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ORIGINAL ARTICLE

Hepatitis B virus immunization with an adjuvant containing vaccine after liver transplantation for hepatitis B-related disease: failure of humoral and cellular immune response

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Summary

Long-term hepatitis B reinfection prophylaxis after liver transplantation with hepatitis B immunoglobulin (HBIG) and nucleoside analogues is expensive and inconvenient. Studies evaluating humoral immune responses to hepatitis B virus (HBV) vaccines showed conflicting results. Best results were achieved under continuous HBIG administration with an adjuvant–containing HBsAg vaccine. In the present study, 8 patients who had been HBsAg positive and HBV DNA negative prior to liver transplantation were immunized with HBsAg-vaccine containing the adjuvant 3-deacylated monophosphoryl-lipid-A. Vaccination was started after discontinuation of HBIG. Six vaccinations were administered at weeks 0, 2, 4, 12, 16 and 24. Humoral (anti-HBs titres) and cellular (enzyme-linked immunospot assay and fluorescence-activated cell sorting analysis) immune responses were studied. Only one of eight patients responded with a humoral immune response (maximum anti-HBs titre 561 U/l). In this patient, decrease of anti-HBs titre before vaccination was significantly slower than in the other seven patients and anti-HBs did not become negative before first vaccination. A T-cell response to HBsAg could not be detected in any of the patients. The responder was the only patient who showed a T-cell response to HBcAg. In conclusion, the adjuvant-containing vaccine did not induce a humoral or a detectable cellular immune response in most patients. Patient-related preconditions and concomitant HBIG administration should be further investigated as possible predictors for response.

Introduction

Combined treatment with nucleoside or nucleotide analogues (pre- and post-transplantation) and hepatitis B immunoglobulin (HBIG) (post-transplantation) is currently the gold standard for hepatitis B reinfection prophylaxis after orthotopic liver transplantation (OLT) [1,2]. With an increasing number of patients receiving an infinite combination prophylaxis, costs are escalating [1,3–5]. Therefore, safe and cost-saving concepts for termination of HBIG treatment are needed.

The most promising strategy emerging during recent years is the active immunization with HBsAg-containing vaccines. However, study results are conflicting. The first publication by Sanchez-Fueyo *et al.* [6] presented the new strategy with stimulating results using a standard HBsAg

vaccine after discontinuation of HBIG. In contrast, another report published by Angelico *et al.* [7] showed a very low response rate to vaccination despite frequent intramuscular and intradermal vaccine application. The controversial discussion weighing risks and benefits of vaccination protocols were strikingly revitalized by the study of Bienzle *et al.* [5]. In that study, the highest response rate to date was reported and notably, this was achieved without terminating the protective HBIG treatment. Unfortunately, the reason for the high response rate remains unclear. A large number of variables, as listed in Table 2, could contribute to influencing the outcome of a vaccination trial. The continuation of HBIG treatment and the adjuvant contained in the vaccine were discussed as the most probable constitutive variables for the good response.

It is unclear to what extent cellular immune responses may contribute to protection from hepatitis B virus (HBV) reinfection. No data has been published investigating the HBV-specific T-cell responses in patients undergoing active immunization after liver transplantation. Therefore, we intended to show whether a cellular immune response could be detectable and possibly contribute to preventing HBV reinfection even in the absence of a humoral immune response. This analysis becomes even more relevant in our study, as patients were vaccinated with an HBsAg vaccine containing monophosphoryl lipid A (MPL) as an adjuvant, featuring a stimulating effect on cellular immune responses. This adjuvant is supposed to be a substantial immunostimulating component of the vaccine which was used in the most successful study by Bienzle *et al.* [5] too. In contrast to the Bienzle *et al.* [5] study, in our study HBIG treatment was stopped prior to vaccination.

Patients and methods

Study protocol

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in *a priori* approval by the appropriate institutional review committee. All patients gave their informed consent. The trial was designed as an open phase II study.

Inclusion criteria

Only patients between 18 and 70 years of age, transplanted due to HBsAg-positive end-stage liver cirrhosis at least 18 months before enrollment, being HBsAg and HBV DNA negative, receiving HBV reinfection prophylaxis with low-dose HBIG and receiving low-dose immunosuppression were enrolled.

