Mechanism and therapeutic potential of DNA-based immunization against the envelope proteins of hepatitis B virus in normal and transgenic mice

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SUMMARY

Two plasmid DNA vectors, pCAGGS(S) encoding the genes of the major envelope protein of hepatitis B virus (HBV), and pCAGGS(S+ preS2) encoding the genes of the middle envelope protein were used to study the mechanism and therapeutic potential of DNA-based immunization. Injection of these plasmids into the regenerating bilateral tibialis anterior muscle (TA) of normal C57BL/6 mice induced hepatitis B surface antigen (HBsAg)-specific humoral and cellular immune responses. Seventy-two hours after injection of pCAGGS(S), infiltrating cells including antigen-presenting dendritic cells (DC) were localized around the injection site and HBsAg was expressed by both muscle cells and infiltrating cells. Spleen DC from the mice were exposed to HBsAg for up to 32 weeks after a single injection of pCAGGS(S), because these DC induced the proliferation of HBsAg-specific memory lymphocytes in culture without exogenous HBsAg. A single injection of pCAGGS(S) or pCAGGS(S+preS2) resulted in the clearance of HBsAg in 28 out of 30 HBV-transgenic (Tg) mice. In contrast, more than 7 monthly injections of an HBsAg-based vaccine were required for the clearance of HBsAg in 6 out of 29 HBV-Tg mice. Infiltrating DC at the DNA vaccine injection site may have a role in initiating HBsAg-specific immune response, whereas the persistence of HBsAg exposed spleen DC may contribute to long-lasting immunity. This study also suggested that DNA-based vaccines may be a potent tool for treating chronic HBV carriers.

INTRODUCTION

Infection with hepatitis B virus (HBV) causes acute as well as chronic necroinflammatory liver disease and there are about 350 million chronic HBV carriers world wide. Many HBV carriers eventually develop serious complications such as liver cirrhosis or hepatocellular carcinoma, and these carriers also represent a permanent source of HBV infection.

The prospects for controlling new HBV infection depend on the availability of safe, effective, and affordable vaccines.[1,2] Recently, antigen-based vaccines have been used to treat human HBV carriers[3,4] and have been studied in HBV-transgenic (HBV-Tg) mice, a murine model of HBV carriage.[5–8]

The successful therapeutic vaccination reported in murine HBV-Tg mice might be related to the fact that hepatitis B surface antigen (HBsAg) transgene expression in these mice is blocked by methylation.[9,10]

Although currently available antigen-based vaccines are used for both prophylactic and therapeutic purposes, a new field of immunological research with applications to vaccine development has developed around the direct transfer of naked DNA, which leads to the *in situ* synthesis and accumulation of antigenic proteins followed by induction of an immune response. Immunization with plasmid DNA vectors encoding the small and middle viral envelope proteins of HBV has been shown to elicit strong and durable humoral and cell-mediated immunity in both normal mice[11–15] and HBV-Tg mice,[16,17] but several aspects of DNA vaccine still require further study before its wider application.

There is little information available regarding the mechanism of induction of immunity by DNA vaccines, so it is not known why a single injection of DNA vaccine can induce long-lasting immunity. Moreover, no study has made a parallel comparison of the therapeutic potential of DNA vaccines and antigen-based vaccines in human or murine HBV-carriers.

In the present study, we used a plasmid vector with a promoter designed for expression in muscle. We first constructed two plasmid HBV DNA vectors, one encoding the

genes of the major envelope protein and the other encoding the genes of the middle envelope protein. Injection of these vectors into the regenerating tibialis anterior muscle (TA) induced HBsAg-specific humoral and cellular immune responses in normal C57BL/6 mice. Next, we used immunohistochemical methods to localize infiltrating cells, including antigen-presenting dendritic cells (DC), and to assess the expression of HBsAg at and around the site of injection of DNA vaccines. To evaluate the mechanism of long-lasting immunity following DNA vaccination, we analysed the function of splenic DC at 32 weeks after a single injection of DNA vaccine in C57BL/6 mice. We also compared the therapeutic potential of a DNA vaccine and the currently available antigen-based vaccine in HBV-Tg mice.

MATERIALS AND METHODS

Animals

The animals used were normal C57BL/6 mice and HBV-Tg mice (official designation: 1·2HB-BS10) that were seropositive for HBsAg, hepatitis B e antigen, and HBV DNA as well as expressing HBV-related mRNAs in their tissues.[18] All animals received humane care and the study was carried out with the permission of the Committee on Animal Experimentation of Ehime University School of Medicine (Ehime, Japan).

Plasmid DNA vectors

The major and the middle envelope genes of HBV were amplified by PCR from plasmid BSadr4 × 2(R), which contained two copies of the full HBV genome. Polymerase chain reaction (PCR) products were digested with *Eco*RI and were inserted into the multiple cloning site of the parental plasmid, pCAGGS, exactly as described by Niwa *et al.*[19] Plasmid DNA vectors were grown in *Escherichia coli* HB101 (Takara, Tokyo, Japan) and were purified using a Qiagen Plasmid Purification Kit (Qiagen, Hilden, Germany).The quantity and quality of the purified plasmid DNA were assessed from the optical density at 260 and 280 nm. The insertion sites of the plasmid DNAs were confirmed using restriction enzymes and DNA sequencing. The pCAGGS(S) vector encoded the genes of the major envelope protein of HBV, whereas the vector, pCAGGS(S + preS2) encoded the genes of the middle envelope proteins.

