



## Improved cell mediated immune responses after successful re-vaccination of non-responders to the hepatitis B virus surface antigen (HBsAg) vaccine using the combined hepatitis A and B vaccine

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### ABSTRACT

We successfully re-vaccinated hepatitis B virus (HBV) vaccine non-responders using a double dose of the combined hepatitis A virus (HAV) and HBV vaccine. The hope was to improve priming of hepatitis B surface antigen (HBsAg)-specific cell mediated immune response (CMI) by an increased antigen dose and a theoretical adjuvant-effect from the local presence of a HAV-specific CMI. A few non-responders had a detectable HBsAg-specific CMI before re-vaccination. An *in vitro* detectable HBsAg-specific CMI was primed equally effective in non-responders (58%) as in first time vaccine recipients (68%). After the third dose a weak, albeit significant, association was observed between the magnitude of HBsAg-specific proliferation and anti-HBs levels. This regimen improves the priming of HBsAg-specific CMIs and antibodies.

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### 1. Introduction

Since the development of hepatitis B virus (HBV) vaccine, its efficacy for protection against hepatitis B virus infection has been well documented [1]. However, previous studies have shown that 5–10% of HBV vaccine recipients produce less than 10 international units (mIU/mL) of antibodies to hepatitis B surface antigen (HBsAg, anti-HBs) following a standard course of immunization [1]. These subjects have been designated vaccine non-responders despite the fact that this most likely not reflects an absolute non-responder status. It is likely that they reflect different degrees of low response to HBsAg. The reason for a non-responder status following HBV vaccination is poorly understood, although several factors are known to affect the response such as smoking, age, overweight, gender, and route of administration. Early studies in inbred mice suggested that different major histocompatibility alleles (MHC) could be linked to a high antibody responder status or a low- or non-responder [2–4]. A similar type of range in anti-HBs responses is

seen in vaccinated humans suggesting that the level of CD4+ T helper (Th) cell priming, or activation of a cell mediated immune response (CMI), may play a role also here. Several factors have been associated with a non-responder status to HBsAg, such as certain human leukocyte antigen (HLA) alleles [5–7], and complement factor 4A [8,9]. However, the precise mechanism of this immunological non-responsiveness to HBsAg remains controversial. It has been suggested that HBV vaccine non-responsiveness may be due to a defect in HBsAg-reactive T cells [10] or in the antigen presenting cell [9], whereas this has been disputed by others [11,12]. Several different explanations for the absence of a response have been proposed, albeit none of these seems to alone offer the full explanation [13]. A number of studies have tried to correct the non-responder status by addition of vaccine adjuvants [14,15], altered doses [15,16], different routes of administration [17], or by the inclusion of additional HBV sequences [18]. All these approaches have shown different degrees of success. This certainly suggests that both the dose and additional immune-stimulating factors may be beneficial for improving response rates.

We recently completed a new re-vaccination schedule using a double dose of the combined hepatitis A virus (HAV) and HBV vaccine [19]. The rationale comprised both an increased level of antigen and the priming of an irrelevant immune response at the same site whereby, theoretically, a positive bystander effect might

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be gained. This regimen proved to be highly effective since 42 out of 44 (95%) previous non-responders developed protective levels of anti-HBs after the double dose of the combined HAV and HBV vaccine [19].

