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ORIGINAL ARTICLE

Successful hepatitis B vaccination in liver transplant recipients with donor-specific hyporesponsiveness

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Summary

Currently, patients are prescribed lifelong treatment with hepatitis B immunoglobulin (HBIg) after liver transplantation (LT) for hepatitis B virus (HBV)-related diseases in order to prevent reinfection with HBV. Active immunization with an HBV vaccine would be a preferable alternative; however, the immunosuppressive environment in LT recipients is believed to elicit a poor response to vaccination. Minimizing the exposure of the HBV-infected LT recipients to immunosuppressants would be beneficial in inducing adaptive immunity against HBV by vaccination. In this study, in addition to efforts to minimize immunosuppression, prophylaxis with HBV vaccination combined with continuous HBIg administration was performed in 17 LT recipients who had undergone transplantation attributable to HBV-related diseases. During the observation period, the overall response rate to HBV vaccination was 64.7%. The immune status of the recipients was evaluated by a mixed lymphocyte reaction assay in response to allostimulation. Patients showing a donor-specific hyporesponse with a well-maintained response to the third-party stimulus always achieved a sustained immune response to the vaccine, whereas patients showing a hyporesponse to both the donor and the third-party stimulus were unable to do so. Thus, inducing an anti-donor-specific immunosuppressive status by minimizing immunosuppression should enable post-transplant HBV vaccination to be a promising prophylactic strategy.

Introduction

Patients face a high risk of endogenous hepatitis B virus (HBV) reinfection in the absence of postoperative prophylaxis after liver transplantation (LT) caused by HBV-related disease. Combined treatment with either a nucleoside or nucleotide analog and hepatitis B immunoglobulins (HBIg) has been the gold standard for prophylaxis of HBV reinfection after LT [1–3]. According to current recommendations, HBIg should be administered indefinitely after LT [4–6]. However, indefinite prophylaxis with HBIg has substantial drawbacks, such as increasing costs [7] and the risk of emergence of HBV envelope protein mutations [8,9]. Therefore, induction of an active immune response against the hepatitis B surface antigen (HBsAg), leading to the continuous production of specific antibodies would be

an enormous advantage, and it would eliminate the need for lifelong replacement with HBIg [10,11].

Several groups have attempted vaccination of LT recipients against HBV [11–20]. In most of these studies, relatively low seroconversion rates as well as serum anti-HBs concentrations were observed among chronic HBV-infected LT recipients; only a minority of vaccinees developed stable antibody levels >100 IU/l, the maintenance of which is required for prevention of HBV reinfection [21]. The poor response to vaccination was probably because of the immunosuppressive environment in LT recipients. Minimizing the exposure of HBV-infected LT recipients to immunosuppressants appears to be beneficial in inducing adaptive immunity against HBV by vaccination; however, the relevance of the immune status of LT recipients to the outcome of HBV vaccination remains to be elucidated.

In this study, prophylactic HBV vaccination combined with continuous HBIg administration was performed in 17 LT recipients who had undergone transplantation because of an HBV-related disease and had not experienced signs of recurrence for at least 12 months after treatment with HBIg. The immune status of these patients was evaluated by a mixed lymphocyte reaction (MLR) assay in response to anti-donor and third-party allostimulation using an intracellular carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeling technique.

Patients and methods

Patients

In this study, we included 17 living donor LT recipients at the Hiroshima University Hospital. All patients had normal liver function without any virologic and biochemical evidence of HBV recurrence. The following were the inclusion criteria: (i) at least 3 months of HBIg plus lamivudine (100 mg/day) with/without adefovir (10 mg/day) administration and (ii) no findings of recurrent infection and negativity for HBsAg and hepatitis B viral deoxyribonucleic acid (HBV DNA) (by PCR) at the time of vaccination. For prophylaxis against reinfection, all transplanted patients were on a stable schedule of 1000–2000 IU of intravenous HBIg every 4 weeks in order to maintain an anti-HBs titer of >100 IU/l. We attempted to minimize immunosuppression in all patients with good liver function by adopting the policy of tapering off the immunosuppressants. The study protocol was approved by the Ethics Committee of Hiroshima University, and all patients provided informed consent before entering into the trial. None of the vaccinees showed clinical evidence of recurrence of HBV graft infection and the episode of rejection throughout the follow-up period, and all of them were persistently negative for both HBsAg and HBV DNA, except for one vaccinee (Patient #3) who showed temporarily positive for HBV DNA.