Cell transfection, expression, and antigen production

COS I cells (SV40 transformed African green monkey kidney cells, ATCC No. CRL 1650, American Type Culture Collection, Rockville, MD) and G8 cells (mouse myoblast cells, ATCC No. CRL 1456) were transiently transfected with plasmid HBV DNA vectors using Lipofectin (Gibco, Gaithersburg, MD). Expression of HBsAg was confirmed in the these cells by immunostaining. Culture supernatants of the transfected cells were harvested every 24 hr for 15 consecutive days and the HBsAg level in each supernatant was measured.

DNA immunization

Damage to the TA was produced by injecting 0·25% bupivacaine (100 µl in phosphate-buffered saline, Sigma, St. Louis, MO). Five days after bupivacaine injection, the regenerating TA was injected with plasmid DNA vectors in 100 µl of phosphate-buffered saline using a 1·0-ml syringe and a 27-g needle fitted with a collar of polyethylene tubing to limit the depth of the penetration to 2 mm. All intramuscular injections were carried out under anaesthesia.

Treatment of HBV-Tg mice with DNA-based or antigen-based vaccines

At the age of 6–7 weeks, HBV-Tg seropositive for HBsAg and HBV DNA were randomized to receive either a single intramuscular injection of a DNA vaccine (pCAGGS(S) or pCAGGS(S + preS2), or the parental plasmid, pCAGGS) or several intraperitoneal injections of an antigen-based vaccine containing 10·0 µg of HBsAg (Foundation for Microbial Diseases of Osaka University, Osaka, Japan) emulsified in complete Freund's adjuvant (CFA) at entry into the study and once in every month for 12 consecutive months. Some of the mice were only injected with CFA once a month for 12 consecutive months and other mice were left untreated, as described previously.[5–8]

Cell preparations

Whole spleen cells, T cells, and DC were isolated from mouse spleens as described previously.[6,7,20,21] T cells were enriched by applying a T-cell recovery kit (Biotex, Lab. Inc. Alberta, Canada) to Sephadex G-10 non-adherent splenic T/B cells, from which B cells had been depleted during passage through the affinity column. DC were isolated by culturing low-density adherent spleen cells for 18 hr at 37°, after which these cells became non-adherent. Purified DC were isolated by treating Fc-receptor-negative cells with a mixture of Thy-1·2 (clone 5a-8) and Lyt-1·2 (clone CG16; Cedarline, Ontario, Canada) plus CD45R (clone RA-3-6B2; Pharmingen, San Diego, CA) and low-toxicity complement (Cedarline) to eliminate contaminating T and B cells. CD4$^+$ T cells were isolated from mouse spleen cells using a commercial CD4 isolation kit (Biotex).

Preparation of antigen-specific memory lymphocytes

The procedure for preparation of HBsAg-specific memory lymphocytes has been described previously.[6,7,21] In brief, normal C57BL/6 mice were injected twice intraperitoneally with 10 µg of HBsAg in alum at an interval of 2 weeks. Serial assessment revealed that 3 months after booster immunization, lymphocytes from HBsAg-injected mice showed proliferation when cultured with HBsAg plus professional antigen-presenting cells like spleen DC, but not when cultured with HBsAg alone or with spleen DC alone.

Cell proliferation assay and culture

Whole spleen cells, purified T cells, and CD4$^+$ T cells were cultured with HBsAg or DC plus HBsAg for 120 hr, as described previously.[6–8,20,21] The incorporation of [^{3}H]thymidine (1 µCi/ml) during the final 12 hr of culture was measured as counts per minute (c.p.m.). HBsAg-specific T-lymphocyte proliferation was determined from the stimulation index (SI), which was calculated as the c.p.m. of cultures containing HBsAg divided by the c.p.m. of cultures without HBsAg. An SI value of > 2·0 indicated significant HBsAg-specific proliferation.

Detection of HBV-related antigens and antibody

Samples were screened for HBsAg and anti-HBs by the reverse passive haemagglutination method and passive

haemagglutination method, respectively, using commercial kits (Mycell, Tokyo Institute of Immunology, Tokyo, Japan). With these methods, samples showing haemagglutination at a dilution $> 2^2$ were considered positive. HBsAg, pre S2 antigen, and anti-HBs were quantified by a chemiluminescence enzyme immunoassay using a commercial kit (Tokyo Institute of Immunology, Tokyo, Japan). The detection limits of HBsAg and anti-HBs with these methods were 0·3 ng/ml and 1·25 mIU/ml, respectively.

Histology and immunohistochemistry

Muscle tissue from the injection sites was fixed in formalin or snap frozen. Infiltrating cells were localized by haematoxylin–eosin staining. To localize HBsAg-and CD11c-positive DC, muscle specimens were incubated overnight at 4° with appropriately diluted primary antibodies as described previously.[22,23] After washing in phosphate-buffered saline, the streptavidin–biotin method was employed for immunostaining. Colour was developed using true blue (KPL, Baltimore, MD) after peroxidase-conjugated streptavidin for HBsAg and

using New Fuchsin (DAKO, Glostrop, Denmark) after alkaline phosphatase-conjugated streptavidin for CD11c.

