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Combination of an Antiviral Drug and Immunomodulation against Hepadnaviral Infection in the Woodchuck Model[▽]

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The essential role of multispecific immune responses for the control of hepatitis B virus (HBV) infection implies the need of multimodal therapeutic strategies for chronic HBV infection, including antiviral chemotherapy and immunomodulation. This hypothesis was tested in the woodchuck model by a combination of lamivudine pretreatment and subsequent immunizations of woodchucks chronically infected with woodchuck hepatitis virus. The immunizations were performed with DNA vaccines or antigen-antibody immune complexes (IC)/DNA vaccines. Immunizations with IC/DNA vaccines led to an anti-woodchuck hepatitis virus surface antibody response and significant reductions of viral load and antigenemia, suggesting that such a strategy may be effective against chronic HBV infection.

Approximately 400 million people worldwide are chronically infected with hepatitis B virus (HBV). Therapy with interferon or nucleoside/tide analogs is not satisfactory due to the low responder rate and resistance development (16, 18, 20, 33). To date, immunotherapy against chronic hepatitis B has not yet achieved satisfactory results (7, 14, 17, 31, 32, 41, 44). Given the crucial role of cytotoxic T lymphocytes in the control of HBV infections, new therapeutic vaccines with the ability to stimulate vigorous, broad HBV-specific cytotoxic T lymphocyte responses are needed (3, 11, 26, 38, 39).

Several studies of therapeutic vaccinations have been carried out in the woodchuck model (reviewed in references 24, 25, 34, and 35) and demonstrated collectively that B- or T-cell responses to viral antigens could be induced in chronic woodchuck hepatitis virus (WHV) carriers (12, 13, 23, 30). Yet, none of these studies have demonstrated the capability of vaccines to suppress viral replication. Here, we carried out a proof-of-principle experiment using DNA vaccines alone and DNA vaccines combined with immune complexes (IC) in the woodchuck model. IC of HBsAg and anti-HBs have been tested in patients and in transgenic mice (42, 43, 45, 46). IC are more efficiently taken up by antigen-presenting cells than free antigens, leading to an improved presentation to T cells. DNA vaccines are potent inducers of T-cell responses. They could stimulate HBV-specific immune responses in humans and prevent hepadnaviral infections in the animal model (21, 22, 37). In addition, woodchucks were pretreated with lamivudine, a potent antiviral drug against HBV with the ability to enhance T-cell responses in chronically HBV-infected patients (1, 2).

A total of 10 chronically WHV-infected woodchucks (from Northeastern Wildlife, Ithaca, NY) were first treated with 15 mg of lamivudine daily and randomly divided into three groups: the control group ($n = 2$), a group vaccinated with WHV surface antigen (WHsAg)-IC and DNA ($n = 4$), and a group vaccinated with DNA ($n = 4$). The immunization schedule is presented in Fig. 1.

The DNA vaccines consisted of an equimolar mixture of three plasmids, pWHsIm, pWHcIm, and pWIFN, expressing WHsAg, WHV core antigen (WHcAg), and woodchuck gamma interferon, respectively, as described previously (21, 37). To produce IC for woodchucks, antibodies to WHs (anti-WHs) and WHsAg were titrated by the checkerboard method to determine the stoichiometry of the antigen and antibodies. The appropriate concentration of WHsAg was chosen and incubated with antibodies at 37°C for 30 min and then incubated overnight at 4°C, resulting in a preparation with a final concentration of 80 μg WHsAg/ml in complex with anti-WHs. The vaccine consisted of 20 μg WHsAg-IC and 250 μg plasmid pWHsIm DNA per 0.5-ml dose. The DNA vaccines and IC-DNA vaccines were administered by intramuscular injections (21, 22).

The following parameters were determined by the indicated methods: serum WHV DNA concentrations as genome equivalents (GE) by real-time PCR with the LightCycler DNA Master Sybr green kit (Roche) (22), WHsAg concentrations by the electroimmunodiffusion technique using a polyvalent anti-WHV antiserum (10, 40), anti-WHs concentrations by enzyme-linked immunosorbent assay (23), and lymphoproliferative responses to WHV proteins and peptides by immunofluorescence (29).

