India
Author contributions: Velu V, Thyagarajan SP, Kulkarni PS, Jadhav SS designed the experiments; Velu V, Kulkarni PS organized the figures and wrote the manuscript; Velu V, Saravanan S, Nandakumar S performed the research, analyzed the data; Shankar EM and Vengatesan A performed the statistical analysis.
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Correspondence to: Dr. Vijayakumar Velu, Department of Medical Microbiology, University of Madras, Chennai 600113, India. vvjai2000@yahoo.com
Telephone: +91-44-22542929 Fax: +91-44-2542939
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Abstract

AIM: To investigate the cellular defects by analyzing the (Th1/Th2) cytokine levels in vaccine responders and non-responders.

METHODS: Peripheral blood mononuclear cell (PBMC) from responders and non-responders were stimulated with or with out recombinant HBsAg or PHA. Broad spectrum of cytokines viz (Th1) IFN-γ, IL-2, TNF-α, IL-12 and (Th2) IL-10, IL-4 were measured after *in vitro* stimulation with recombinant HBsAg and were compared with respective antibody titers.

RESULTS: A significant decrease (P = 0.001) in Th1 and Th2 cytokines namely, IL-2, INF-γ, TNF-α and IL-10 in non-responders was observed. The level of IL-4 was not significant between the three groups. Furthermore, despite a strong Th1 and Th2 cytokine response, the level of IL-12 was elevated in high-responders compared to other groups (P = 0.001) and demonstrated a positive correlation with anti-HBs titers and Th1 cytokine response.

CONCLUSION: Our findings suggest that unresponsiveness to recombinant hepatitis B vaccines (rHB) is multifactorial, including specific failure of antigen presentation or the lack of both T helper Th1 and Th2 response.

Key words: Hepatitis B vaccine; Cytokines; Humoral response; T cell response; Adult vaccines

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INTRODUCTION

Hepatitis B is one of the world's major health problems[1]. It is estimated that more than 2 billion people are infected with hepatitis B virus (HBV) globally, and more than 400 million are chronic carriers[2]. The infection is supposed to be causally related to 1 to 2 million deaths per year worldwide[2]. Vaccination with the surface antigen of HBV (HBsAg) is considered the main strategy for effective control of the infection and viral transmission[3,4]. Recombinant hepatitis B vaccines (rHB) are recommended for Universal vaccination of neonates, as well as the high-risk healthy adult individuals[5]. Even though conventionally it is believed that the available HB vaccines induce only circulating humoral immunity, occasional reports suggest the possibility of induction of cell mediated immune response by HB vaccines.

The HB vaccine induces a protective antibody response (anti-HBs antibody ⩾ 10 mIU/mL) in the majority of individuals after three dose regimen. However, about 10% of healthy recipients fail to generate protective levels of antibodies to the vaccine after standard immunization[6,7]. Furthermore, non-responders remain susceptible to infection with HBV[8].

Inadequate immune response to HBsAg could be attributable to a variety of mechanisms including defect in the generation of primary HBsAg-specific T-cell[9] or B-cell repertoires[10], expression of certain human leukocyte antigens (HLA) and haplotypes[11,12], destruction of HBsAg-specific B-cells by antigen-specific cytotoxic T cells[13], immunologic tolerance[14,15] and imbalance in T-helper (Th) cell function[16].

HBsAg is a glycoprotein antigen with T-cell dependent characteristics. Induction of specific antibody to this antigen requires coordinated secretion of Th1 and Th2 cytokines leading to maturation and differentiation of the HBsAg-specific B-cell clones. Therefore, defective T-helper (Th) cell function, either Th1 or Th2, could result in failure of immune response to this antigen.