Statistical analysis

Data were expressed as the mean ± standard deviation (SD). Statistical analysis was done using Student's *t*-test, Fisher's exact test, and the Mann–Whitney *U*-test, as indicated. In all statistical comparisons, $P < 0.05$ was taken to indicate a significant difference.

RESULTS

Two plasmid DNA vectors, pCAGGS(S) and pCAGGS(S + preS2), respectively, were constructed that contained the genes of the major and middle envelope proteins of HBV. To verify protein expression, COS I cells and G8 cells were transfected with these plasmids and with the parental plasmid (pCAGGS). Immunohistochemical staining confirmed the expression of HBsAg in COS I cells and G8 cells transfected with these plasmids (Fig. 1a–c), but not in cells transfected with pCAGGS
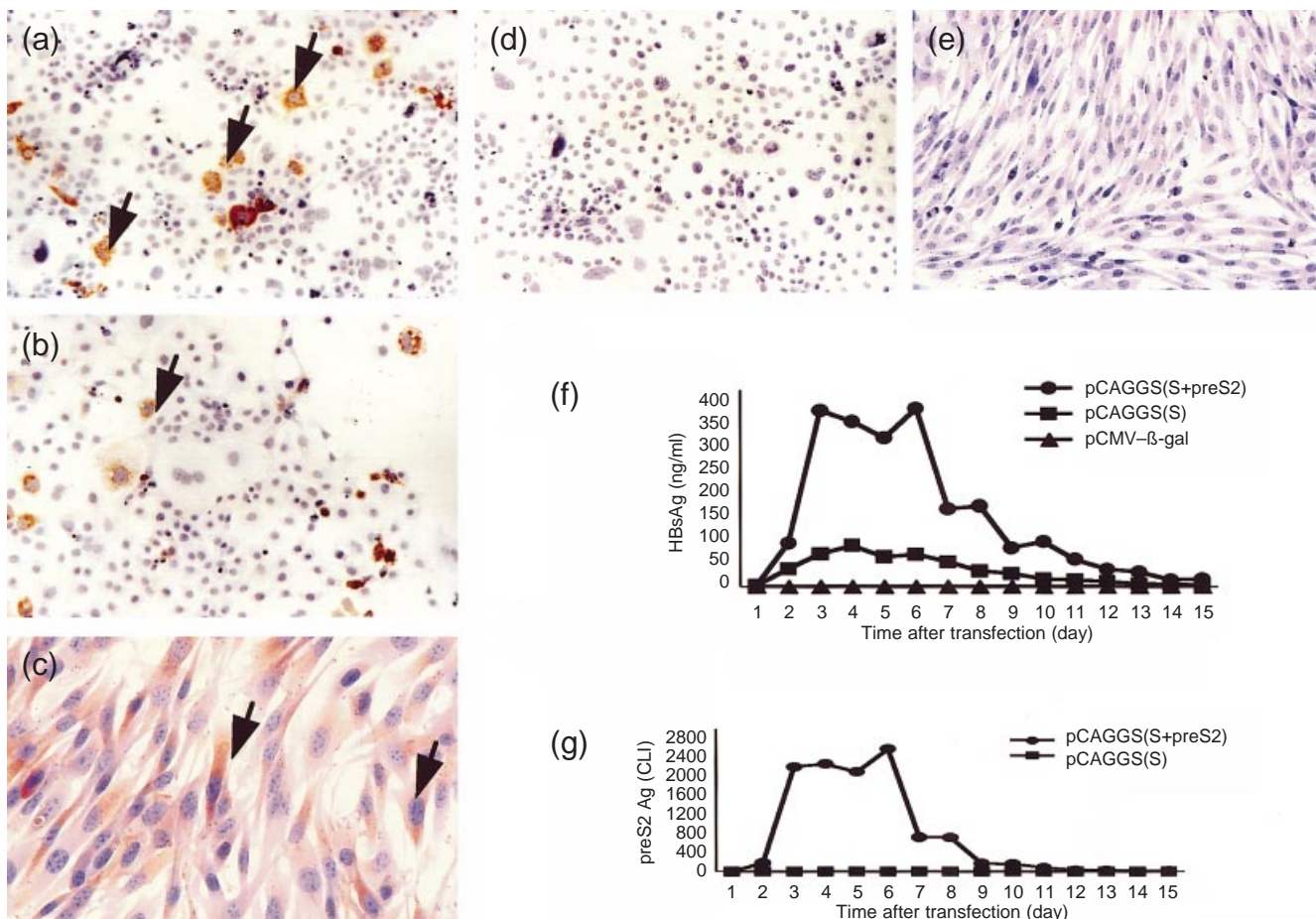

Figure 1. COS I cells were transfected with pCAGGS(S) or pCAGGS(S + preS2) and G8 cells were transfected with pCAGGS(S). Expression of HBsAg was localized by immunohistochemistry. HBsAg expression was seen in COS I cells transfected with pCAGGS(S) (a) or pCAGGS (S + preS2) (b), as well as in G8 cells transfected with pCAGGS(S) (c), but was not seen in COS I cells or G8 cells transfected with the parental plasmid vector (pCAGGS) (d, e). (f) Time course of HBsAg secretion into the culture supernatant of COS I cells transfected with pCAGGS(S) or pCAGGS(S + preS2). (g) Time course of preS2 antigen secretion by COS I cells transfected with pCAGGS(S + preS2). HBsAg levels are shown as ng/ml and preS2 levels are shown as CLI (chemiluminescence intensity unit).