Vaccination protocol

All participants received a yeast-derived recombinant, adsorbed HBV vaccine (Bimmugen®; Chemotherapy and Serotherapy Laboratories Inc., Kumamoto, Japan) subcutaneously every 4 weeks at a dose of 10–20 μg (0.5–1.0 ml) in combination with HBIg and lamivudine/adefovir. HBIg immunoprophylaxis was continued during primary immunization (dose, 1000–2000 IU every 4 weeks). The response to vaccination was defined as (i) a confirmed increase in the anti-HBs titer to >100 IU/l that could not be explained by HBIg administration and (ii) sustained anti-HBs titer to >100 IU/l after discontinuation of combined administration of the vaccine and HBIg. If the anti-HBs titer exceeded the responsive increasing level, HBIg substitution and vaccine administration were discontinued. Lamivudine/adefovir prophylaxis was additionally discontinued, if the anti-HBs titer was maintained effectively without HBIg administration. The vaccine was continuously and indefinitely administered till acquired immunity was elicited.

Serologic markers and virologic assays

Serum HBsAg, hepatitis Be antigen (HBeAg), hepatitis B core antibody (HBcAb), and anti-HBsAb were measured monthly using an enzyme-linked immunoassay (Abbott Diagnostics, Chicago, IL, USA). HBV DNA was detected by the Amplicor HBV monitor test (Roche Diagnostics, Tokyo, Japan). The measurement range of the assay is $10^{2.6}$–$10^{7.6}$ copies/ml (2.6–7.6 log copies/ml). These quantitative assays of HBV DNA were performed at the Special Reference Laboratory, Tokyo, Japan. Positive levels of HBV DNA were defined as levels >2.6 log copies/ml. HBV recurrence was diagnosed on the basis of appearance of HBsAg or HBV DNA.

Immune monitoring by *in vitro* CFSE-MLR assay

For patients who showed completely normal liver function, CFSE-MLR was performed to determine whether immunosuppression could be further minimized. In patients with hyporesponse of anti-donor T cells, immunosuppression was successfully reduced.

For CFSE-MLR, the peripheral blood mononuclear cells prepared from the blood of the LT recipients (autologous control), donors, and healthy volunteers with same blood type as the donors (third-party control) for use as the stimulator cells were irradiated with 30 Gy and those obtained from the recipients for use as the responder cells

were labeled with 5 μM CFSE (Molecular Probes Inc., Eugene, OR, USA), as described previously [22]. The stimulator and responder cells (2×10^6 each) were incubated in 24-well flat-bottomed plates (BD Labware, Franklin Lakes, NJ, USA) in a total volume of 2 ml of culture medium at 37 °C under 5% CO_2 for 5 days. After culture for MLR, the harvested cells were stained with either phycoerythrin (PE)-conjugated anti-human CD4 or PE-conjugated anti-human CD8 monoclonal antibodies (mAbs; BD Pharmingen, San Diego, CA, USA) and subjected to analysis by flow cytometry (FCM). All analyses were performed on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). Dead cells were excluded from the analysis by forward scatter or propidium iodide gating. T-cell proliferation was visualized by serial-halving of the fluorescence intensity of CFSE. $CD4^+$ and $CD8^+$ T-cell proliferation and stimulation index (SI) were quantified using a previously described method [23,24]. Briefly, the number of division precursors was extrapolated from the number of daughter cells of each division, and the number of mitotic events in each $CD4^+$ and $CD8^+$ T-cell subset was calculated. Using these values, the mitotic index was calculated by dividing the total number of mitotic events by the total number of precursors. The SIs of allogeneic combinations were calculated by dividing the mitotic index of a particular allogeneic combination by that of self-control.

