

T cell responses to hepatitis B surface antigen are detectable in non-vaccinated individuals

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after the third vaccination. Surprisingly, these individuals showed response even before the first vaccination. T cell response to control antigens and mitogens was similar in all groups.

CONCLUSION: Our data suggest that there is no general immune deficiency in non-/low-responders. Thus, we hypothesize that the induction of anti-HBsAg responses by vaccination is significantly dependent on the pre-existing T cell repertoire against the specific antigen rather than the presence of a general T cell defect.

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Abstract

AIM: To evaluate, whether humoral hepatitis-B-vaccine non-responders also fail to mount a T cell response and to compare these results to normal vaccines.

METHODS: Forty-seven health care employees were enrolled in this study including all available non-responders ($n = 13$) with an anti-HBsAg titer < 10 kU/L and all available low-responders ($n = 12$) with an anti-HBsAg titer < 100 kU/L. Also, 12 consecutive anti-HBsAg negative pre-vaccination subjects were enrolled as well as 10 subjects (+7 from the vaccinated group) with titers > 1000 kU/L as controls. PBMC from all subjects were analyzed by IFN- γ and IL-4 ELISPOT assays for the presence of hepatitis B surface antigen (HBsAg) reactive T cells.

RESULTS: Non-responders and low-responders had no or only very limited T cell responses, respectively. Individuals responding to vaccination with the induction of a high anti-HBsAg titer showed a strong T cell response

INTRODUCTION

Worldwide, hepatitis B virus (HBV) infections are a growing problem with a high prevalence of over 8% hepatitis B surface antigen (HBsAg) positive individuals in Africa, South America, parts of Eastern Europe, South Asia, and Canada^[1]. It is estimated that approximately 350 million people are chronic carriers of HBV with 1-1.5 million dying from liver cirrhosis and primary liver cancer^[2]. Nowadays, protective repeated vaccinations with recombinant HBsAg are not only recommended to all health care workers and travellers, but have recently been included in the childhood and adolescence immunization schedule. Hepatitis B vaccinations prevent HBV infections as well as its complications in most of the vaccines^[3,4]. However, 5% to 10% fail to produce protective anti-HBsAg titers after three vaccinations irrespective of the source of the antigen, which is a problem not only for health care workers, and represents a major medical as well as economic challenge^[5-7]. Low- (HBsAg titer 10-99 kU/L) or

non-responsiveness (HBsAg titer < 10 kU/L) to vaccination are associated with certain human leukocyte antigen (HLA)-class II alleles. DRB1*0301, DRB1*0701, and DQB1*0201^[5,8,9] were shown to have a higher prevalence in non-responders, whereas other antigens (DRB1*0101, *1301, *1501, and DPB1*0401) seem to mediate strong immune responses^[9-12]. Higher age, obesity, male gender, smoking, and chronic dialysis are risk factors for a non-/low-responsiveness^[8,9,13-15].

Monitoring of vaccination efficacy is currently performed by measurements of humoral immune responses (antibody titers) using ELISA assays, which does only indirectly reflect antigen-specific T cell responses. However, the T cell response plays an important role in the immune defence against viral infections. Patients with defects in T-cell function or repertoire such as human immunodeficiency virus (HIV) or transplant patients often suffer from opportunistic infections with viruses including cytomegalovirus (CMV), Epstein-Barr virus (EBV), herpes simplex viruses (HSV), and varicella zoster virus (VZV). It remains unclear, if vaccines with no or low anti-HBsAg titer similarly have a compromised T cell response to HBsAg^[16-18]. Thus, it remains an open question, whether a B cell non-responder is also a T cell non-responder. To clarify this issue, we initiated a study to analyze whether HBsAg non- or low-responders are able to mount a significant T cell response against the HBsAg and compared them to "normal" vaccines.