(Fig. 1d, e). HBsAg was detected in the culture supernatant of COS I cells transfected with pCAGGS(S) and pCAGGS(S + preS2). PreS2 antigen was detected in the culture supernatant of COS I cells transfected with pCAGGS(S + preS2) but not in culture supernatant of COS I cells transfected with pCAGGS(S) (fig. 1f–g). The levels of HBsAg were higher in culture supernatant of COS I cells transfected with pCAGGS(S + preS2) compared with their levels in culture supernatant of COS I cells transfected with pCAGGS(S). This is possibly due to the fact that chemiluminescence enzyme immunoassay method for HBsAg detected both HBsAg and pre S2 antigen. HBsAg was also detected in the culture supernatant of G8 cells transfected with pCAGGS(S) and pCAGGS(S + preS2) (data not shown).

Humoral and cellular immune responses to HBsAg in normal C57BL/6 mice

Injection of normal C57BL/6 mice with $100 \cdot 0$ µg and $50 \cdot 0$ µg of pCAGGS(S) and pCAGGS(S + preS2) resulted in the detection of anti-HBs in the serum after only 2 weeks. In most of the mice, the maximum anti-HBs levels were seen at 8 or 12 weeks after injection. The anti-HBs level declined thereafter, but it remained detectable until 32 weeks in most of the mice. HBsAg-specific T-cell proliferation was detected in all of the normal mice injected with pCAGGS(S) that became seropositive for anti-HBs as well as in two out of five normal mice injected with pCAGGS(S) that were seronegative for anti-HBs (Table 1). On the other hand, normal C57BL/6 mice injected with the control plasmid did not show HBsAg-specific T-cell proliferation.

Infiltration of antigen-presenting DC at the injection site and expression of HBsAg by muscle cells and infiltrating cells

In normal mice, infiltrating cells increased over time at the injection site of pCAGGS(S) in the TA (Fig. 2a–d). CD11c-positive DC were also found among these infiltrating cells (Fig. 3a–b). HBsAg was expressed by muscle cells at the injection site(Fig. 3c), and also by infiltrating cells (Fig. 3d).

HBsAg-pulsed DC in the spleens of DNA-vaccinated mice

Spleen DC from normal mice at 32 weeks after the injection of pCAGGS(S), but not spleen DC from untreated normal mice, induced the proliferation of T cells from DNA-vaccinated mice

Table 1. HBsAg-specific cellular immune response in vaccinated normal C57BL/6 mice

Mouse	Numbers	Positivity (%)	SI
DNA-vaccinated normal mouse (serum anti-HBs (+ve))	10	10 (100%)	$5 \cdot 9 \pm 1 \cdot 2$*
DNA-vaccinated normal mouse (serum anti-HBs (–ve))	5	2 (40%)	$2 \cdot 9$, $3 \cdot 1$

Normal C57BL/6 mice were injected with a single injection of 100 µg of pCAGGS(S). Spleen cells from 10 mice seropositive for anti-HBs and 5 mice seronegative for anti-HBs were cultured with HBsAg for 120 hr. The incorporation of [^{3}H]thymidine during the final 12 hr of culture were measured as c.p.m. HBsAg-specific T lymphocyte proliferation was determined from the stimulation index (SI), which was calculated as the c.p.m. of cultures containing HBsAg divided by the c.p.m. of cultures without HBsAg. An SI value of $> 2 \cdot 0$ indicated significant HBsAg-specific proliferation.
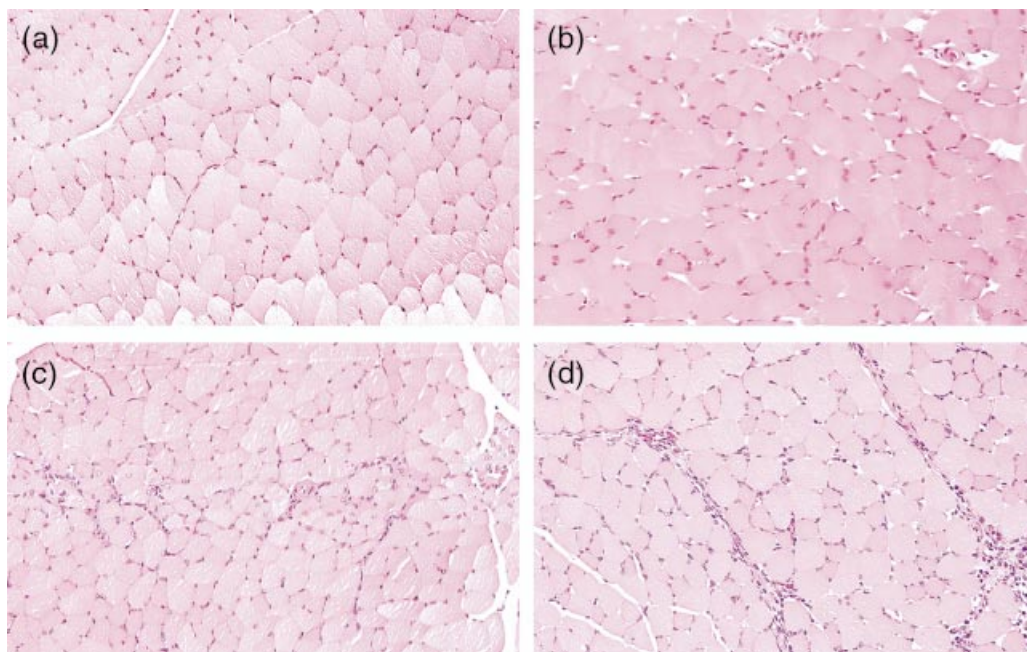
*Mean $\pm$ SD.