We compared three rHB vaccines; GeneVac-B® (Serum Institute of India Ltd, Pune), Engerix-B® (SmithKline Beecham Biologicals, Belgium) and Shanvac-B® (Shantha Biotechnics, India) in 400 healthy adults[17]. All three vaccines induced similar humoral immune (anti-HBs) response in the vaccines. However, a proportion of approximately 2% of the vaccines did not show adequate antibody response (> 10 mIU/mL) to the vaccines. In order to better understand the non-responsiveness to these vaccines, we evaluated, for the first time in south Indian population, the broad spectrum of Th1 and Th2 cytokines levels in healthy adults vaccinated with rHB vaccines.

MATERIALS AND METHODS

Subjects

The subjects were healthy volunteers aged between 25 to 40 years. All subjects received intramuscular injections of three doses of 20 μg of any of the three rHB vaccines with 0, 1 and 2 month regimen. Four weeks after the three dose of vaccination, serum anti-HBs levels were quantified using commercially available anti-HBs kit. Based on the anti-HBs titers, subjects were identified and recruited in the study as non-responders (< 10 mIU/mL), hypo-responders (10 to 100 mIU/mL) and high-responders > 100 mIU/mL (Figure 1). Four weeks following the third dose, blood samples were obtained and peripheral blood mononuclear cells (PBMCs) were separated from 27 volunteers that comprised of 7 non-responders, 10 hypo-responders and 10 high-responders to rHB vaccines for *in vitro* stimulation of the lymphocytes with HBsAg and PHA.

Measurement of anti-HBs antibody response

Vaccines were screened for anti-HBs titers 4 wk after vaccination using the commercial Monolisa anti-HBs kit (BioRad, Belgium). Anti-HBs titers were quantified by extrapolation from a standard curve constructed using a serum sample with known concentration of antibody, provided by the manufacturer. All these volunteers were also tested for anti-HCV and anti-HIV antibodies to rule out the possibility of immunosuppression.

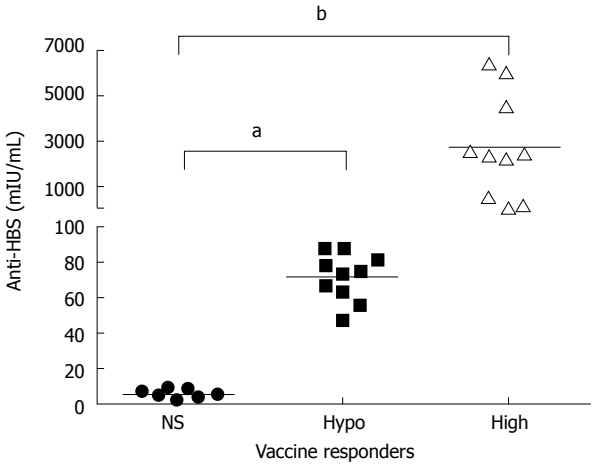

Figure 1 Comparison of anti-HBs levels from vaccine non-responders (NS), hyporesponders (Hypo) and high-responders (High) after booster HBV vaccination. [a]$P < 0.05$; [b]$P = 0.001$.

In vitro stimulation of PBMC

PBMC were separated from EDTA-anticoagulated venous blood by density gradient centrifugation on Ficoll-Paque (Amersham Biosciences, NJ, USA). After washing in RPMI-1640 medium (Himedia, India), PBMC were suspended in complete culture medium containing RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (FCS) (Himedia India), 2 mmol/L L-glutamine (Himedia, India), 100 μg/mL penicillin and 100 μg/mL Gentamicin (Gibco BRL, Scotland). PBMC were seeded at 1×10^6 cells/mL in a 24-well sterile tissue culture plate (Nunc, USA) in the presence or absence of 5 μg/mL of purified rHBsAg provided by Serum Institute of India, and 5 μg/mL of PHA (Gibco BRL, Gaithersburg, MD) were used as positive controls and un stimulated cells act as a negative control. The plates were incubated for 72 h at 37℃ in a humidified CO_2 (5%) incubator (Nuaire, USA). Culture supernatants were collected and stored at -70℃ until use.