Statistical analysis

The values are presented as the median and the range. The Mann–Whitney *U*-test was performed to analyze whether the age of the vaccinees at the time of vaccination, the time elapsed since LT, the anti-HBsAb titers at the start of the vaccination, the median tacrolimus trough levels, and the SI in anti-donor and anti-third-party MLR differed significantly between the good and poor responders and also between the moderate and poor responders. A Fisher's exact test was performed to determine whether there were differences between both the above groups with regard to gender, indication for LT, ratio of HBV DNA and HBeAg negative before LT, ratio of donor HBc and HBsAb positive before LT, and immunosuppressive monotherapy at the time of vaccine administration. *P*-values below 0.05 were considered statistically significant.

Results

Demographics

A total of 17 HBV vaccinees (four female- and 13 male subjects; age range, 20–65 years; median age, 49 years) participated in this study. The demographic and clinical data of the participants are shown in Table 1. Of them, 14 patients underwent LT for HBV-related cirrhosis and three underwent transplantation for HBV-related fulminant hepatic failure. Among the 17 vaccinees, five (29.4%) had been HBV DNA positive before LT with levels >2.6/ml, and five (29.4%) had been HBeAg positive before LT. Immunosuppressive treatment comprised either cyclosporine or tacrolimus monotherapy in 11 patients (64.7%) and additional steroid therapy (methylprednisolone, 2–4 mg/day) in six patients. Steroids were withdrawn at after a median duration of 13 months (range, 1–50 months) after LT. At the time of vaccination, a median duration of 21 months (range, 3–41 months) had elapsed since LT. The median follow-up time after commencement of vaccination was 26 months (range, 8–72 months). At the start of vaccination, a median anti-HBsAb titer was 161.4 (range, 37.7–328.4) IU/l.

Response to vaccination

During the observation period, 11 of the 17 HBV vaccinees (64.7%) achieved a sustained immune response to the HBV vaccine, which was defined as a confirmed increase in the anti-HBs titer to >100 IU/l that could not be explained by HBIg administration and no decrease in the anti-HBs titer to <100 IU/l even after discontinuation of combined administration of the vaccine and HBIg (Table 1). Within 1 year, 5/11 responders responded to the vaccine, and other six responded after 1 year from the commencement of vaccination (Fig. 1a and b). The other six HBV vaccinees did not respond to the vaccine during the study period (Fig. 1c). When the subjects were divided into three distinct groups, i.e., patients who responded to the vaccine within 1 year after commencement of vaccination (good responders), patients who responded to the vaccine after 1 year since commencement of vaccination (moderate responders), and patients who did not respond to the vaccine within 1 year and still remain receiving the vaccine (poor responders), the following factors did not exhibit statistically significant differences between the good and poor responders and also between the moderate and poor responders: age, gender, indication for LT, HBV viremia, donor HBcAb and HBsAb before LT, immunosuppressive regimen and tacrolimus trough levels and anti-HBsAb titers at the time of vaccination, duration between vaccination and transplantation and also duration between steroid withdrawal and transplantation. (Table 2) (Fig. 2).

Estimation of immunosuppressive status during vaccination by CFSE-MLR assay

Eleven patients (#1, 2, 4, 5, 7, 9, 11, 12, 13, 14 and 17) and their donors consented to be subjected to an

Table 1. The demographic and clinical characteristics of patients.