MATERIALS AND METHODS

Healthy individuals included in the study

Forty-seven healthy employees of the University of Cologne (median age 32 years, range: 19-61 years, 21 male, 35 female) were enrolled in this study. These 47 employees including 12 consecutive health care employees, who received the first hepatitis B vaccine, were included as well as 10 vaccinated subjects with an anti-HBsAg titer > 1000 U/L. Documented non- and low-responders were contacted and asked to participate in the study, of whom 25 agreed. Of these 25, 13 individuals were non-responders as defined by an anti-HBsAg titer of 0-9 IU/L after the third of three vaccinations and 12 were low-responders (titer 10-99 IU/L). In this study, 3 different groups were analyzed. Non-responders ($n = 13$, titer 0-9 U/L), low-responders ($n = 12$, titer 10-99 U/L), vaccines before the first and after the third vaccination ($n = 12$), and high-responders. The last group comprises 10 selected individuals with an anti-HBs titer > 1000 U/L plus 7 vaccines after the third vaccine with a titer > 1000 U/L (total $n = 17$). Therefore, the patient number ($n = 47$) and the subjects analyzed in the 3 groups ($n = 54$) do not match.

Cell separation and ELISPOT analysis

PBMC were isolated by Ficoll density centrifugation from 15 mL of blood and stored in liquid nitrogen until performance of ELISPOT assays. Membrane-bottomed 96-well plates (MAHA, Millipore) were coated overnight with 50 μ L of anti-IFN- γ or anti-IL-4 antibodies (Hölzel, Cologne, Germany) at a concentration of 10 mg/L car-

bonate coating buffer (0.1 mol/L Na₂CO₃, 0.1 mol/L NaHCO₃, pH 9.6) at 4°C. Plates were washed three times with RPMI 1640 and incubated with CellGenix medium (CellGenix, Freiburg, Germany) supplemented with 100 mL/L fetal calf serum (FCS) for 1 h at 37°C. Triplets of 2×10^5 PBMC in 100 μ L CellGenix medium containing Glutamax I (Gibco BRL, Karlsruhe, Germany) were added per well and incubated with medium only, 5 mg/L yeast-derived HBsAg (subtype adw, Biotrend, Cologne, Germany), 5 LF/mL tetanus toxoid, or 10 mg/L pokeweed mitogen (PWM) at 37°C and 5% CO₂. After 72 h, plates were extensively washed with PBS/0.05% Tween and incubated with 100 μ L/well of 10 mg/L biotinylated anti-IFN- γ or anti-IL-4 antibodies (Hölzel, Cologne, Germany) for 2 h at 37°C and 5% CO₂. After washing with PBS, plates were incubated with 100 μ L/well of 1:2000 diluted streptavidin-ALP for 1 h at 37°C and 5% CO₂. Development of spots was performed with 50 μ L/well of chromogenic alkaline phosphatase substrate (BCIP/NBT, Sigma Aldrich, Germany). The reaction was terminated after approximately 5 min by rinsing plates with tap water. Spots were counted after drying of plates with an automated AELVIS Plate Elispot reader (AELVIS GmbH, Hannover, Germany). Specific T cell frequencies were calculated by subtracting mean background + 2 times standard deviation of background from counted spots. Therefore, the absolute numbers of spots are relatively low compared to data from other groups.

Statistical analysis

All statistical calculations were performed with the software package SPSS V12.0 for Windows (Chicago, IL). T cell responses between groups were compared by using a 2-sided Student *t*-test. Probabilities $P < 0.05$ were considered as statistically significant.

RESULTS

Subjects with high anti-HBsAg titers show a strong T cell response

All subjects with an anti-HBsAg titer > 1000 IU/L were analyzed for T-cell responses against the HBsAg and tetanus. This group comprised of a total of 17 subjects (6 male, 11 female, including 7 subjects from the vaccinee group) with a median age of 31 years (range: 20-44 years). The average IFN- γ spot count for HBsAg was 5.4 ± 5.1 , the average IL-4 spot count was 1.0 ± 2.1 . The average IFN- γ spot count for tetanus Ag was 9.1 ± 6.2 , the average IL-4 spot count was 3.0 ± 3.7 .