Lamivudine therapy alone. In both control animals, the viral loads decreased slightly during treatment and reached 8.1×10^9 GE/ml for woodchuck WH17496 and 5.6×10^8 GE/ml for woodchuck WH17498 at week 21, corresponding to reductions

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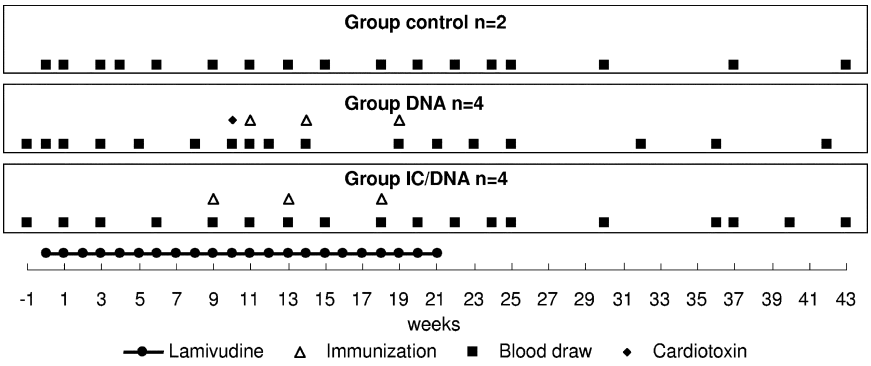


FIG. 1. Schema of treatment and immunizations. Ten woodchucks with chronic WHV infection were divided into three groups. For 21 weeks, 15 mg of lamivudine was given orally to all woodchucks. The first group ($n = 2$) served as the control and did not receive any vaccinations. The second group ($n = 4$) was immunized with the combination of three plasmids expressing WHsAg, WHcAg, and woodchuck gamma interferon. The third group ($n = 4$) received three immunizations with WHsAg-IC and plasmid pWHsIm.

of 0.7 log and 0.32 log, respectively (Fig. 1; Table 1). Following the discontinuation of lamivudine treatment, the viral loads rebounded and increased to peak values of 9. 7×10^{10} GE/ml (WH17496) and 8.8×10^9 GE/ml (WH17498) at week 25 and then returned to the pretreatment levels for both animals. The serum WHsAg concentrations in WH17496 and WH17498 were 438 and 239 μg/ml, respectively, prior to the lamivudine treatment. The WHsAg concentrations in the control animals decreased to 281 μg/ml and 140 μg/ml at week 21 and rebounded to 516 μg/ml and 170 μg/ml, respectively, at week 25. These results are consistent with the published data (28). No anti-WHs antibodies were detected in either control woodchuck.

DNA vaccination. Four woodchucks, WH17490, WH17491, WH17495, and WH17502, had initial WHV loads at 7.87×10^8, 3.74×10^9, 1.08×10^{10}, and 2.28×10^9 GE/ml, respectively (Fig. 2B). Pretreatment with cardiotoxin was performed at week 10 and at the DNA immunizations at weeks 11, 14, and 19. The viral loads decreased slightly in all four woodchucks without relation to vaccinations and rebounded at week 21 with the end of lamivudine treatment (Fig. 2B). In woodchuck WH17491, the WHV DNA concentration was reduced from 1.5×10^{10} to 7.2×10^8 GE/ml from weeks 11 to 21. In woodchuck WH17502, the viral load declined to 5.4×10^7 GE/ml after the start of lamivudine treatment but showed no response to vaccinations. The serum WHsAg concentrations in WH17490, WH17491, and WH17502 showed decreases of 25%, 3%, and 64%, respectively, at week 21. For unknown reasons, WHsAg increased in WH17495 to 118 μg/ml, corresponding to 147.5% of the baseline. No anti-WHs antibodies were detected in these woodchucks.