Patient	Age*	Gender	Underlying disease	HBV DNA before LT	Recipient HBeAg before LT	Donor HBcAb before LT	Donor HBsAb before LT	Time of vaccination†	Time of steroid withdrawal†	Duration of follow-up‡	Immuno-suppressive drugs*	Tac/CsA trough level (ng/ml)*	Anti-HBsAb titer (IU/l)*
Patients who responded to the vaccine within 1 year after commencement of vaccination (good responders)													
1	62	M	Cirrhosis/HCC	<2.6	Negative	NA	Negative	41	3	34	CsA 50 mg	39.3 (CsA)	152.6
2	54	M	Cirrhosis/HCC	<2.6	Negative	NA	NA	26	2	35	CsA 50 mg	15.0 (CsA)	189.1
3	58	M	Cirrhosis	6.4	Positive	Negative	Negative	9	2	43	Tac 2 mg	4.6	161.0
4	43	M	Cirrhosis/HCC	3.4	Negative	Negative	NA	35	45	20	Tac 3 mg + mPSL 2 mg	1.5	220.6
5	57	M	Fulminant	<2.6	Negative	Negative	Negative	9	1	15	Tac 2 mg	3.4	37.7
Patients who responded to the vaccine after 1 year since commencement of vaccination (moderate responders)													
6	34	M	Fulminant	<2.6	Negative	NA	NA	3	7	72	Tac 6 mg + mPSL 4 mg	4.2	152.1
7	38	M	Cirrhosis/HCC	<2.6	Negative	NA	NA	35	7	49	Tac 1 mg	4.4	146.6
8	57	M	Cirrhosis/HCC	<2.6	Negative	NA	Negative	40	50	31	Tac 2 mg + mPSL 4 mg	4.4	68.3
9	46	F	Cirrhosis/HCC	4.6	Positive	Negative	Positive	17	2	33	Tac 3 mg	4.7	93.4
10	46	F	Cirrhosis	<2.6	Negative	Positive	Positive	20	1	22	Tac 1 mg	4.2	214.9
11	53	M	Cirrhosis/HCC	<2.6	Negative	Positive	Positive	13	4	15	Tac 2 mg	4.2	160.5
Patients who did not respond to the vaccine during the study period (poor responders)													
12	20	M	Fulminant	>7.6	Positive	Negative	Positive	18	29	20	Tac 3 mg + mPSL 2 mg	5.0	222.7
13	46	M	Cirrhosis	<2.6	Negative	Negative	Negative	16	1	20	Tac 1 mg	6.6	188.9
14	58	F	Cirrhosis/HCC	<2.6	Negative	NA	NA	18	20	11	Tac 2 mg + mPSL 2 mg	1.5	92.3
15	65	M	Cirrhosis/HCC	4.5	Positive	NA	NA	12	21	13	Tac 4 mg + mPSL 4 mg	8.0	328.4
16	45	F	Cirrhosis/HCC	<2.6	Negative	NA	Negative	25	23	8	Tac 0.5 mg	3.7	193.3
17	54	M	Cirrhosis/HCC	<2.6	Positive	Positive	Positive	13	1	9	Tac 2 mg	2.9	122.2

LT, liver transplantation; Tac, tacrolimus; CsA, cyclosporine; mPSL, methylprednisolone; NA, not available.

*At the time of vaccination.

†Months after liver transplantation.

‡Months after commencement of vaccination.