Cytokine assays

Supernatants from the PBMC proliferation assays were harvested after 72 h and cytokine levels (IFN-γ, IL-2, TNF-α, IL-12, IL-10 and IL-4) were measured using commercial sandwich ELISA kits (Biosource Europe, SA) as per manufacturer's instructions. Briefly, culture supernatants distributed in 96-well plates coated with corresponding anti-cytokine antibodies were used to detect cytokine anti-cytokine complexes. The reaction was developed with TMB in 0.1 mol/L sodium acetate solutions and H_2O_2. Optical density was read at 450 nm. The concentration of cytokines in culture supernatants were calculated from the standard curve for each cytokine plotted on a log-log paper. The sensitivities of the assays for IFN-γ, IL-2, TNF-α, IL-12, IL-10 and

Table 1 Levels of cytokines secreted *in vitro* from PBMC of high, hypo and non-responder adults following stimulation with HBsAg

Cytokines/units	Stimulations	High-responders	Hypo-responders	Non-responders	*P* value	Multiple comparison[2]
IFN-γ (U/mL)	HBsAg	18 ± 5.3	13.4 ± 5.6	6.8 ± 3.6	0.001[1]	Ns *vs* Hy *vs* Hi
IL-2 (U/mL)	HBsAg	15.9 ± 5.5	8.4 ± 1.6	5.1 ± 1.8	0.001[1]	Ns *vs* Hi
TNF-α (pg/mL)	HBsAg	201.5 ± 86.9	64.8 ± 49.0	21.4 ± 4.5	0.001[1]	Ns *vs* Hi
IL-12 (pg/mL)	HBsAg	512.8 ± 213.6	117.6 ± 58.2	23.8 ± 7.6	0.001[1]	Ns *vs* Hi
IL-10 (pg/mL)	HBsAg	260.8 ± 128.8	60 ± 49.7	47 ± 38.7	0.001[1]	Ns *vs* Hi
IL-4 (pg/mL)	HBsAg	83.2 ± 72.3	62.8 ± 44.4	49.2 ± 39.9	-	Not significant

[1]Significant; Test of significance was One way ANOVA *F*-test; [2]Multiple comparison by Bonferroni *t*-test.

Table 2 Comparison of cytokine levels secreted after *in vitro* stimulation with HBsAg and PHA

Cytokines	Stimulation	High-responders	Hypo-responders	Non-responders
IFN-γ (U/mL)	HBsAg	18+ ± 5.3	13.4+ ± 5.6	6.8 ± 3.6
	PHA	21.2 ± 5.0	21.9 ± 2.8	18 ± 3.8
	P	0.19	0.001	0.001
IL-2 (U/mL)	HBsAg	15.9 ± 5.5	8.4 ± 1.6	5.1 ± 1.8
	PHA	19.8 ± 2.3	19 ± 3	16.8 ± 3.4
	P	0.06	0.001	0.001
TNF-α (pg/mL)	HBsAg	201.5 ± 86.9	64.8 ± 49.01	21.4 ± 4.5
	PHA	680.2 ± 138.4	626.4 ± 168.6	621.8 ± 198.7
	P	0.001	0.001	0.001
IL-12 (pg/mL)	HBsAg	512.8 ± 213.6	117.6 ± 58.2	23.8 ± 7.6
	PHA	611.3 ± 140.0	597.7 ± 141.8	491 ± 130.76
	P	0.24	0.001	0.001
IL-10 (pg/mL)	HBsAg	260.8 ± 128.8	60 ± 49.7	47 ± 38.7
	PHA	651.6 ± 132.07	606 ± 165.88	519.1 ± 152.2
	P	0.001	0.001	0.001
IL-4 (pg/mL)	HBsAg	83.2 ± 72.3	62.8 ± 44.4	49.2 ± 39.9
	PHA	610 ± 135.63	607.9 ± 163.4	569 ± 109.34
	P	0.001	0.001	0.001

Test of significance was student independent *t* test.