TABLE 1. Viral loads and WHsAg concentrations in the treated woodchucks

Group	Woodchuck	Baseline viral load (10^8 GE/ml)	Reduction of WHV DNA concn[a] (log)	Baseline WHsAg concn (μg/ml)	Reduction of WHsAg concn[a] (%)
Control	17496	400	0.7	438	36
	17498	12	0.32	239	25
DNA	17490	7.87	0.42	528	25
	17491	37.4	0.72	779	3
	17495	108	0.44	80	+47.5[b]
	17502	22.8	0.85	383	64
IC and DNA	17492	70	1.3	158	92
	17493	25	2.9	556	71
	17494	7.3	2.2	535	67
	17497	490	1.44	250	85

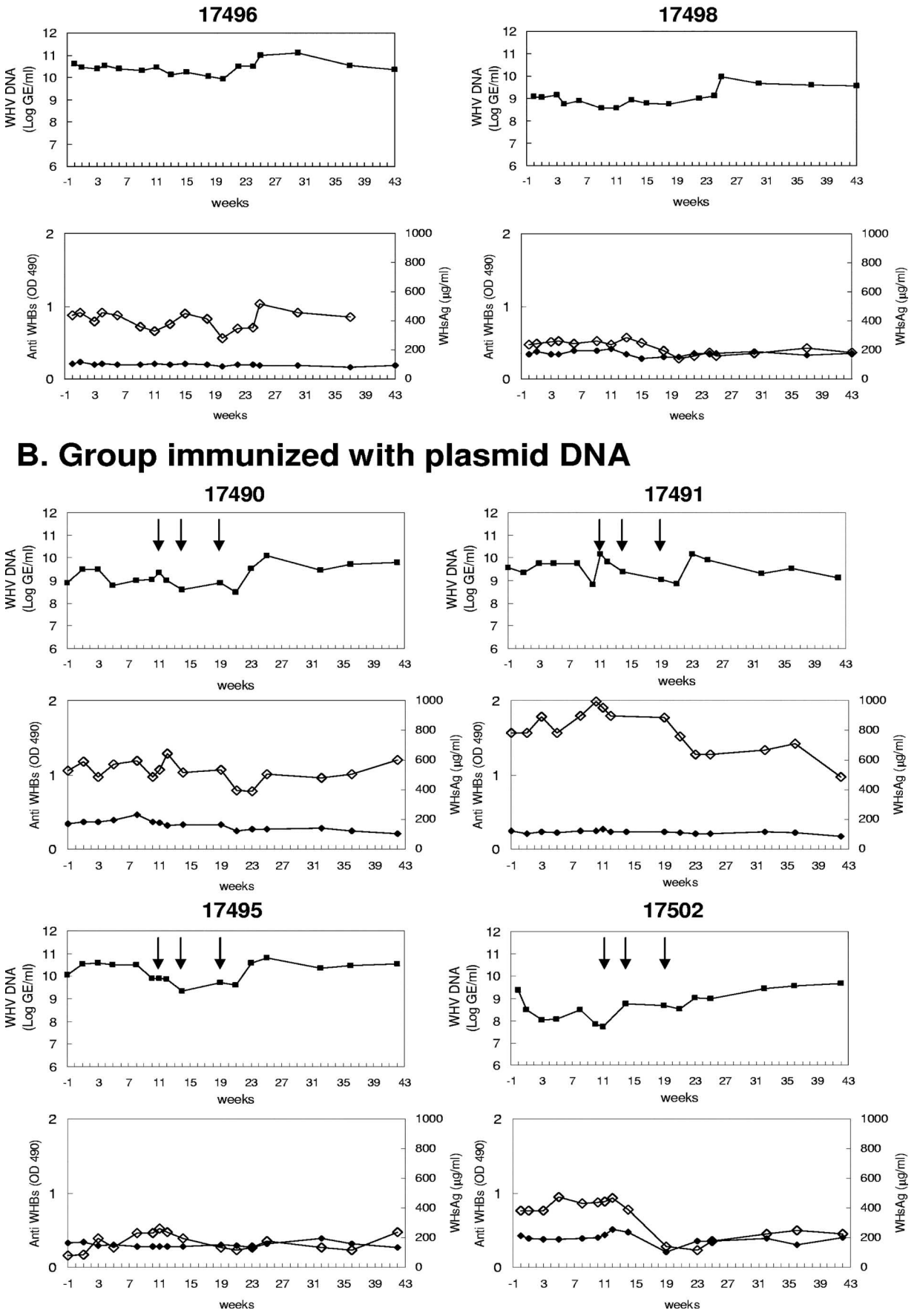
[a] The reductions of the viral loads and WHsAg concentrations in the woodchucks were calculated using the baseline values measured before the treatment and the values measured from weeks 18 to 21 after the completion of three vaccinations with DNA or IC and DNA.