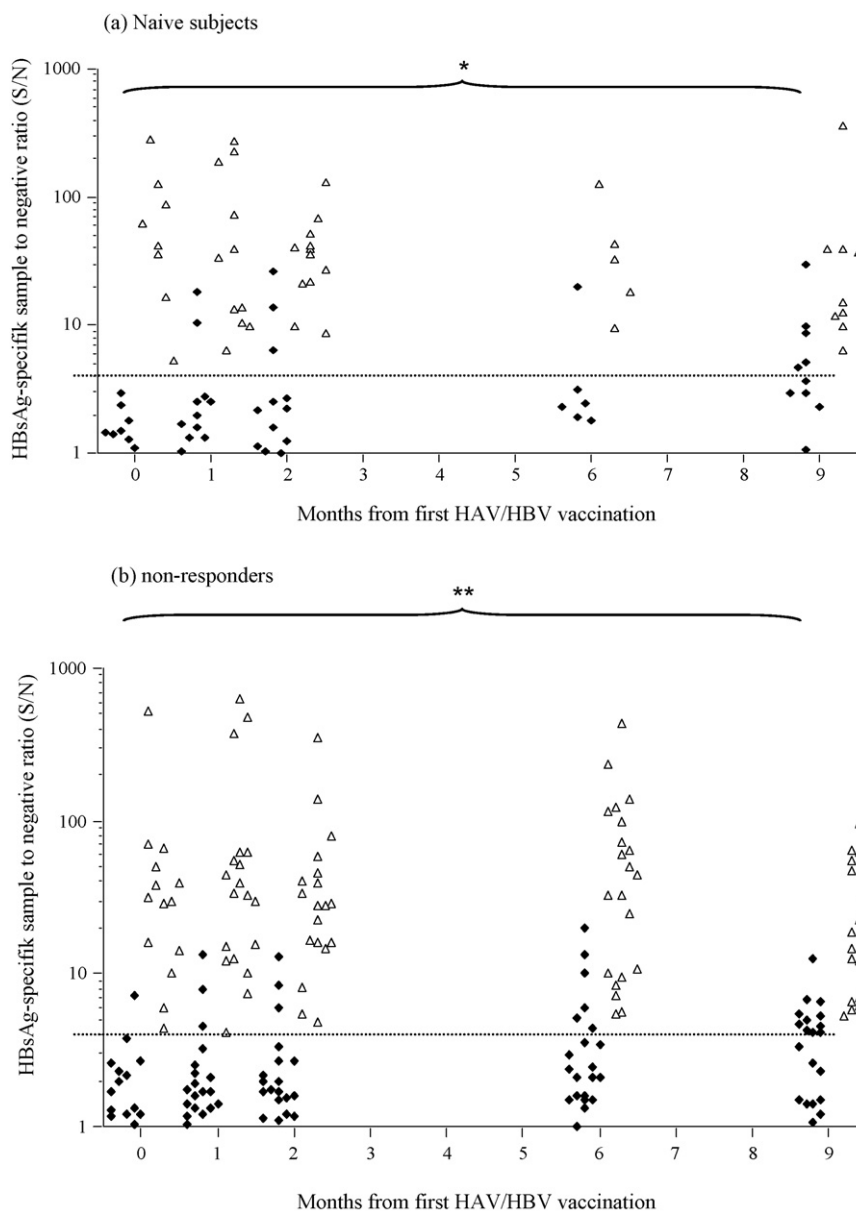
In the current study we wanted to determine if these humoral responses were paralleled by a detectable activation of HBsAg-specific CD4+ and CD8+ T cells, which indeed was the case.

## 2. Material and methods

### 2.1. Study subjects

Forty-four previously known non-responders, failing to mount an anti-HBs response after a previous standard vaccination schedule with three doses of recombinant HBV vaccine 0.1 mL

intradermal and at least one extra booster dose of vaccine, were asked to participate in a re-vaccination study [19]. Peripheral blood mononuclear cells (PBMC) were obtained from 41 of these individuals. The subjects were all re-vaccinated intramuscularly three times (at 0, 1, and 6 months) with a double dose (2.0 mL) of the combined HAV and HBV vaccine (Twinrix®, GlaxoSmithKline, Rixensart, Belgium) [19]. As a control group PBMCs were obtained from 19 out of 20 naive HB vaccinees selected by random from the medical staff at Linköping University Hospital [19]. Blood was drawn at months 0, 1, 2, 6 and 9. PBMC were isolated by gradient centrifugation using Ficoll-paque (Pharmacia, Uppsala, Sweden) and stored in liquid nitrogen until analysis. The detection of anti-HBs using a commercially available kit (Abbott Laboratories, Chicago, IL) has been reported [19]. The study protocol was approved by the Ethics Committee, Health University, Linköping, Sweden. Background data showed that 57/64 subjects