IL-4 were 0.1 U/mL, 0.1 U/mL, 3 pg/mL, 1.5 pg/mL, 1 pg/mL, 2 pg/mL, respectively.

Statistical analysis

The data generated were analyzed using the statistical package for social sciences, (SPSS, version 13.0, Chicago, IL, USA). Anti-HBs response to all volunteers is expressed as geometric mean titers statistical difference was obtained by student *t* test. Differences in cytokine concentrations between the three groups of subjects were analyzed with the one-way ANOVA *F* test. Comparison of three groups between two different stimulations were analyzed by using multiple comparisons by Bonferroni "*t*" test. Correlations were calculated using Pearson's test. *P* values $\leqslant$ 0.05 were considered as significant.

RESULTS

Booster vaccination to HBV elicited broad spectrum of cytokines in high-responders

Booster HB vaccination induces a strong humoral response; however, approximately 2% of healthy adults in our study fails to induce Ab response. Based on the production of the antibody levels volunteers were classified in to non responders and responders (Figure 1). There was a general correlation between serum anti-HBs level and the levels of Th1 and Th2 type cytokine response *in vitro*. A significant increase in the production of Th1 and Th2 cytokines was observed following stimulation of PBMC from high-responders with HBsAg except the Th2 cytokine IL-4. On the other hand, PHA induced two to three fold higher levels of all the cytokines in all the groups compared to HBsAg stimulation (Tables 1 and 2). This difference was significant for TNF-α, IL-10, and IL-4. However, there was no difference in the IFN-γ, IL-2 and IL-12 levels. In addition, there was a several fold difference between the production of all cytokines between HBsAg and PHA stimulation in non-responders and hypo-responders (Table 2) which clearly demonstrate that the non-responsiveness is restricted to HBsAg specific response.

Deficient Th1 cytokine levels in non-responders after booster immunization

The overall Th1 cytokine profile was elevated in high-responders compared with other two groups (Figure 2A). IFN-γ production was significantly higher in high and hypo-responders compared to non-responders ($P = 0.001$). However, high-responders secreted higher levels of IL-2 and TNF-α compared with hypo-