Figure 2. Muscle samples were removed from the site of injection of the DNA vaccine before as well as 24, 48 and 72 hr after injection of pCAGGS(S), encoding the major envelope protein of HBV. Haematoxylin–eosin staining showed the localization of infiltrating cells at the site of DNA vaccination before (a), as well as 24 hr (b), 48 hr (c), and 72 hr (d) after injection.

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(Table 2a) and the proliferation of HBsAg-specific memory T cells (Table 2b) in cultures without exogenous HBsAg. These findings indicate that DC from pCAGGS(S)-vaccinated mice were already exposed to HBsAg *in vivo* and that no further stimulation by this antigen was needed to induce a specific cellular immune response from HBsAg-specific memory T cells. This was an antigen-specific phenomenon because spleen DC from pCAGGS(S)-injected mice did not induce the proliferation of keyhole limpet haemocyanin-specific T cells (data not shown).

When spleen DC from pCAGGS(S)-injected mice were cultured in the presence of recombinant mouse granulocyte–macrophage colony-stimulating factor (R & D Systems,

Minneapolis, MN) for 14 days, the HBsAg level was were 12 ng/ml, 25 ng/ml, and 43 ng/ml in three separate cultures.

Therapeutic value of DNA and HBsAg-based vaccines in HBV-Tg mice

A single injection of pCAGGS(S) (50 or 100 μg/mouse) resulted in the clearance of HBsAg from 14 out of 15 HBV-Tg mice and the HBsAg level decreased in the other mouse (Table 3). Anti-HBs was detected in 4 out of 15 HBV-Tg mice at 12 weeks after vaccination (Table 3). The outcome was similar in HBV-Tg mice injected with pCAGGS (S + preS2) (Table 4). These HBV-Tg mice remained anti-HBs-positive

Table 2. HBsAg-pulsed dendritic cells in the spleens of DNA-vaccinated mice

DC	T cells	HBsAg	Numbers	SI*
(a)				
Normal untreated mice	DNA-vaccinated mice	–	5	1·0
Normal untreated mice	DNA-vaccinated mice	+	5	6·9±1·2
DNA-vaccinated mice	DNA-vaccinated mice	–	5	5·3±2·1
DNA-vaccinated mice	DNA-vaccinated mice	+	5	9·3±1·2
(b)				
Normal untreated mice	HBsAg-specific memory T cells	–	5	1·0
Normal untreated mice	HBsAg-specific memory T cells	+	5	4·7±0·9
DNA-vaccinated mice	HBsAg-specific memory T cells	–	5	4·9±0·8
DNA-vaccinated mice	HBsAg-specific memory T cells	+	5	8·7±1·5