**Fig. 1.** The HBsAg-specific (black diamonds) and PHA-induced (open triangles) proliferative responses in PBMC from naive subjects (a) and non-responders (b) have been shown. Frequencies of proliferative responses were compared using the Fisher's exact test and significant differences have been indicated (a "\*" sign indicates  $p < 0.05$ , and a "\*\*" sign indicates  $p < 0.01$ ). The dotted line represents the cut-off of  $S/N \geq 4$  developed from the mean of the first sample from the naive subjects plus three times their standard deviation.

were female and that there were no difference in body mass index (BMI) between the groups, but the non-responders were of a higher age than the naive subjects [19].

2.2. *In vitro* restimulation of PBMCs

PBMCs were quickly thawed and single cell suspensions were prepared in complete RPMI 1640 medium and proliferation assays were performed as previously described [20]. In brief cells were plated in microplates together with serial dilutions of serum-derived HBsAg (from 0.1 µg/mL to 0.1 µg/mL; subtype adw; kindly provided by Prof. D.L. Peterson, Virginia Commonwealth University, VA), or synthetic peptides (10 µg/mL) overlapping the HBs gene which were produced as previously described [21]. Phytohemagglutinin (PHA; Sigma) and tetanus toxin (TT; Sigma) were used as positive controls in each experiment. Supernatants were removed at 44 h for detection of cytokines. For measuring the T cell proliferation the plates were incubated for 96 h with the addition of 1 µCi [<sup>3</sup>H] thymidine (TdR; Amersham, UK) for the last 16 h and the level of [<sup>3</sup>H] TdR incorporation was determined in a β-counter [20]. Since the pre-vaccination samples of the naive subjects had never been in contact with HBsAg we used these samples to calculate a reliable cut-off value using the mean sample to negative (S/N) ratio plus three times the standard deviation. This yielded a cut-off at an S/N of 3.9 whereby we used S/N ≥ 4 as a cut-off. To ensure a high quality of the data, only assays where the phytohemagglutinin gave an S/N ≥ 4 were used for calculations since this showed that the cells were viable. All other assays were discarded. However, this also means that the number of patients included in calculations at each time point differ.

ELISpot assays were performed essentially as previously described [22]. In brief, nitrocellulose bottom 96-well plates (ELI-IPOSSP, Millipore Co., Bedford, MA) were coated with IFN-γ mAb (anti-IFN-γ 3420-3, Mabtech AB, Stockholm, Sweden) overnight at 4 °C medium at 37 °C. The following day restimulation was performed using 2 × 10<sup>5</sup> PBMCs per well with peptides, proteins, tetanus toxin or PHA and the plates left undisturbed for 44 h at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>. Production of IFN-γ was detected by a biotin-conjugated anti-IFN-γ mAb (Mabtech AB, Sweden), streptavidine-alkaline phosphatase (Mabtech AB) and BCIP/NBT substrate solution (Bio-Rad Laboratories, Richmond, CA). The number of spots were scored using Aid ELISpot reader system Version 2.6 (Autoimmun Diagnostika, Germany). Again, to ensure a high quality of the data, only assays where the PHA control gave ≥ 100 spot forming cells (SFCs)/10<sup>5</sup> PBMC were used

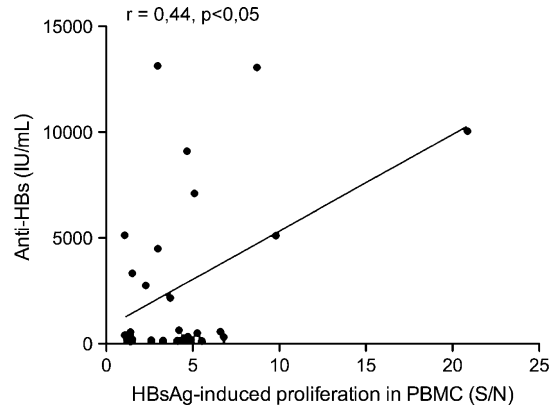


Fig. 2. Relation between the anti-HBs titres (x-axis) and HBsAg-induced S/N levels (y-axis) at 9 months after first immunization in both non-responders (n = 19) and naive subjects (n = 10; r = 0.0442, p < 0.05, ANOVA).

for calculations since this showed that the cells were viable. All other assays were discarded. However, this also means that the number of patients included in calculations at each time point differ.