Non-/low responders lack a sufficient T cell response against HBsAg

Twenty-five subjects had an anti-HBsAg titer < 100 IU/L after at least 3 vaccinations, of whom 13 were non-responders (anti-HBsAg titer of 0 IU/L) and 12 were low-responders (mean anti-HBsAg titer 36 ± 16 IU/L). The median age of non-responders and low-responders was 40 years (range: 20-57 years) and 32 years (range: 19-61 years), respectively. Of the 13 non-responders, 4 were male, 9 female, of the 12 low-responders 4 were male, 8

Table 1 Number of spots in IFN- γ and IL-4 ELISPOT assays for all individuals (*n*)

	<i>n</i>	Mean anti-HBsAg titer (IU/L)	IFN-g response (# of spots)		IL-4 response (# of spots)	
			HBsAg	TT Ag	HBsAg	TT Ag
Non-responders	13	0 \pm 0	1.0 \pm 1.3	9.1 \pm 4.3 (6)	0.2 \pm 0.3	3.5 \pm 3.9 (6)
Low-responders	12	36 \pm 16	1.7 \pm 2.6	7.8 \pm 6.5 (7)	0.5 \pm 1.0	6.2 \pm 10.1 (4)
High-responders	17	> 1000	5.4 \pm 5.1	9.1 \pm 6.2	1.0 \pm 2.1	1.7 \pm 3.9 (13)
Vaccines before 1st vx	12	0 \pm 0 (9)	4.5 \pm 5.7	11.2 \pm 7.3 (11)	0.3 \pm 0.5 (9)	4.5 \pm 4.9 (5)
Vaccines after 3rd vx	12	10 \times > 1000, 1 \times 211, 1 \times 394	7.1 \pm 6.2	10.0 \pm 8.4	2.7 \pm 5.6	3.0 \pm 4.4 (8)

were female. PBMCs were stimulated with medium only, HBsAg, tetanus Ag, or pokeweed mitogen (PWM) to evaluate T-cell specific cytokine release of IFN- γ and IL-4. When assessing T cell responses against the mitogen PWM, there was no difference between non-responders, low responders, or high responders. A strong cytokine production occurred in all samples after stimulation with PWM (positive control). Similarly, when assessing antigen-specific T cell responses against tetanus Ag, we observed comparable T cell responses in all three groups. Non-responders showed a mean IFN- γ response against tetanus Ag of 9.1 \pm 4.3 spots (*n* = 6) and a mean IL-4 response of 3.5 \pm 3.9 spots (*n* = 6). Low-responders had an average count of 7.8 \pm 6.5 IFN- γ spots (*n* = 7) and 6.5 \pm 10.0 IL-4 spots (*n* = 4). For high-responders, T cell response against tetanus Ag was 9.1 \pm 6.2 IFN- γ spots (*n* = 11) and 4.5 \pm 4.9 IL-4 spots (*n* = 5) before vaccination and 10.0 \pm 8.4 IFN- γ spots (*n* = 17) and 1.7 \pm 3.9 IL-4 spots (*n* = 13, Table 1).

In contrast, when assessing responses to the HBsAg, we observed clear differences between the three groups. Non-responders had a mean anti-HBsAg IFN- γ T cell response of 1.0 \pm 1.3 spots and a mean IL-4 T cell response of 0.2 \pm 0.3 spots. Low-responders showed a slightly higher T cell response with mean IFN- γ responses of 1.7 \pm 2.6 spots and a mean IL-4 T cell response of 0.5 \pm 1.0 spots) compared to high-responders with an average IFN- γ spot count for HBsAg of 5.4 \pm 5.1, and an average IL-4 spot count of 1.0 \pm 2.1.

Taken together, although all individuals tested showed strong responses to the mitogen PWM and significant responses to tetanus Ag, there was a clear difference between non-responders and high responders (*P* = 0.006 for IFN- γ results, *P* = ns for IL-4) with low-responders showing intermediate values. These data suggest that antibody titers indeed correlate with T cell responses as assessed by ELISPOT analysis.