DC were isolated from mouse spleens of normal untreated C57BL/6 mice or pCAGGS(S)-injected C57BL/6 mice. T cells were isolated from C57BL/6 mice at 32 weeks after a single injection of 100 μg of pCAGGS(S) (DNA-vaccinated mice). The procedure for preparation of HBsAg-specific memory T cells was described in Methods. Different cells were cultured with or without HBsAg for 120 hr. The mean cpm in cultures containing DC from normal untreated mice and T cells from DNA-vaccinated mice (a) or the mean cpm in cultures containing DC from normal untreated mice and T cells from HBsAg-specific memory T cells (b) were considered as stimulation index (SI) of 1·0. An SI value of >2·0 indicated significant HBsAg-specific proliferation.
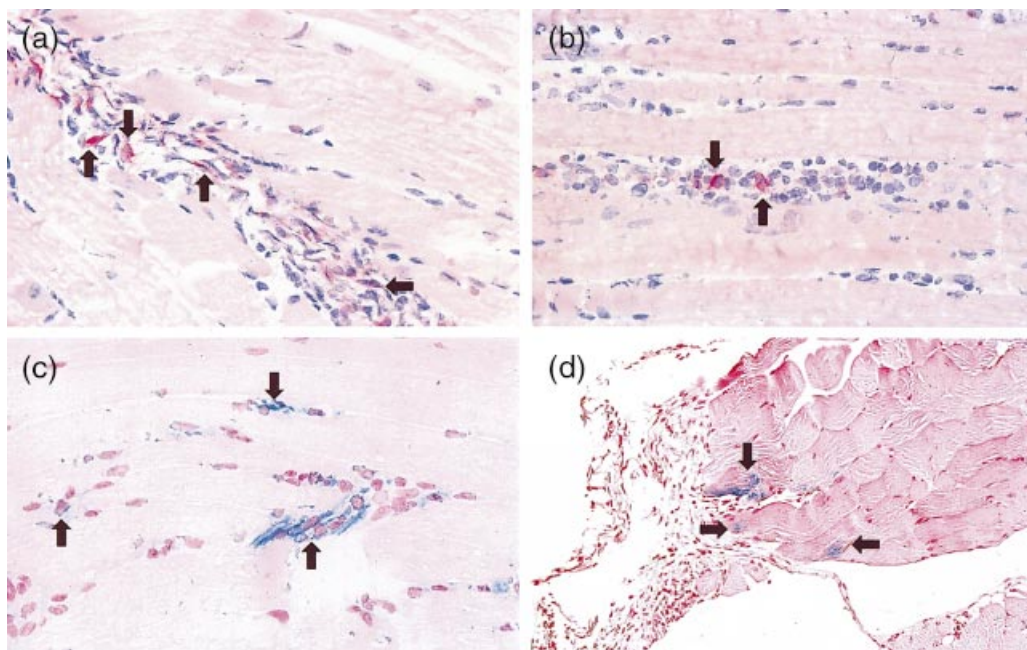
*Mean±SD of five separate experiments.

Figure 3. Muscle samples were taken from the site of vaccination at 72 hr after injection of pCAGGS(S) (encoding major envelope protein of HBV) were immunohistochemically stained to localize CD11c-positive DC and HBsAg. DC were found among the infiltrating cells (arrowhead a, b). HBsAg was expressed both by the muscle cells (c) and the infiltrating cells (d).

throughout the observation period of 32 weeks (Table 4). The HBsAg level showed almost no change in HBV-Tg mice injected with pCAGGS, the parental vector (data not shown).

The changes of HBsAg positivity in 29 HBV-Tg mice injected with the HBsAg-based vaccine are shown in Fig. 4. None of the mice became negative for HBsAg until 7 months after the start of therapy. Six of the 29 mice became negative for

Table 3. Therapeutic value of vaccination of pCAGGS(S) in HBV-Tg mice

	HBsAg (ng/ml)						Anti-HBs (mIU/ml)					
	Before	2 weeks	4 weeks	8 weeks	12 weeks	32 weeks	Before	2 weeks	4 weeks	8 weeks	12 weeks	32 weeks
(a) 100 µg/mouse												
Female HBV-Tg mice												
No.1	50	<0·3	<0·3	<0·3	<0·3	<0·3	<1·25	<1·2	<1·25	<1·25	<1·25	<1·25
No.2	48	<0·3	<0·3	<0·3	<0·3	<0·3	<1·25	<1·25·	<1·25	11	82	65
No.3	52	<0·3	<0·3	<0·3	<0·3	<0·3	<1·25	<1·25	<1·25	17	69	56
No.4	29	<0·3	<0·3	<0·3	<0·3	<0·3	<1·25	<1·25	<1·25	<1·25	<1·25	<1·25
No.5	57	<0·3	<0·3	<0·3	<0·3	ND	<1·25	<1·25	<1·25	<1·25	<1·25	ND
Male HBV-Tg mice												
No.1	120	<0·3	76	45	ND	<0·3	<1·25	<1·25	<1·25	<1·25	ND	<1·25
No.2	210	68	45	ND	<0·3	<0·3	<1·25	<1·25	<1·25	ND	<1·25	<1·25
No.3	208	<0·3	98	48	ND	<0·3	<1·25	<1·25	<1·25	<1·25	ND	<1·25
No.4	105	<0·3	<0·3	<0·3	<0·3	<0·3	<1·25	<1·25	<1·25	<1·25	5·0	<1·25
No.5	154	<0·3	52	25	<0·3	<0·3	<1·25	<1·25	<1·25	<1·25	7·0	<1·25
(b) (50 µg/mouse): Female HBV-Tg mice												
No.1	56	<0·3	<0·3	<0·3	<0·3	<0·3	<1·25	<1·25	<1·25	<1·25	<1·25	<1·25
No.2	52	<0·3	<0·3	<0·3	<0·3	<0·3	<1·25	<1·25	<1·25	<1·25	<1·25	<1·25
No.3	54	<0·3	<0·3	<0·3	<0·3	<0·3	<1·25	<1·25	<1·25	<1·25	<1·25	<1·25
No.4	50	<0·3	<0·3	<0·3	<0·3	<0·3	<1·25	<1·25	<1·25	<1·25	<1·25	<1·25
No.5	56	<0·3	12	8	7	ND	<1·25	<1·25	<1·25	<1·25	<1·25	ND

At the age of 6–7 weeks, 15 HBV-Tg mice (female 10 and male 5) were injected with a single intramuscular injection of 100 µg of pCAGGS(S) (a) or 50 µg of pCAGGS(S) (b). Serum HBsAg and anti-HBs levels were evaluated before and at 2, 4, 8, 12 and 32 weeks after vaccination by chemiluminescence enzyme immunoassay method. The detection limits of HBsAg and anti-HBs by these methods were 0·3 ng/ml and 1·25 mIU/ml, respectively.