[b] The WHsAg concentration was increased in WH17495; therefore the change is indicated with +.

Vaccination with WHs-IC and plasmid DNA. WH17492, WH17493, WH17494, and WH17497 had initial viral loads of 7.0×10^9, 2.5×10^9, 7.3×10^8, and 4.9×10^{10} GE/ml, respectively, and received the combined IC/DNA vaccine at weeks 9, 13, and 18. In woodchucks WH17492 and WH17497, the viral loads were reduced to 3.6×10^8 and 1.33×10^9 GE/ml, respectively, after the second boost (Fig. 2C). The viral loads in WH17493 and WH17494 dropped strongly to 3×10^6 and 4×10^6 GE/ml, respectively, at week 18. The maximal reduction levels of the viral loads in woodchucks after vaccinations with IC/DNA ranged between 1.3 and 2.9 log (Table 1). A rebound of the viral loads occurred after the discontinuation of lamivudine treatment at week 21.

Uniformly, the vaccinations with IC and DNA led to significant decreases of the serum WHsAg concentrations of 67% to 92% compared with the baseline in all four woodchucks (Fig. 2C; Table 1). After the start of lamivudine treatment, the serum WHsAg concentrations fell from 159 and 250 μg/ml in WH17492 and WH17497 to 12.5 and 37.7 μg/ml, respectively, after three vaccinations. WH17493 and WH17494 had serum WHsAg concentrations over 500 μg/ml at the beginning. The WHsAg concentrations reached about 200 μg/ml in these two animals after three vaccinations.

Strikingly, woodchucks WH17492, WH17493, and WH17494 developed a detectable anti-WHs antibody response after boosts, though the antibody response was not sustained (Fig. 2C). One woodchuck, WH17497, did not show any detectable anti-WHs antibody response despite the reduction of serum WHsAg concentrations. A reciprocal correlation between the amount of anti-WHs and WHV DNA or WHsAg was clearly observed in animals WH17493 and WH17494. With the rise of anti-WHs antibodies at week 13, the WHV DNA and the


A. Control group
17496
17498
B. Group immunized with plasmid DNA
17490
17491
17495
17502
WHV DNA (Log GE/ml)
Anti WHBs (OD 490)
WHsAg (μg/ml)
weeks