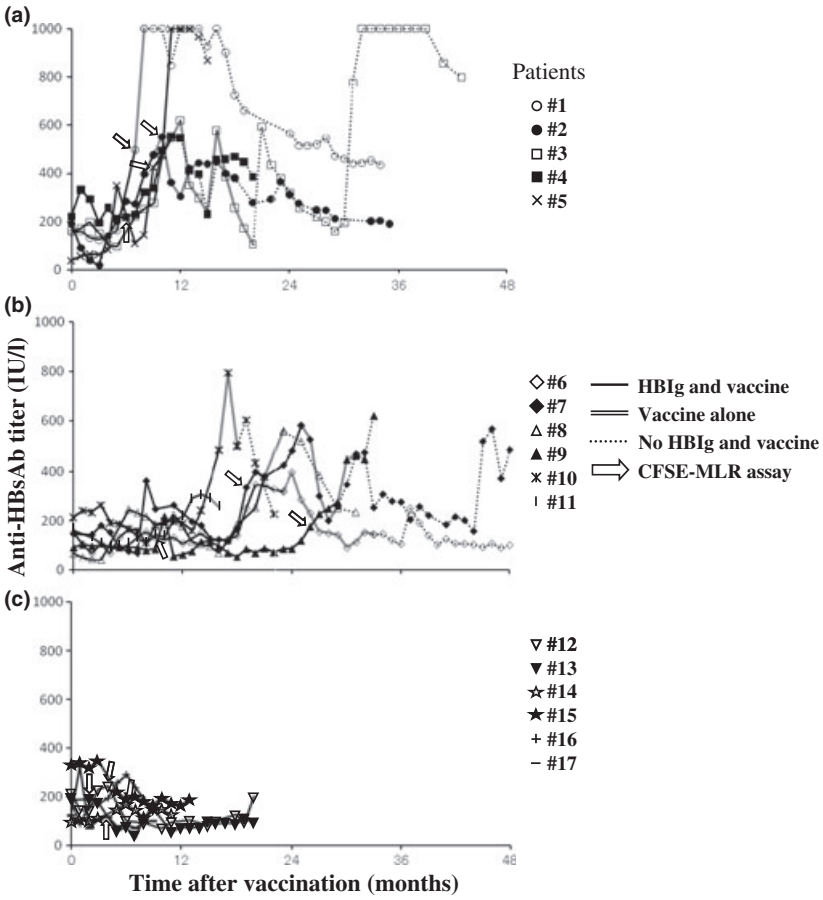

Figure 1 Anti-HBs titer kinetics in patients who responded to the vaccine within 1 year after commencement of vaccination (good responders) (a), in patients who responded to the vaccine after 1 year since the commencement of vaccination (moderate responders) (b), and in patients who did not respond to the vaccine (poor responders) (c).

Table 2. Age, gender, indication for LT, HBV viremia, immunosuppresive regimen, duration between vaccination and transplantation, and duration between steroid withdrawal and transplantation.

	Good responders (n = 5)	Moderate responders (n = 6)	Poor responders (n = 6)	P-value
Age at vaccination (years)*	55 (43–62)	46 (34–57)	48 (20–65)	NS
Gender (male/female)	5/0	4/2	4/2	NS
Indication for LT (fulminant hepatitis/cirrhosis)	1/4	1/5	1/5	NS
HBV DNA before LT (positive/negative)	2/3	2/4	2/4	NS
Recipient HBeAg before LT (positive/negative)	1/4	1/5	3/3	NS
Donor HBcAb before LT (positive/negative)	0/3	2/1	1/2	NS
Donor HBsAb before LT (positive/negative)	0/3	3/1	2/2	NS
CsA or Tac monotherapy/combination with steroid†	4/1	4/2	3/3	NS
Duration between vaccination and transplantation (months)*	24 (9–41)	21 (3–40)	17 (12–25)	NS
Duration between steroid withdrawal and transplantation (months)*	11 (1–45)	12 (1–50)	16 (1–29)	NS
Anti-HBsAb titer (IU/l)*†	152 (38–221)	139 (93–215)	191 (92–328)	NS

NS, not significant; LT, liver transplantation; CsA, cyclosporine A; Tac, tacrolimus.
*Median (range).
†At the time of vaccination.

MLR assay using a CFSE-labeling technique. In all the seven patients who responded to the HBV vaccine, limited $CD4^+$ T-cell proliferation was observed in the anti-donor MLR assay as compared with the anti-third-party MLR assay, i.e., a hyporesponse in the anti-donor MLR assay and a normal response in the anti-third-party MLR assay (Fig. 3). In these patients, the average of SIs for $CD4^+$ T cells in response to anti-third-party stimulation was >2 (average value in healthy volunteers without any immunosuppressive treatment). In contrast,

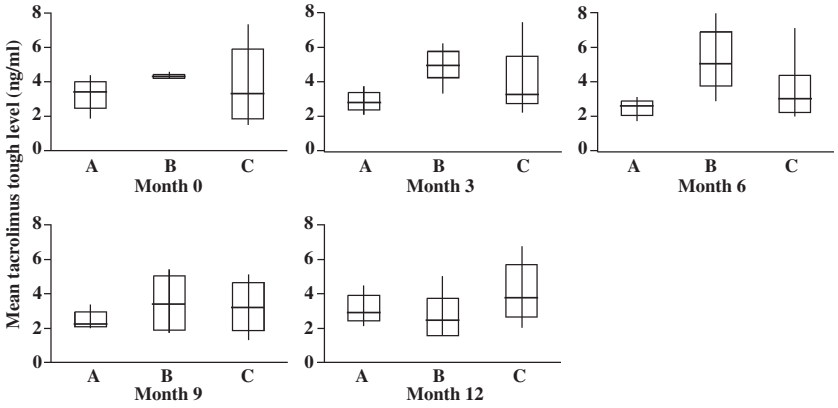

Figure 2 Tacrolimus trough levels in patients who responded to the vaccine within 1 year after commencement of vaccination (good responders) (A), in patients who responded to the vaccine after 1 year since the commencement of vaccination (moderate responders) (B), and in patients who did not respond to the vaccine (poor responders) (C). The Mann–Whitney *U*-test was used to compare the tacrolimus trough levels between the good and moderate responders with those of poor responders. The box plot represents the 25th to 75th percentile, the dark line is the median, and the extended bars represent the 10th to the 90th percentile. Statistical analyses at none of the time-points at 0, 3, 6, 9 and 12 months were significant.