T cell responses against HBsAg are measurable in vaccines before the first immunization

To further corroborate the above findings, we assessed T cell responses and anti-HBsAg titers before the first and after the third vaccination in 12 individuals. The median age was 31 years (range: 23-50 years), 3 subjects were male, 9 were female. All had a negative anti-HBsAg titer before the first and an anti-HBsAg titer > 200 IU/L after the third vaccination suggesting all to be within the intermediate or high-titer group. The average T cell response against HBsAg was 4.5 \pm 5.7 IFN- γ spots and 0.3 \pm 0.5 IL-4 spots

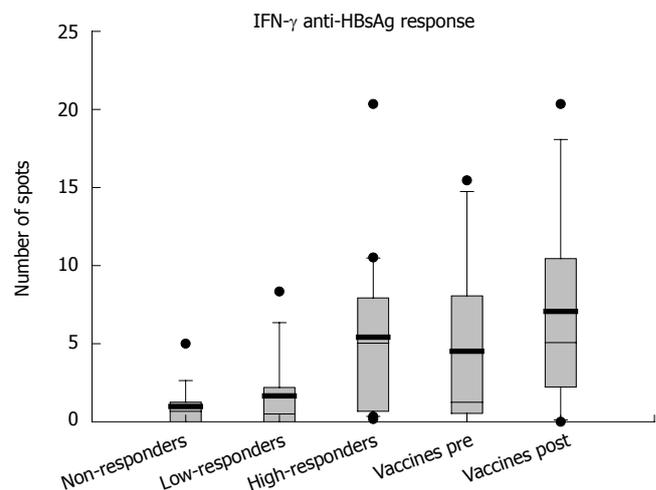


Figure 1 Box and whisker plots of number of IFN- γ spots from ELISPOT assays of PBMC stimulated with HBsAg. The black dots show the outliers.

before the first vaccination and 7.1 \pm 6.2 IFN- γ spots and 2.7 \pm 5.6 IL-4 spots after the third vaccination, respectively. The difference between pre- and post-vaccination anti-HBsAg response was not significant in a Student *t*-test (*P* = 0.08 for IFN- γ , *P* = 0.10 for IL-4, Table 1 and Figure 1).

DISCUSSION

Approximately 5% to 10% of the healthy population fails to mount a protective anti-HBsAg antibody titer after three vaccinations. However, it has not been evaluated, whether patients with a low antibody titer are also incapable of producing an HBsAg specific T cell response. Our data clearly show that humoral non- and low-responders have no or only a limited T cell repertoire reacting to HBsAg compared to responders. Only 1 out of 13 non-responders and 2 out of 12 low-responders had (IFN- γ) T-cell responses that were equal to or exceeded the average T cell response of the high-responder group. Two other studies have reported decreased IL-2, IFN- γ , and IL-10 cytokine production in ELISA assays of non-responders to HBsAg stimulation and conclude that these subjects may have a defect in either the primary HBsAg-specific T cell repertoire or antigen presentation^[16,17]. Our ELISPOT data clearly indicate that non- and low-responders do not have a general immune defect as their T cell response to control antigens (e.g tetanus toxoid) is comparable to that of “normal” vaccines. Also, their lymphocytes were capable of strong cytokine secretion after PWM stimulation.

So what prevents a non-responder from developing a protective HBsAg titer? As non-responsiveness to HBsAg is associated with certain HLA-haplotypes, it has been hypothesized that antigen presenting cells of non-responders are unable to adequately present this specific antigen^[19], although a recently published trial showed that HLA-DR0301 non-responders are not deficient in their HBsAg-presentation and do not lack B7 co-stimulatory molecules^[20]. Other studies suggested that non-responsiveness was caused by the presence of suppressor T cells^[21,22] or the absence of the Th1 cells or cells with TCR specificity for HBsAg^[23]. Salazar *et al*^[24] reported that non-responders show a defect in HBsAg reactive CD4⁺ helper T cells. Our data extend these hypotheses suggesting that a preexisting T cell repertoire exists in the majority of vaccines that may be critical for strong post vaccination T cell responses. Except for 2 out of 12 individuals, all vaccines had a (IFN- γ) T cell response before vaccination. In contrast, non-/low-responders lacked a significant T cell response. Thus, we hypothesize that a pre-existing immunologic T cell cross-reactivity against the HBsAg is necessary in order to respond to vaccinations.