ND, not determined.

Table 4. Therapeutic value of vaccination of pCAGGS(S + preS2) in HBV-Tg mice

	HBsAg (ng/ml)						Anti-HBs (mIU/ml)					
	Before	2 weeks	4 weeks	8 weeks	12 weeks	32 weeks	Before	2 weeks	4 weeks	8 weeks	12 weeks	32 weeks
(a) 100 µg/mouse												
Female HBV-Tg mice												
No.1	52	<0·3	<0·3	<0·3	<0·3	<0·3	<1·25	20·0	25·0	32	156	124
No.2	50	<0·3	<0·3	<0·3	<0·3	<0·3	<1·25	18	21	24	148	72
No.3	51	<0·3	<0·3	<0·3	<0·3	<0·3	<1·25	<1·25	<1·25	<1·25	<1·25	<1·25
No.4	62	28	26	29	25	24	<1·25	<1·25	<1·25	<1·25	<1·25	<1·25
No.5	58	<0·3	<0·3	<0·3	<0·3	ND	<1·25	<1·25	<1·25	<1·25	<1·25	ND
Male HBV-Tg mice												
No.1	202	54	<0·3	<0·3	<0·3	<0·3	<1·25	<1·25	<1·25	<1·25	<1·25	<1·25
No.2	260	68	<0·3	<0·3	<0·3	<0·3	<1·25	<1·25	<1·25	<1·25	<1·25	<1·25
No.3	278	75	<0·3	<0·3	<0·3	<0·3	<1·25	<1·25	<1·25	<1·25	<1·25	<1·25
No.4	150	52	<0·3	<0·3	<0·3	<0·3	<1·25	<1·25	<1·25	<1·25	<1·25	<1·25
No.5	280	80	<0·3	<0·3	<0·3	<0·3	<1·25	<1·25	<1·25	<1·25	<1·25	<1·25
(b) 50 µg/mouse: Female HBV-Tg mice												
No.1	56	30	<0·3	<0·3	<0·3	<0·3	<1·25	<1·25	<1·25	<1·25	<1·25	<1·25
No.2	53	35	<0·3	<0·3	<0·3	<0·3	<1·25	<1·25	<1·25	<1·25	<1·25	<1·25
No.3	54	<0·3	<0·3	<0·3	<0·3	<0·3	<1·25	<1·25	<1·25	<1·25	21	68
No.4	51	<0·3	<0·3	<0·3	<0·3	<0·3	<1·25	<1·25	<1·25	<1·25	<1·25	<1·25
No.5	57	<0·3	<0·3	<0·3	<0·3	ND	<1·25	<1·25	<1·25	<1·25	<1·25	ND

At the age of 6–7 weeks, 15 HBV-Tg mice (female 10 and male 5) were injected with a single intramuscular injection of 100 µg of pCAGGS(S + preS2) (a) or 50 µg of pCAGGS(S + preS2) (b). Serum HBsAg and anti-HBs levels were evaluated before and at 2, 4, 8, 12 and 32 weeks after vaccination by chemiluminescence enzyme immunoassay method. The detection limits of HBsAg and anti-HBs by these methods were 0·3 ng/ml and 1·25 mIU/ml, respectively.
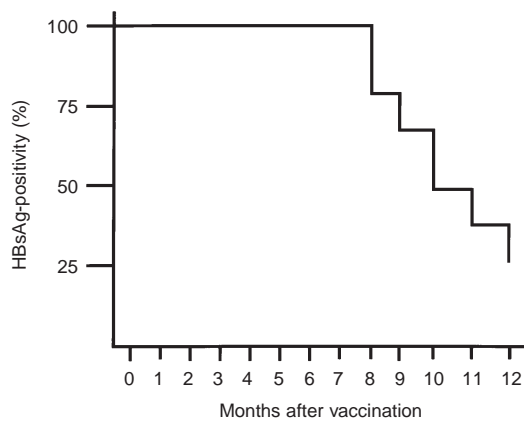
ND, not determined.

Figure 4. HBsAg-based vaccine therapy in HBV-Tg mice. Twenty-nine HBV-Tg mice were assigned to receive injections of a vaccine containing 10 µg of HBsAg emulsified in CFA at the start of the study and then once a month for 12 consecutive months. No HBV-Tg mice became negative for HBsAg until 7 months after the start of vaccination. Six of the 29 HBV-Tg mice became negative for HBsAg after receiving eight vaccinations with HBsAg in CFA, and 21 mice became negative for HBsAg after 12 months.

HBsAg after receiving 8 monthly vaccinations and 21 were negative after 12 months. There was no alteration of the HBsAg titre in HBV-Tg mice injected with CFA alone or in control HBV-Tg mice (data not shown).

The CD4$^+$ T-cell response against HBsAg in HBV-Tg mice vaccinated with pCAGGS(S) or HBsAg-based vaccines are shown in Table 5. Both of these vaccines induced the HBsAg-specific proliferation of CD4$^+$ T cells in the mice.