Exclusion criteria

Any of the following criteria precluded patients from being enrolled: decompensated liver function, histologically proven acute or chronic rejection in liver biopsy, pregnancy or lactation, clinical signs of febrile illness, clinically relevant co-morbidity.

According to the protocol, low-dose HBIG treatment was discontinued and replaced by a daily treatment with 100 mg lamivudine. Vaccines were injected intramuscularly in the upper deltoid region at weeks 0, 2, 4, 12, 16 and 24, ±3 days, with an overall window of ±2 weeks at week 24. Adverse events at the time of vaccination and during the subsequent week were monitored and scored as mild (local signs as swelling or redness), moderate (interfering with normal everyday activities) or severe (hindering everyday activities). HbsAg-, anti-HBs- and drug levels of immunosuppression as well as biochemical and haematological safety laboratory parameters were monitored at regular intervals.

Vaccine

The vaccine consisted of recombinant purified HBsAg combined with the adjuvant system AS04, containing 3-deacylated MPL, a detoxified derivative of lipid A, a component of *Salmonella minnesota* lipopolysaccharide (LPS). Each 0.5 ml of vaccine dose contained 20 μg HBsAg and 50 μg MPL and aluminium salt 0.5 mg (Glaxo-Smith-Kline Biologicals, Rixensart, Belgium).

Statistical analysis

Time of anti-HBs titre decline before first vaccination from 100 to <10 IU/l was calculated (Fig. 1) in all patients. Mean and SD of anti-HBs titre drop was calculated for nonresponders and compared with the responder's value. All analyses were performed with SPSS software version 11.0 (SPSS, Chicago, IL, USA).

Methods

Hepatitis serology

Anti-HBs, HBsAg and HBV-PCR were performed as described previously [4]. Cellular immune response: peripheral blood mononuclear cells (PBMC) from all patients were isolated by Ficoll–Hypaque gradient centrifugation before first, fourth and sixth vaccination. A total of 300 000 cells per sample were stimulated *in vitro* with 1 μg of recombinant HBcAg and HBsAg (Austral Biologicals, San Ramon, CA, USA), PHA (Sigma, St Louis, MO, USA) and medium for 20–24 h. Interferon-producing cells (IFN) were detected by enzyme-linked immunospot (ELISPOT) assay, as described previously [8]. At least three wells per condition were measured at different time points per patient, mean with SD was calculated (Fig. 2a).

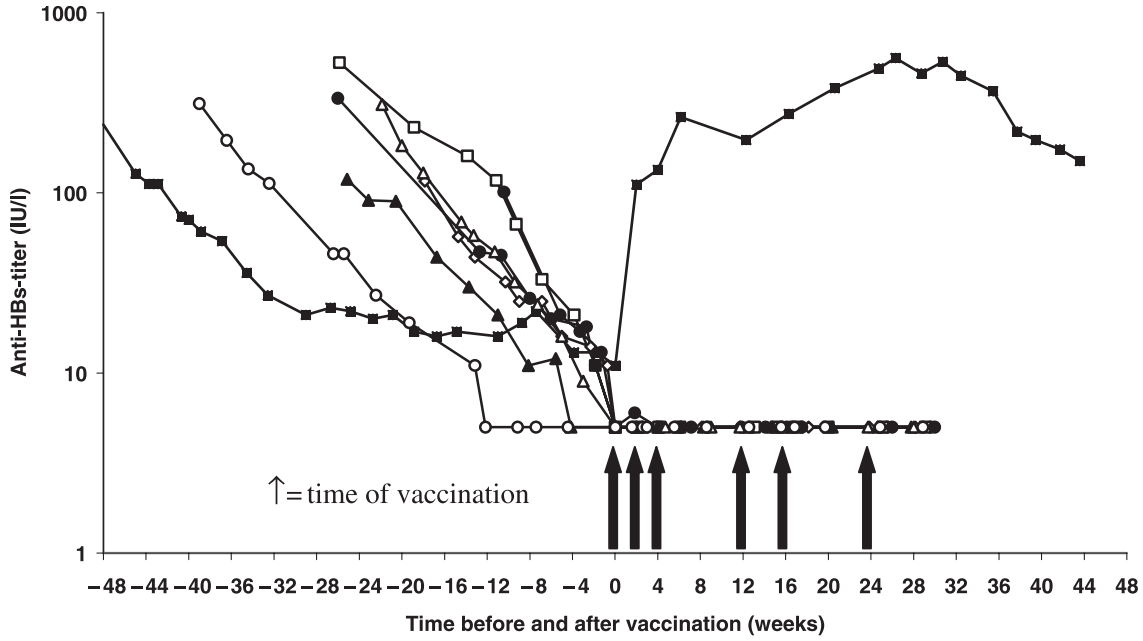

Figure 1 Humoral immune response: anti-HBs titres before and after vaccination. Titres <10 IU/l were rated as negative.