The cytokines IL-2, IL-4, IL-5, IFN-γ, and IL-10 were assayed for in the 44-h culture supernatants using the Luminex assay (Biosource, Nivelles, Belgium). The Th1/Th2 Five-Plex antibody bead kit was used and was performed according to the manufacturers instructions. No patient had detectable IL-2, IL-4, IL-5, or IFN-γ (data not shown) by the Luminex assay, whereas IL-10 was frequently detected. Again, to ensure a high quality of the data, only IL-10 assays where the PHA control gave ≥ 80 pg of cytokine/mL were used for calculations since this showed that the cells were viable. To standardize between the sample a sample to negative (media control) ratio was used and a cut-off of ≥ 3 (mean + 3 standard deviations) was calculated from the HBsAg-stimulated samples obtained before vaccination of the naive subjects. All other assays were discarded.

2.3. Statistical analysis

Frequencies were compared using the Fishers exact test, and group means were compared using Student's t-test and Mann-Whitney using the InStat version 3.0b software (GraphPad, La Jolla, CA).

Table 1

Number of (%) subjects out of those tested with a detectable HBsAg-induced proliferation, IFN-γ production as spot forming cells (SFCs), or IL-10 production from PBMC, during and after re-vaccination with a double dose of a combined HAV and HBV vaccine

Group	Assay	Months from first re-vaccination					Number of patients positive at any time point tested
		0	1	2	6	9	
Naive	Proliferation	0/8 (0)	1/11 (9)	1/12 (8)	1/6 (17)	5/10* (50)	5/13 (38)
	ELISpot	0/13 (0)	0/15 (0)	0/15 (0)	2/14 (14)	4/13 (31)	5/18 (28)
	IL-10	0/3 (0)	0/2 (0)	1/4 (25)	2/2 (100)	3/3 (100)	4/6 (67)
Total TP and/or ELISpot and/or IL-10		0/17 (0)	1/17 (6)	2/19 (11)	5/17 (29)	10/16** (62)	11/19 (58)
Non-responders	Proliferation	1/14 (7)	2/19 (11)	3/19 (16)	6/21 (29)	10/19** (53)	17/33 (52)
	ELISpot	2/31 (6)	0/33 (0)	3/30 (10)	5/32 (16)	3/31 (10)	11/39 (28)
	IL-10	0/1 (0)	5/6 (83)	3/7 (42)	3/7 (42)	3/4 (75)	7/12 (58)
Total TP and/or ELISpot and/or IL-10		3/33 (9)	6/36 (17)	9/35 (26)	14/35* (40)	15/35** (43)	28/41 (68)

\* p < 0.05, Fisher's exact test, as compared to the pre-vaccination sample.

\*\* p < 0.01, Fisher's exact test, as compared to the pre-vaccination sample.