DISCUSSION

We produced two HBV DNA-encoding plasmid vectors, pCAGGS(S) and pCAGGS(S+preS2) that expressed their gene products in muscle tissue. COS I cells and G8 cells transiently transfected with these two vectors expressed and produced HBsAg, with higher levels of HBsAg being detected after injection of pCAGGS(S+preS2) (Fig. 1).

Detection of anti-HBs is generally being used to assess the HBsAg-specific immune response due to DNA vaccination. However, two of the HBV DNA-vaccinated normal C57BL/6 mice were negative for anti-HBs, but showed an HBsAg-specific cellular immune response (Table 1). Davis *et al.* have shown that HBV DNA-vaccinated mice simultaneously secrete HBsAg and produce anti-HBs.[24,25] Due to this, low levels of anti-HBs may not be detectable in some DNA-vaccinated mice due to circulating HBsAg. Under this circumstances, it is important to always evaluate the HBsAg-specific cellular immune response, in addition to serum anti-HBs level.

Muscle cells and infiltrating mononuclear cells were found to express HBsAg both at and around the site of DNA vaccination. CD11c-positive DC were also found at these locations. Although the expression of HBsAg on DC in the muscle tissue has not been confirmed in this model, DC located near the injection site may take up HBsAg secreted by the muscle cells. Indeed, DC may undergo direct transfection by these plasmids.[26] Whatever the mechanism involved, according

Table 5. HBsAg-specific CD4 + T cell responses in vaccinated HBV-Tg

Mouse	Numbers	Positivity (%)	SI*
DNA-vaccinated HBV-Tg mice (anti-HBs(+ve))	5	5 (100%)	4·1 ± 0·4
HBsAg-vaccinated HBV-Tg mice (anti-HBs(+ve))	5	5 (100%)	3·6 ± 0·5

HBV-Tg were randomized to receive either a single intramuscular injection of 100 µg of pCAGGS(S) or several intraperitoneal injections of 10·0 µg of HBsAg in CFA at entry and once in every month for 12 consecutive months. CD4$^+$ T cells were isolated from spleens of HBV-Tg mice seropositive for anti-HBs in the sera using an affinity column (Biotex). CD4$^+$ T cells and spleen DC were cultured without or with HBsAg for 120 hr. HBsAg-specific T lymphocyte proliferation was determined from the stimulation index (SI), which was calculated as the cpm of cultures containing HBsAg divided by the c.p.m. of cultures without HBsAg. An SI value of >2·0 indicated significant HBsAg-specific proliferation.
* Mean ± SD of five separate experiments.

to the general principles of antigen presentation, DC should capture HBsAg and migrate to regional lymph nodes or secondary lymphoid tissues like the spleen to induce an HBsAg-specific immune response. A single injection of DNA vaccine has been reported to induce a long-lasting HBsAg-specific immune response. We also detected anti-HBs for up to 32 weeks after a single injection of DNA vaccine in some mice. HBsAg-specific memory lymphocytes may be a source of anti-HBs in HBV DNA-vaccinated mice, but it is important to clarify how these memory lymphocytes are activated *in vivo* to produce anti-HBs. DC isolated at 32 weeks after a single injection of pCAGGS(S) had already been exposed to HBsAg because these DC were able to induce the proliferation of HBsAg-specific memory lymphocytes when cultured in the absence of HBsAg (Table 2b). We also detected HBsAg in the cultures of purified splenic DC from pCAGGS(S)-injected mice. Although the underlying mechanism needs to studied in detail, the persistence of HBsAg in the spleens of DNA-vaccinated mice may have had a cardinal role.

The present experiments also assessed the therapeutic potential of DNA-based versus HBsAg-based vaccines. So far, we have conducted six placebo-controlled trials of HBsAg-based vaccination in HBV-Tg mice.[5–8] Consistent with our previous observations, monthly injection of HBsAg-based vaccine for 7 consecutive months did not achieve HBsAg-negativity in any of our HBV-Tg mice (Fig. 4). Only 6 of the 29 (21·0%) HBV-Tg mice became negative for HBsAg after receiving 8 monthly injections of HBsAg in CFA (Fig. 4). At the end of 12 months, however, 21 out of 29 HBV-Tg mice (70%) were negative for HBsAg. In sharp contrast, most of the HBV-Tg mice (28 out of 30) became negative for HBsAg at 2–4 weeks after a single injection of DNA vaccine (Tables 3 and 4). DNA-vaccinated HBV-Tg mice not only showed a rapid anti-HBs response, but these mice revealed an HBsAg-specific CD4$^+$ T-cell proliferative response (Table 5).

In summary, we showed that DNA vaccines encoding HBV envelope protein genes can efficiently elicit both humoral and cellular immune responses in normal mice. The presence of HBsAg-pulsed DC in the spleen at 32 weeks after a single injection of DNA vaccine and the production of HBsAg by spleen DC indicate that these cells may play a cardinal role in long-lasting HBsAg-specific immune responses following

a single injection of DNA vaccine. Finally, a DNA-based vaccine may be a better therapeutic choice for the treatment of HBV carriers than an antigen-based vaccine.

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