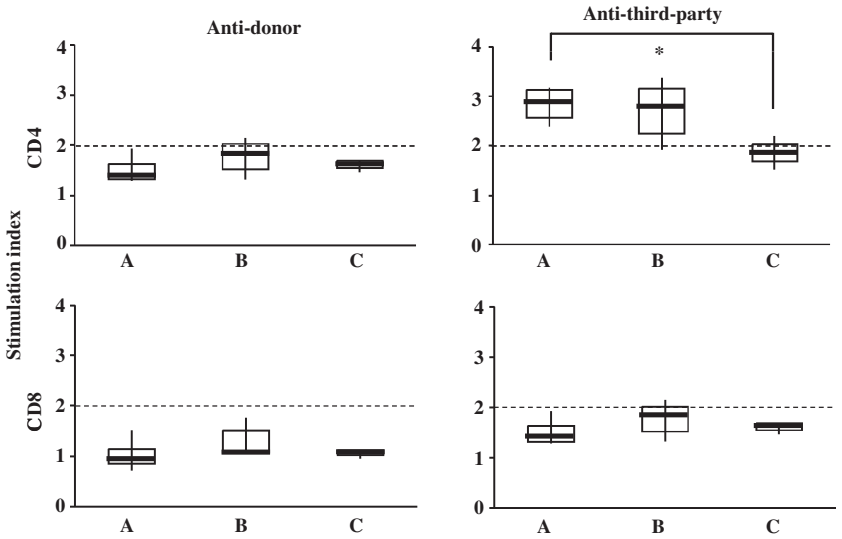

Figure 3 Stimulation indices (SIs) of each of the $CD4^+$ and $CD8^+$ T-cell subsets in the anti-donor and anti-third-party MLR in patients who responded to the vaccine within 1 year after commencement of vaccination (good responders) (A), in patients who responded to the vaccine after 1 year since the commencement of vaccination (moderate responders) (B), and in patients who did not respond to the vaccine (poor responders) (C). $CD4^+$ and $CD8^+$ T-cell proliferation and their SIs were quantified as follows. The number of division precursors was extrapolated from the number of daughter cells of each division, and the number of mitotic events in each of the $CD4^+$ and $CD8^+$ T-cell subsets was calculated. Using these values, the mitotic index was calculated by dividing the total number of mitotic events by the total number of precursors. The SIs of allogeneic combinations were calculated by dividing the mitotic index of a particular allogeneic combination by that of the self control. The Mann–Whitney *U*-test was used to compare the tacrolimus trough levels between the good and moderate responders with those of poor responders. The box plot represents the 25th to 75th percentile, the dark line is the median, and the extended bars represent the 10th to the 90th percentile. $*P = 0.04$.

in the four patients who did not respond to the HBV vaccine, limited $CD4^+$ and $CD8^+$ T-cell proliferation was observed in both the anti-donor and the anti-third-party MLR assay, i.e., a hyporesponse in both cases. In these patients, the average of SIs for $CD4^+$ T cells in response to both anti-donor and anti-third-party stimulation was <2. Thus, the SIs for $CD4^+$ T cells in response to anti-third-party stimulation in good responders was higher than that of poor responders ($P = 0.04$) (Fig. 3).