### 3. Results

#### 3.1. HBsAg-specific proliferative responses in PBMC

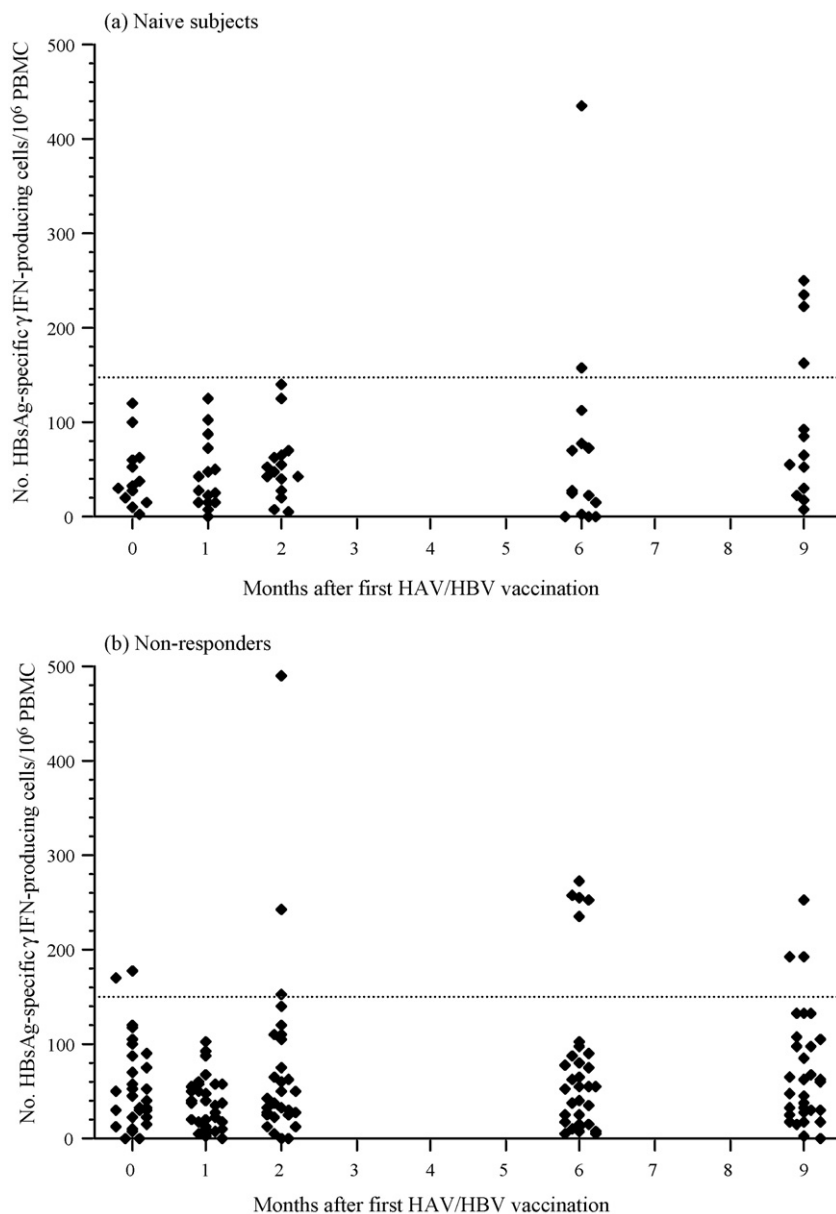
We first determined the proliferative responses to HBsAg following vaccination. The proliferative responses increased significantly with time in both the new vaccinees and in the non-responders (Fig. 1 and Table 1). There was no difference in the PHA-induced responses between groups or between different time points (Fig. 1). This supports that the sampling, storage and freeze-thawing conditions of the samples during the study period were appropriate.

The number of subjects who developed an HBsAg-specific proliferative response in PBMC increased at 9 months after the first vaccination in both new vaccinees and non-responders ( $p < 0.05$  and  $p < 0.01$ , respectively, Fishers exact test; Fig. 1 and Table 1). Interestingly, an HBsAg-specific response could be detected in 3 (9%) out of 33 tested non-responders before initiation of re-vaccination