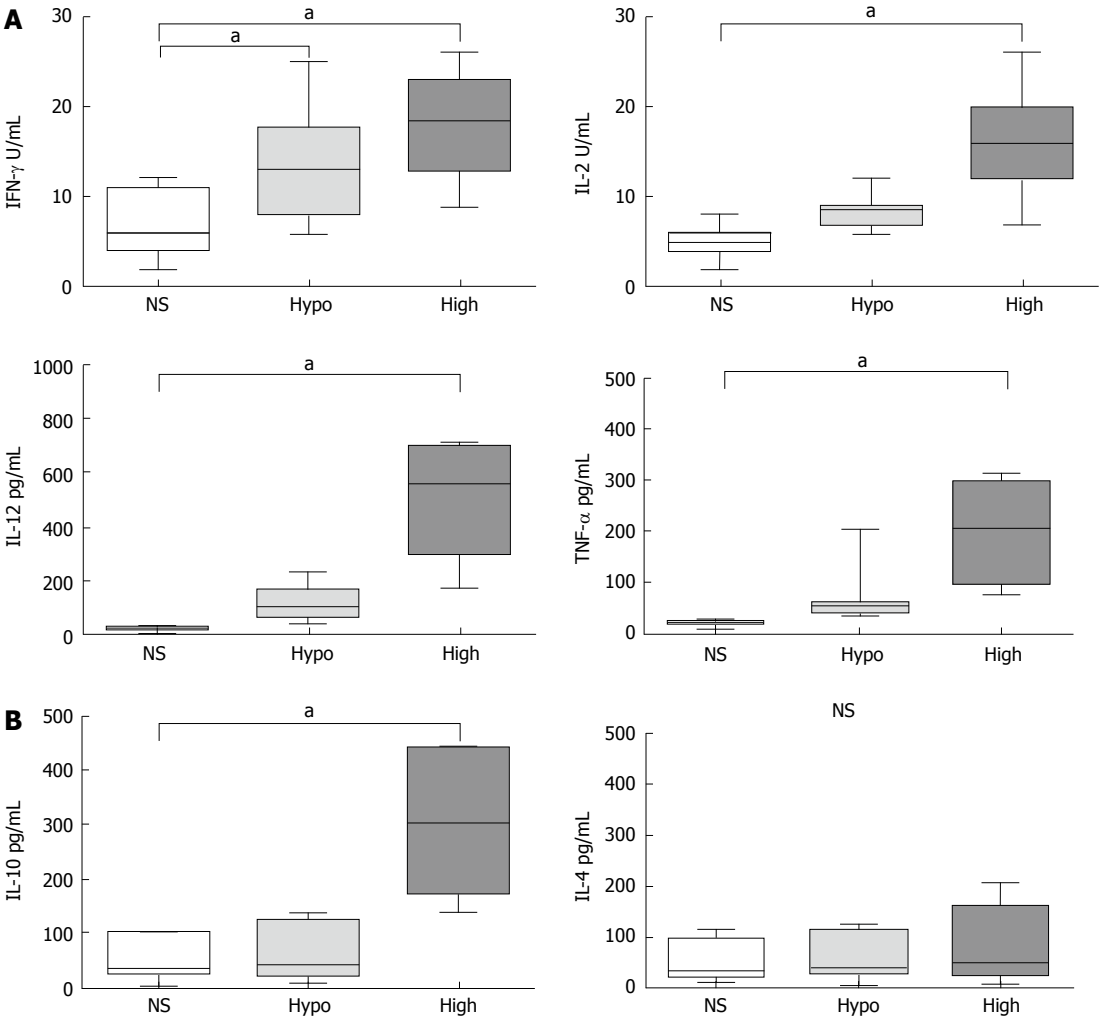

Figure 2 Cytokine secreted by PBMC's from high-responders, hypo-responders and non-responder adults after *in vitro* stimulation with HBsAg. **A**: Comparison of Th1 cytokines (IFN-γ, IL-2, TNF-α, and IL-12) between the three groups of vaccine responders; **B**: Comparison of Th2 cytokines (IL-10 and IL-4) between the three groups of vaccine responders. [a]$P < 0.05$.

responders and non-responders (Table 1 and Figure 2A). IFN-γ and IL-2 levels with HBsAg in high-responders were comparable with those of PHA, however, which was not observed in hypo or non-responders. A stronger positive correlation was observed between Th1 cytokine IFN-γ ($r^2 = 0.254$) and IL-2 ($r^2 = 0.424$) production when compared with the anti-HBs response between the three groups of volunteers (Figure 3A). On the contrary, a weak correla-tion was observed with TNF-α ($r^2 = 0.183$) production. Furthermore, the levels of IFN-γ and IL-2 strongly correlated with the levels of IL-12 ($r^2 = 0.52$) when compared between the three groups (data not shown).

Modulation of IL-12 production in non-responders and high-responders

There was a significant enhancement in production of IL-12 ($P = 0.001$) in high responders compared with hypo-responders and non-responders (Figure 2A). Furthermore, a strong correlation ($r^2 = 0.436$) was observed between the *in vivo* anti-HBs and *in vitro* IL-12 levels (Figure 3A). On the whole, our results suggest the existence of a strong association between the levels of IL-12 and Th1 cytokines, which strongly implies that IL-12 may play a major regulatory role in the modulation of Th1 cytokine production in high-responders.

Elevated levels of Th2 cytokine response in high-responders after booster immunization

PBMC from high-responders produced significantly more IL-10 levels than those of hypo-responders and non-responders ($P = 0.001$). The levels of IL-4 were very low and were comparable between the three groups of volunteers. However, they were significant when compared with PHA stimulations ($P = 0.001$). No significant correlation was observed between anti-HBs production and IL-4 response ($r^2 = 0.088$) (Figures 2B and 3B). In addition, a strong negative correlation was observed between the Th1 cytokine production and the IL-4 response (data not shown). In contrast, a weakly significant correlation was observed between IL-10 production and anti-HBs response ($r^2 = 0.25$) in the volunteers (Figure 3B).