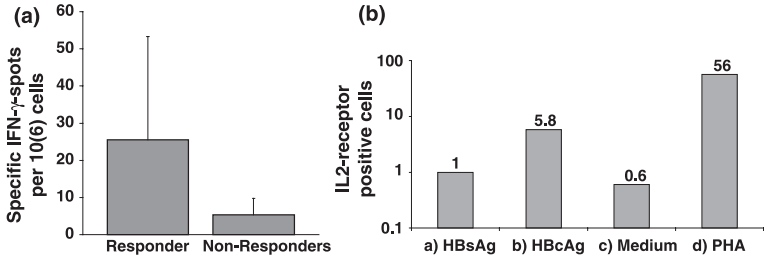

Figure 2 (a) Cellular immune response: mean of cells with interferon-γ-response in enzyme-linked immunospot assay to HBcAg stimulation of peripheral blood mononuclear cells. Response in <10 cells was rated as negative. (b) Cellular immune response: percentage of interleukin-2 (IL-2) receptor-positive cells in fluorescence-activated cell sorting analysis of patient no. 7. IL-2 receptors were labelled with anti-CD25 antibody after (a) stimulation with HBsAg; (b) stimulation with HBcAg; (c) medium, no stimulation (negative control); (d) stimulation with PHA (positive control).

To confirm positive ELISPOT results with a second independent method, cryopreserved cells were thawed and stimulated for 5 days with identical concentrations of antigens in 10% AB medium. Subsequently, upregulation of the high-affinity interleukin 2 (IL-2) receptor CD25 on $CD4^+$ cells was determined by flow cytometry. Cells were stained with 1 µl of a FITC-conjugated CD25 antibody (Clone2A3; Becton Dickinson, Heidelberg, Germany) and $CD25^+$ cells were counted by fluorescence-activated cell sorting (FACS) as described previously [9].

Results

Enrolled patients and adherence to study protocol

Eight patients were enrolled. Median time from OLT to inclusion was 60 months. Median age at time of first vaccination was 50 years. All patients were anti-HDV and HDV RNA negative. One hepatitis C-coinfected patient was HCV RNA positive throughout the study period. Further patients' characteristics are shown in Table 1. Patients adhered to vaccination protocol, except no. 5 whose subsequent vaccinations following the first vaccination were postponed 2 weeks because of an acute respiratory infection.

Decline of anti-HBs levels before vaccination

Mean time of anti-HBs decline from 100 to <10 IU/l before first vaccination in nonresponders was 15.0 ± 3.8 weeks (range 10.3–19.6). Anti-HBs titre of responder decreased significantly slower (42.2 weeks) than those of nonresponders and it did not become negative before first vaccination in the responder.

Table 1. Demographic, clinical and virological characteristics of individual patients.

Patient no.	Age*	Sex	Concomitant diseases	Current immunosuppression	Time of first vaccination after OLT (months)
1	68	M	–	MMF 2 g/day	67
2	46	F	–	Tac 5 ng/ml + PDN 2.5 mg/day	73
3	36	M	HCV infection	CsA 120 ng/ml	42
4	48	M	–	Tac 7 ng/ml	26
5	52	F	–	MMF 2 g/day + PDN 7.5 mg/day	52
6	59	M	–	MMF 2 g/day + PDN 5 mg/day	66
7	39	M	Renal transplant	MMF 2 g/day + PDN 5 mg/day + CsA 60 ng/ml	90
8	67	M	–	MMF 1 g/day + Tac 8 ng/ml	28

MMF, mycophenolate mofetil; CsA, cyclosporin A; Tac, tacrolimus; HCV, hepatitis C virus; PDN, prednisolone; OLT, orthotopic liver transplantation.
*At time of first vaccination in years.

Humoral immune response to vaccination

Patients received six vaccinations as scheduled above. Only one of eight patients responded with a maximum anti-HBs level of 561 IU/l at week 27 measured 2 weeks after the sixth vaccine dose (Fig. 1).