This cross-reactivity is probably triggered by a common infectious agent, which is processed by antigen presenting cells to peptides similar in structure to parts of the HBsAg. In certain HLA-haplotypes MHC/peptide complexes do or do not induce crossreactive effector or regulatory T cells. This could be the explanation, why non-/low-responsiveness is linked to certain MHC/HLA-types. What we do not know, is whether this cross-reactive T cell response alone yields any protection against a HBV infection. But because HBsAg vaccinations are a very effective measure against a new infection^[25,26], it seems that a concurrent B cell response (*i.e.* protective anti-HBsAg titer) is primarily necessary to prevent HBV replication.

In our trial, IFN- γ was used to study TH1 response and IL-4 for TH2 response. However, the number of spots were relatively low, which has been reported before^[27]. It is especially surprising that the TH2 response was very low in our responders although the hepatitis B vaccine is considered to be an immunization primarily based on a strong TH2/B-cell response. However, our data are in good concordance with other studies demonstrating a dominant TH1 cell response after hepatitis B vaccination^[27-30]. Bauer *et al* recently showed that 15 hepatitis B individuals, who had been successfully vaccinated, but had lost anti-HBs titers were able to mount a significant TH1 response similar to our responders^[27]. This again shows that non- and low-responders significantly differ from responders, even if the latter lose their anti-HBsAg titers.

In light of the burden of HBV infection several strategies are currently under study to increase the efficacy of HBsAg vaccination, e.g. by applying double vaccination dose^[31], using intradermal boosters^[32-34], or adding adjuvants such as monophosphoryl lipid A (MPLA) or influenza vaccine^[35,36]. Although these measures were more effective than the yeast derived hepatitis B vaccine alone, not all non-responders showed seroconversion. Currently, early studies using immunostimulatory CpG oligodeoxynucleotides (CpG ODN) suggest a breakthrough in hepatitis B prevention. Not only do vaccines develop considerably higher titers after

three vaccinations with this new combination vaccine, but most of them already mount protective titers after the first vaccination compared to none of the controls immunized with HBsAg alone^[37]. It will be interesting to study whether the seroconversion after HBV-CpG ODN vaccination is also accompanied by the induction of T cell responses as shown here and whether previous non-responders will now mount comparable T cell responses as a result of vaccination. As the safety of a CpG-ODN vaccine is still to be evaluated, a possible future strategy could be to identify possible non-/low-responders with an ELISPOT assay and administer CpG-ODN admixed vaccine for these individuals. The majority of people could still be vaccinated with the common hepatitis B vaccine.

COMMENTS

Background

Non-responsiveness to hepatitis B vaccine is a problem often experienced by professional health-care workers. It is believed that non-responders lack certain properties in their immune system or are even immunocompromised.

Innovations and breakthroughs

Here, we find that all subjects responding to the hepatitis B vaccine already had a T-cell response against the hepatitis Bs antigen before their first vaccination. We hypothesize that the induction of anti-HBsAg responses is dependent on the pre-existing T-cell repertoire against the specific antigen, which may be expanded by the cross-reaction to a ubiquitous antigen.

Applications

By new adjuvants as CpG-ODN, it may be possible in the future to induce a sufficient anti-HBs antigen response in all vaccines.

Terminology

Hepatitis Bs Ag is the hepatitis B surface antigen (HBsAg). Antibody titers against this antigen are an indicator of a good immune response against the pathogen.

Peer review

In this well written article authors find that all subjects responding to the hepatitis B vaccine already had a T-cell response against the hepatitis Bs antigen before their first vaccination, so they hypothesize that the induction of anti HBsAg responses by vaccination is significantly dependent on the pre-existing T-cell repertoire against the specific antigen rather than the presence of a general T-cell defect, which timely contribute to us.

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