DISCUSSION

Correlation between the humoral response *in vivo* and the

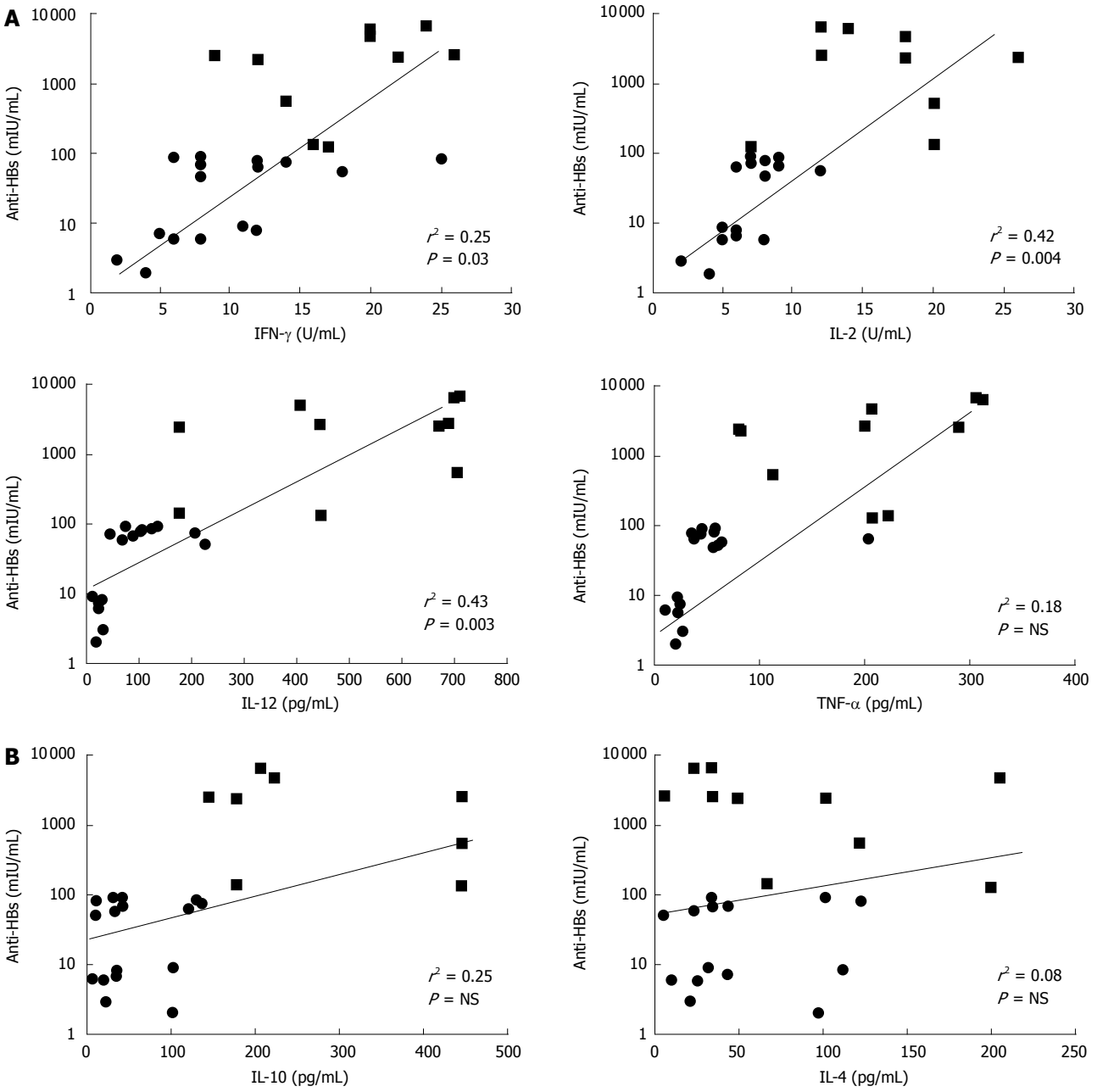

Figure 3 Correlation between anti-HBs titers and cytokine levels secreted after *in vitro* stimulation of PBMC with HBsAg from high-responder, hypo-responder and non-responder adults. **A**: Correlation of anti-HBs antibody response and the Th1 cytokine (IFN-γ, IL-2, IL-12 and TNF-α) response to HBsAg; **B**: Correlation of anti-HBs antibody response and the Th2 cytokine (IL-10 and IL-4) response to HBsAg.

cellular response *in vitro* to HBsAg is dependent on the cytokine secretion profile of activated T lymphocytes[18]. Protective immune response to HBsAg is associated with the production of HBsAg specific neutralizing antibody[19,20]. The process of antibody production to this HBsAg is T-cell dependent and requires Th cell activation[20]. Secretion of Th2-like cytokines, such as IL-4, IL-5, IL-6, IL-10 and IL-13, is thought to be detrimental for B-cell differentiation and production of specific antibodies, whereas secretion of Th1-like cytokines, namely IL-2, IFN-γ, TNF-α and transforming growth factor (TGF)-β triggers the cell-mediated immune response leading to cure of HBV infected heptocytes or destruction of HBV-infected cells[21-23].

Several investigators have tried to study the pattern of cytokine production in a variety of diseases[20,24], including investigations in unresponsiveness to HBsAg in the recent past[26]. The results have been contradictory in analysis of *in vitro* HBsAg-induced cytokine production. These included absence of Th1 cytokine production in non-responders[9,26,27], lack of Th2 response in both responders and non-responders[27], absence of Th1 and Th2 cytokines in non-responders[27,28] and production of both cytokines in high-responders[28]. However, in contrast to the above findings there are also report showing no correlation between the function and cytokine production of HBsAg specific CD4 T cells[29,30]. In addition, predominant Th2 and Th1 responses have also been reported in high and low-responders, respectively[31].