Discussion

The strategy of HB vaccination after LT to achieve protective immunity and to allow discontinuation of long-term HBIg administration has been investigated in a number of studies [7,11,12,15–20]. However, those attempts to immunize these patients with HB vaccine have been equivocal and generally less than successful. It is common practice to immunize these patients against hepatitis B; however, the response of LT recipients could be below adequate standard. Although the currently available HBV vaccines are extremely safe and have an efficacy of more than 90% in the general population, it has been reported that the response rate is slightly lower in obese individuals, smokers, and men and is significantly lower in patients with cirrhosis or chronic renal failure, patients undergoing long-term hemodialysis, organ transplant recipients, and immunocompromised patients [21]. In particular, because of the impairment in T-cell-dependent functions in cirrhotic patients, the results of vaccination in transplant candidates have been very disappointing [25–29]. Moreover, even in responder patients, immunosuppressive treatment frequently leads to a decrease in the serum antibody titers after transplantation [21]. Among the previous HBV vaccination trials in multiple institutions, most of the results did not show significant promise with regard to HBV vaccine response rates. Each vaccination protocol differed with respect to the dose of vaccine, the time of commencement and frequency of vaccination, the route of vaccination, combination with HBIg, and the immunosuppressive regimen at the time of vaccination. It has been reported that successful vaccination is attributed to the long time-interval that had elapsed after transplant, which allowed them to markedly reduce the immunosuppressive therapy [11]. It has also been proposed that the administration of the vaccine through the intradermal route in preference to the intramuscular route might prove to be more responsive to HB vaccination, because the epidermis is known to be rich with antigen-presenting cells, making it an appropriate target for vaccine delivery [18]. Based on these hypotheses in this study, vaccination through the intradermal route was administered to the LT recipients against HBV with an effort to minimize immunosuppression. In addition to the different vaccination protocols, the difference in the immune status of the subjects likely influences their HBV vaccine response.

In order to evaluate the immune status of the LT recipient vaccinees, we employed a MLR assay using a CFSE-labeling technique [22]. CFSE stably stains intracellular proteins without toxicity, and the fluorescence of each stained cell segregates equally to the daughter cells upon cell division, resulting in sequential halving of cellular fluorescence intensity with each successive generation [30]. When analyzed by FCM, this sequential halving of fluorescence is visualized as distinct peaks or populations of cells and can be used to track cell division in populations of proliferating cells. This, then, allows phenotypic analysis of the proliferating cells and determination of the number of cells produced in each generation by multicolor FCM analysis, i.e., the number of viable $CD4^+$ and $CD8^+$ responder T cells that proliferate in response to allostimulation can be quantified separately. The lack of proliferation of $CD4^+$ T cells in anti-donor MLR reflects the suppression of the anti-donor response [22]. In this study, all of the good responders showed a normal response of the anti-third-party $CD4^+$ T cells (Fig. 3). In contrast, the poor responders showed a hyporesponse of both anti-donor and anti-third-party $CD4^+$ T cells, suggesting an excessively immunosuppressive state. The development of an effective immune response to HB vaccination requires coordinated immune activity comprising the interaction of T cells, cytokines, antigen-presenting cells, and B cells [31]. It is important to note that these immunocompetent cells can be sufficiently activated to acquire immune activity at the time of vaccination even in a state of immunosuppression. T-cell interaction should lead to (i) activation of anti-HBsAg-specific T cells in order to achieve a successful response to vaccination and (ii) suppression of anti-donor-specific T cells to avoid transplant rejection. Patients showing a donor-specific hyporesponse with a well-maintained response to the third-party stimulus always achieved a sustained immune response to the vaccine in this study; based on this observation, we propose a concept that inducing anti-donor-specific immunosuppressive status by minimizing immunosuppression enables post-transplant HBV vaccination to become a promising prophylactic strategy, although further studies are needed to establish the optimal HBV vaccination protocol. A larger and prospective trial might be required to evaluate whether or not the MLR response can actually predict successful HBV vaccination. The higher rate of response to vaccination than that of this study has been shown in a previous report [17]. An adjuvant preparation of vaccine that used in the previous study is thought to attribute to the successful induction of a strong response. It remains to elucidate whether patients with hyporesponse to both anti-donor and anti-third-party $CD4^+$ T cells can respond to such an adjuvant preparation of vaccine.

Authorship

HT, KC, and HO: designed research. HT and YT: performed research. HT, KI, KI, MS, TI, YU, MO, MB,

HT, TI, and TA: collected data. HT, YT, and HO: analyzed data. HT and HO: wrote the paper.

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