Cellular immune response

Peripheral blood mononuclear cells were stimulated with HBsAg, HBcAg, medium and PHA. IFN-γ response in ELISPOT assay to HBsAg was not found in any of the patients, neither before nor after vaccination. A significant IFN-γ response to HBcAg was found only for the anti-HBs responder (patient no. 7) before first and sixth vaccination, but not in the anti-HBs nonresponders at any time point (Fig. 2a). Significant IFN-γ response to PHA stimulation was documented for all patients at each time point as positive control, whereas negative response to stimulation with medium was documented in all patients at any time point as negative control.

The positive IFN-γ response to HBcAg was confirmed by FACS as a second independent method, showing a significant upregulation of CD25 after HBcAg but not after HBsAg stimulation of PBMC (Fig. 2b).

Safety

HBsAg remained negative in all patients over the study period and follow up. No rejections or significant alterations of biochemical or haematological parameters were observed. No serious adverse event occurred. Fifty per cent of patients reported local adverse events (mild in three, moderate in one and severe in zero patients).

Follow up

Reinfection prophylaxis with HBIG was restarted in all nonresponders 4 weeks after the sixth vaccine injection in combination with continued lamivudine treatment.

Discussion

Active immunization against hepatitis B after liver transplantation for HBV-induced liver failure has been controversially discussed in recent years. The studies of Angelico *et al.* [7] and Lo *et al.* [10] showed low response rates and low anti-HBs levels of responders. The data of Sanchez-Fueyo *et al.* [6] and Albeniz Arbizu *et al.* [11] showed a promising overall response rate, but low anti-HBs levels of most responders. In contrast, Bienzle *et al.* [5] demonstrated that a substantial number of patients can benefit from post-transplant HBV vaccination with impressive anti-HBs levels.

Patient-based factors on one hand and vaccine or protocol-related factors on the other hand decide on success or failure of the vaccination. Several parameters have been discussed to be potentially predictive for response: immunosuppression, pretransplant course of HBV infection (acute versus chronic), time between OLT and vaccination, concomitant treatment with nucleoside analogues, level of pretransplant HBV DNA, HCV or HDV coinfections, age, gender, number and sequence of vaccinations, vaccine dosage, adjuvant systems and continuation versus discontinuation of HBIG treatment [5–7,10–12]. Most of these variables do not differ significantly in between published studies as given in Table 2 and our present study, or do not explain the substantially better response of the study of Bienzle *et al.* [5]. Therefore, continuation of HBIG and the adjuvant system have been discussed as distinctive

Table 2. Vaccination trials with HBsAg containing vaccines after OLT for HBV-related liver disease.

	Sanchez-Fueyo *et al.* [6,12]	Angelico *et al.* [7]	Bienzle *et al.* [5]	Albeniz Arbizu *et al.* [11]	Lo *et al.* [10]
Number of patients	22	17	20	12	52
Male/female	15/7	15/2	18/2	NA	48/4
Age, median (range)	38 ± 8†/41 ± 9‡	53 (36–63)	54 (35–69)	NA	47 (17–61)
Acute/chronic HBV	8/14	0/17	2/18	1/11	0/52
Immunosuppression (mono/combination)	11/6	17/0	16/4	12/0	48/4
HBV-DNA negative before OLT (%)	100	100	100	NA	81
Nucleoside analogues	5/22	17/17	4/20	8/12	52/52
Time of vaccination (months after OLT)*	33 (18–76)	48 (25–85)	78 (24–156)	>24	14 (12–68)
Maximum number of vaccinations (per cycle)	3 + 3	3 + 6 + 3	5 + 3	3 + 3 + 3	3 + 3
HBsAg dose (μg) and route of vaccination	40 i.m./40 i.m.	40 i.m./10 i.d./40 i.m.	20 i.m.§/100 i.m.¶	40 i.m.	40 i.m.
Adjuvant to vaccine	No	No	MPL + QS21	No	No
HBIG prophylaxis during vaccination	No	No	Yes	No	No
≥10 IU/l in % (absolute)	63.6 (14)	17.6 (3)	–	75 (9)	7.7 (4)
≥100 IU/l in % (absolute)	22.7 (5)	11.8 (2)	–	23 (3)	1.9 (1)
≥500 IU/l in % (absolute)	9.1 (2)	5.9 (1)	80 (16)	NA	0.0 (0)
Maximum anti-HBs titres of responders*	47 (10–1000)	253 (20–678)	25 344 (1255–83 121)	NA	22 (12–103)

NA, not available; OLT, orthotopic liver transplantation; MPL, monophosphoryl lipid A; HBIG, hepatitis B immunoglobulin.
*Values given are median (range).
†Responders (median ± SD).
‡Nonresponders (median ± SD).
§Ten patients.
¶Ten patients.

parameters that may have led to the much better response to vaccination in the study of Bienzle *et al.* [5].