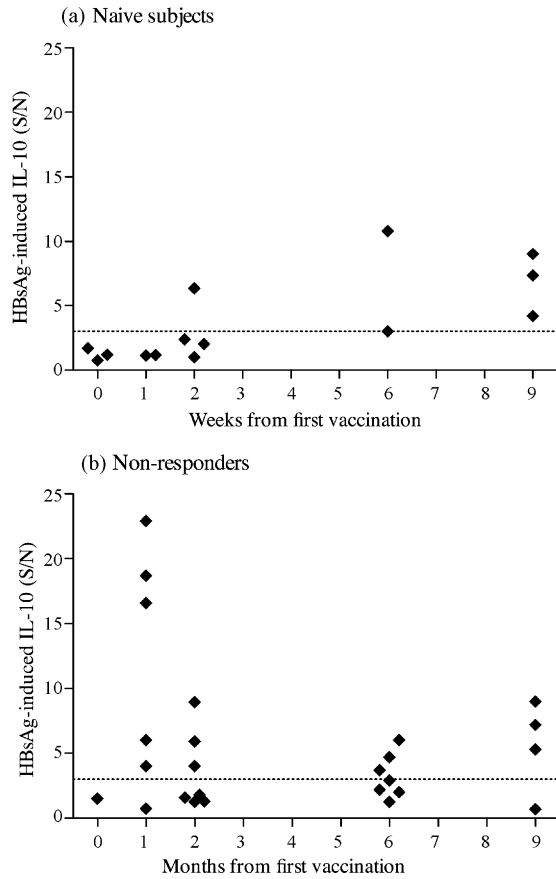
(Fig. 1 and Table 1). This suggests that a minority of non-responders may develop a long-lived CMI in PBMC in the absence of an apparent humoral response. There was at no time point any statistical difference in the CMI between the new vaccinees and the non-responders (Table 1). This implies that the failure to elicit protective levels of anti-HBs during the first rounds of HBV vaccination may be secondary to an inability to prime an effective HBsAg-specific T cell response. This is most likely a prerequisite for a robust anti-HBs response.

#### 3.2. Relation between CMI and antibody levels

We compared if there was any relation between the magnitude of the proliferative responses and anti-HBs titres. We found a weak, albeit statistically significant relation between the HBsAg-induced S/N levels and the anti-HBs titres at 9 months when adding the results from both groups (Fig. 2,  $r = 0.44$ ,  $p < 0.05$ , ANOVA), but not



**Fig. 3.** The number spot forming cells (SFC) producing IFN- $\gamma$  in PBMC in response to HBsAg has been given for vaccinated naive subjects (a) and re-vaccinated non-responders (b). The dotted line represents the cut-off of 150 SFCs/ $10^6$  cells based on the mean number of SFCs/ $10^6$  cells in the first sample from the naive subjects plus three times their standard deviation.



**Fig. 4.** Presence of HBsAg-induced IL-10 in PBMC culture supernatants detected by the Luminex assay in vaccinated naive subjects (a) and re-vaccinated non-responders (b). The dotted line represents the cut-off of  $S/N \geq 3$  based on the mean  $S/N$  of HBsAg-induced IL-10 (mean = 1.2  $S/N$ ) in the first sample from the naive subjects plus three times their standard deviation (S.D.; S.D. = 0.5  $S/N$ ).

when compared separately (ANOVA). No such relation was seen between the HBsAg-induced  $S/N$  levels and the anti-HAV titres, sex, BMI or age. Thus, this supports an association between the CMI and humoral responses after HBV vaccination.

### 3.3. HBsAg-specific IFN- $\gamma$ responses

HBsAg-specific cytokines were determined by the presence of HBsAg-specific IFN- $\gamma$  producing cells by ELISpot using HBsAg and a complete set of peptides spanning HBsAg (Table 1 and Fig. 3). This revealed that, again, only a minority non-responders had evidence of pre-existing HBsAg-specific IFN- $\gamma$  producing cells (Table 1). Also, the appearance of HBsAg-specific IFN- $\gamma$  producing seemed to be less commonly detected than proliferative responses in PBMC (Table 1).

We also determined the number of HBsAg-specific IFN- $\gamma$  producing cells using the five peptide-mixes spanning the complete HBsAg sequence. We could only detect peptide-specific IFN- $\gamma$  producing SFCs in a few subjects (data not shown). The peptides were 25 amino acids long with a 15 residue overlap spanning HBsAg. Thus, they should theoretically be able to detect the presence of both CD4+ Th and CD8+ CTLs. We were therefore unable to determine if these responses represents CD4+ or CD8+ T cells. Despite this we can conclude that the i.m. injection of the double dose of the combined HAV and HBV vaccine is not very effective in priming HBsAg-specific IFN- $\gamma$  producing cells as detected by synthetic peptides.