Our findings of diminished Th1 and Th2 responses

in non-responders confirmed and extended the results reported by others[25,28]. However, those studies assessed only a few cytokines, whereas in our study we looked at a broad spectrum of cytokines which represents Th1/Th2 profiles. Both types of cytokines Th1 cytokines (IL-2, IFN-γ, TNF-α, IL-12) and Th2 cytokines (IL-10, IL-4) were secreted at significantly higher levels in high-responders compared with hypo-responders and non-responders. In addition the regulatory cytokine levels IL-12 were highly elevated in the high-responders compared to hypo-responders and non-responders, furthermore it is demonstrated that IL-12 induction of Th1 response is important for viral clearance in subjects suffering with chronic HB infection[24,27].

The limitations in our study would be a small sample size and the variations in responder's cytokine profiles. However, the overall differences between the three groups of vaccines are pronounced (Table 1 and Figures 1 and 2). The significance of our results is more magnified when analyzed in the context of PHA-induced cytokine production. Despite the production of several-folds higher concentration of all cytokines in response to PHA, as compared with HBsAg, in all three groups of responders (Table 2), no significant differences were observed between the stimulations for IFN-γ, IL-2, IL-12, IL-10, IL-4 and TNF-α (Table 2). These findings strongly agree and suggest the possibility of involvement of a generalized immune dysfunction in the non-responder subjects with regard to HBsAg stimulation and culture condition.