Adjuvants are added to enhance the immunogenicity of HBsAg, due to the fact that classical HBV vaccines are not supposed to be sufficiently immunogenic in immunosuppressed patients with a history of chronic hepatitis B infection prior to transplantation. Oil-in-water emulsions or aluminum salts are used to prolong antigen release and enhance incorporation by macrophages. Furthermore, bacterial components, such as MPL, which have an immune stimulating activity with diverse effects on the cellular elements of the immune system are added to the vaccine proteins. MPL is a chemically modified derivative of LPS of *S. minnesota* with greatly reduced toxicity and has been used extensively in clinical trials as a component in prophylactic and therapeutic vaccines targeting infectious diseases, cancer and allergies. HBsAg is formulated with MPL and QS21 in an oil in water emulsion in the vaccine used by Bienzle *et al.* [5]. QS21, another adjuvant, is a pure fraction of Quil A saponin derived from the plant *Quillaja saponaria* and known to induce the production of IFN-γ and IL-2 as well as antibody formation of the immunoglobulin G2a isotype [13]. However, the impact of the adjuvant system on the good response to vaccination remains debatable, because a control group using a conventional recombinant HBsAg vaccine is missing. Furthermore, our study shows a very low response rate despite using a similar adjuvant system with MPL and aluminum salt.

Contrary to all previously published studies we investigated not only the humoral immune response, but also the cellular immune response to our vaccine. In patients with HBV infection, virus elimination or control depends on cytotoxic T-lymphocyte ($CD8^+$) and T-helper cell response ($CD4^+$) with a subsequent B-cell response. Anti-HBs-specific T-helper cell response is low in chronic hepatitis B patients and may be even weaker in immunosuppressed liver transplant recipients [14]. An HBV-specific response of the host immune system has usually not evolved in chronic hepatitis B patients who received a liver graft. In these patients HBV infection is controlled by the passive administration of HBIG and nucleoside or nucleotide analogues. The rationale to use the novel adjuvant system AS04 is based on the fact that MPL was shown to stimulate mainly the cellular arm of the immunity in animal experiments (Th1 lymphocyte response). Furthermore, AS04 induces a strong and rapid anti-HBs antibody response in human volunteers. The immunogenicity profile of MPL-containing vaccines showed higher seroconversion, seroprotection and geometric mean titre rates in comparison with Engerix B^{TM} (GlaxoSmithKline Biologicals, Rixensart, Belgium) as the control vaccine in several clinical studies [15]. Unfortunately, in the patients under our protocol, the vaccine failed to induce a measurable T-cell

response, neither in the patient with a considerable rise of the anti-HBs level nor in the patients without humoral immune response.

It is interesting to note that the only patient with a measurable anti-HBs antibody response to the vaccination had a significantly different anti-HBs decrease prior to vaccination compared with the other patients. This could probably be assessed as a sign for active anti-HBs production at a low level already masked by the HBIG prophylaxis. Remarkable also, that only this patient showed a T-cell response to stimulation with HBcAg. Both facts may be signs of an increased basic immunological reactivity of this particular patient indicating that the individual precondition of the patient plays an important role for the success of the vaccination apart from the vaccination protocol and the vaccine itself. However, as the strong immune response reported by Bienzle *et al.* [5] in the majority of patients significantly differs from our results, further investigations of predictive factors beyond the individual preconditions appear to be worthwhile. In consideration of the low response rate in our patients despite the use of adjuvant MPL, it may be speculated, whether the successful induction of a strong response in the Bienzle *et al.* [5] study could be attributed to the concomitant HBIG administration rather than to the adjuvant in the vaccine. Due to the fact that the adjuvant composition was slightly different in the Bienzle *et al.* [5] study, further studies are necessary to address this question.

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