### 3.4. HBsAg-specific cytokines in culture supernatants

We determined the presence of IL-2, IL-4, IL-5 and IL-10 in culture supernatants from 44-h PBMC cultures using the Luminex assay. We were unable to detect the presence of any other cytokine than IL-10 in culture supernatants (data not shown and Fig. 4). The presence of IL-10 showed similar pattern to IFN- $\gamma$  as detected by the ELISpot assay, with responses appearing earlier in the re-vaccinated non-responders (Fig. 4 and Table 1). Interestingly, HBsAg-induced IL-10 in culture supernatants was newer detected at the same time as IFN- $\gamma$  by ELISpot, supporting the contrasting roles of these two cytokines. No significant differences between the groups could be detected.

## 4. Discussion

One of the major issues remaining regarding hepatitis B vaccination is that 5–10% of vaccinees fails to develop protective levels of anti-HBs [1]. The original observation that predicted that some humans would fail to mount an anti-HBs response stems from studies in which a number of immunized inbred murine lineages failed to develop a detectable B- and/or T cell response to HBsAg [4]. The existence of non-responders to the HBsAg-based vaccine turned out to be correct, albeit a strong linkage to low responders HLA alleles has not been definitely identified [13]. Hence, a number of other explanations for the inability to develop protective levels of anti-HBs have been suggested [9,10].

Although it is of importance to determine the responsible mechanism, since this may affect how remedies should be designed, existing data suggest some approaches. Previous studies have suggested that both the addition of adjuvant and an increased dose may be beneficial [14,15]. We therefore designed a re-vaccination schedule that encompassed both a double dose and an additional inactivated whole virus antigen, HAV. By this approach we hoped to define whether an absolute non-responder status to HBsAg was common with respect to both humoral and cellular responses. We could herein show that this is certainly not the case. Almost all non-responders developed protective levels of anti-HBs after re-vaccination, 42 of 44 (95%) [19]. We now show that a priming of an HBsAg-specific CMI in peripheral blood paralleled the development of anti-HBs. A trend was that the cytokine responses appeared earlier in the re-vaccinated non-responders as compared to the vaccinated naive subjects. This is in line with previous studies suggesting that most vaccinated subjects developed an HBsAg-specific proliferative or cytokine response during and/or after vaccination [17], supporting that an effective vaccination is needed for the priming of memory responses [23]. Also, we found a weak association between the magnitude of the proliferative responses and the levels of anti-HBs after completed vaccination. Thus, a non-responder status to HBsAg is certainly not absolute, but rather represents a range of low responders to HBsAg. This certainly suggests that the number of non-responders will be reduced by a more potent vaccine.

Moreover, we did find evidence that a few non-responder subjects had a pre-existing response to HBsAg despite the absence of protective levels of anti-HBs. This raises the possibility that at least a few non-responders may have a partial protection against HBV infection. The reason why these CMI responses do not result in protective anti-HBs levels should be investigated further.

The present study would have been strengthened by an analysis of the T cell responses to HAV. However, we were unable to establish a reliable assay due to the lack of appropriate recall antigens. However, this should certainly be investigated in future studies on the combined HAV and HBV vaccine to determine whether the



HAV-specific T cell response indeed can adjuvant an HBV-specific T cell response, as previously suggested [19,24].

In conclusion, a non-responder status to HBsAg is not absolute but seems to encompass subjects with a variable degree of low response to HBsAg. This is supported by the fact that a double dose of the combined HAV and HBV vaccine effectively primed both anti-HBs and HBsAg-specific proliferative responses. These data are fully consistent with the murine observations predicting a range of low responders to HBsAg exist also in humans. Whether this can be linked to particular HLA alleles remains to be determined.

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