In summary, we have demonstrated diminished production of broad range of cytokines IFN-γ, IL-2, TNF-α, IL-12, IL-10 and IL-4 in PBMC from healthy non-responders to HB vaccine, suggesting insufficient or lack of Th1 and Th2 responses. This could be because of a defect in either the primary HBsAg-specific T-cell repertoire[9] or antigen presentation[32]. Our study in non-responder adults strongly suggests the contribution of IL-12 cytokine levels which may lead to the dysfunction of antigen-presenting cells in unresponsiveness to the vaccine.

COMMENTS

Background

Hepatitis B virus (HBV) infection is one of the leading causes of morbidity and mortality world wide. Since there is no effective treatment for HBV, vaccination plays a vital role in preventing the infection. Hepatitis B vaccine induces a protective immune response in the majority of individuals. However, 4%-10% of healthy recipients fail to generate an effective antibody response against HBV after standard immunization. Inadequate immune response could be attributed to a variety of mechanisms. Here we made an attempt to study the cellular defects attributed to HB vaccine non-responders and responders.

Research frontiers

This study was undertaken in order to analyze the cellular (cytokines) defects associated with non-responsiveness to hepatitis B (HB) vaccine in healthy individuals. Here we demonstrated that peripheral mononuclear cells (PBMC) isolated from anti-HBs seropositive subjects after booster injection were able to make both Th1/Th2 cytokines *in vitro* by stimulation with the surface antigen of HBV (HBsAg) in responses. In contrast, under the same conditions, non-responder PBMC failed to produce cytokines *in vitro*, furthermore we also demonstrated Th1 cytokine profile dominant compared to the Th2 profile and a strong positive correlation was obtained when comparing the regulatory cytokine (IL-12) production with the strong anti-HBs response. To the best of our knowledge this is the first study which addressed the lack of Th1/Th2 cytokine profile in the south Indian population.

Innovations and breakthroughs

Vaccination with HBsAg induces protective immunity through T-helper (Th) cell dependent production of anti-HBs antibody. Several studies have been conducted to investigate the precise role of Th1 and Th2 derived cytokines in the immune response to hepatitis B vaccine and to get further insights into the cellular basis of unresponsiveness to HBsAg. Controversial results, however, have been reported. Analyses of *in vitro* HBsAg-induced cytokine production have revealed defects in: Th1 cytokines in non-responder subjects; Th2 response in both responder and non-responder groups; Th1 and Th2 cytokines in non-responders; Different patterns of cytokine production have been observed in T-cell clones isolated from responder subjects, with either predominant Th0 or Th2 response; or Th1 and Th2 responses in high and low responders, respectively. Insufficient production of both types of cytokines in healthy non-responder individuals has been demonstrated. However, in this study we looked into the broad spectrum of cytokines and correlated them with their antibody production. Furthermore we also observed significant difference in the production of IL-12 by HBsAg in high-responders compared with non-responders. The significance is more magnified when analyzed in the context of the PHA induced IL-12 profile. Despite the production of higher concentration of IL-12 in response to PHA, as compared to HBSAg in both responders and non-responders subjects, no significant difference was observed between the groups, these results emphasize the exclusion of the possibility of involvement of generalized immune dysfunction on non-responders adults. Furthermore increase in IL-12 levels also strongly correlated with the induction of Th1 response. Taken together our data suggest that the non-responsiveness is associated with the defective production of both Th1 and Th2 cytokines.

Applications

Our findings of significantly increased production of all cytokines in response to HBsAg as compared to control cultures without stimulation in responder vaccines, together with significantly higher secretion of these cytokines induced by PHA compared to HBsAg in non-responder, but not responder adults, may have important implications. These results suggest that in addition to the serum levels of anti-HBs antibody, the profile of cytokine secretion could also be used as an objective criteria and distinctive parameter to identify hepatitis B vaccine responder and non-responder individuals.

Peer review

The authors compared a broad spectrum of cytokine (Th1/Th2 cytokine) response in hepatitis B vaccine non-responders, hypo-responders and high responders. They also demonstrated that lack of both Th1/Th2 cytokine profiles are associated with the non-responsiveness to hepatitis B vaccine.

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