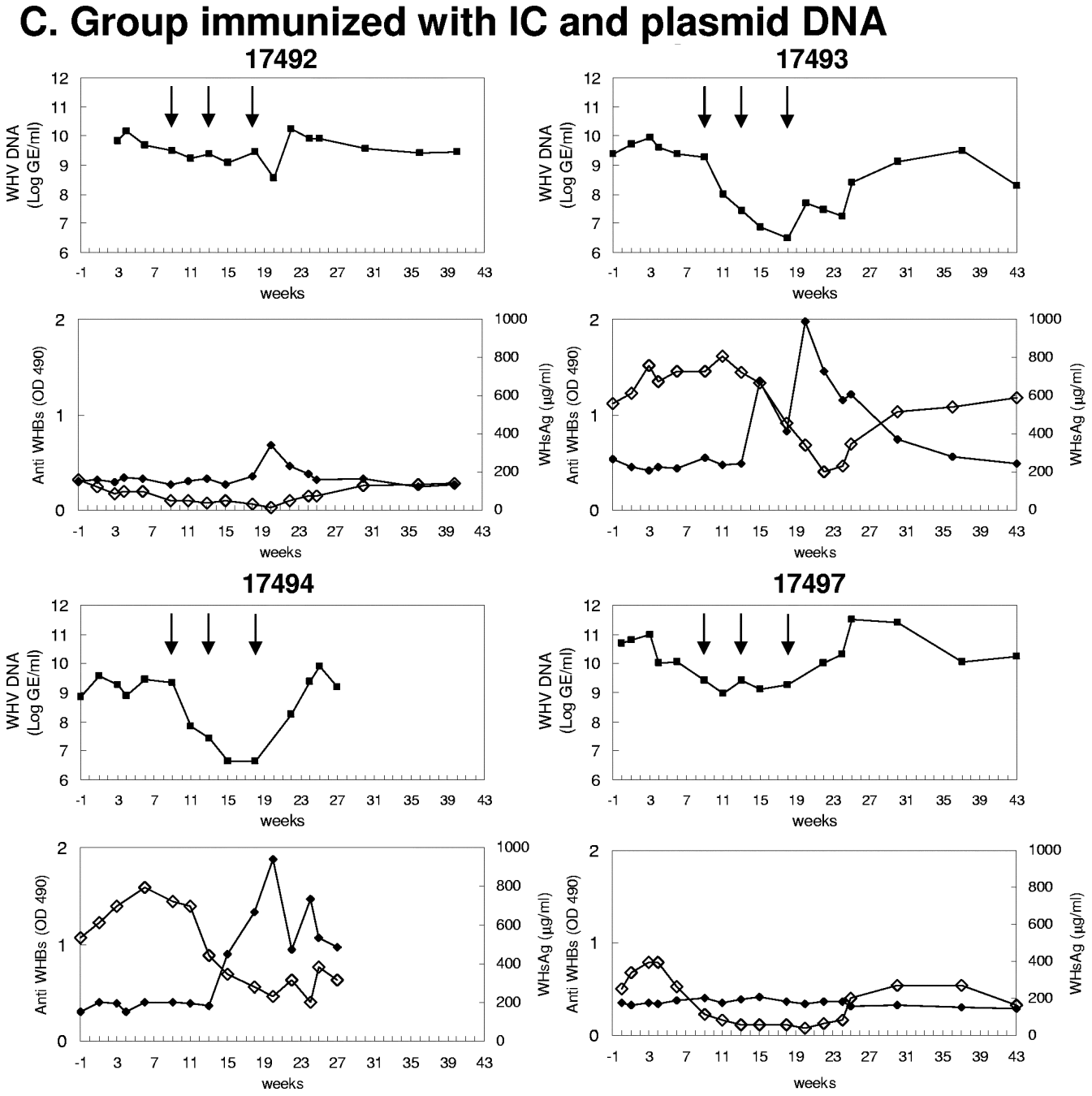


FIG. 2. Therapeutic vaccinations of chronically WHV-infected woodchucks. The serum WHV DNA concentrations (■), anti-WHs antibody response (Anti WHBs) (◆), and serum WHsAg concentrations (◇) in the treated woodchucks were measured. The duration of lamivudine treatment is indicated with a line. (A) Control group; (B) group immunized with plasmid DNA; (C) group immunized with IC and plasmid DNA. The serum WHV DNA concentrations are presented as GE/ml in the log scale. The anti-WHs were measured by enzyme-linked immunosorbent assay and are presented as values of optical density at 490 nm. WHsAg is given as μg/ml. The animal WH17494 was in a bad condition due to the biopsies and therefore was not included in further monitoring after week 27.

serum WHsAg concentrations were reduced to low levels in the woodchucks. Both markers for WHV replication increased again as the anti-WHs antibody titer decreased. Surprisingly, the anti-WHs antibodies were detected at higher levels in woodchucks WH17493 and WH17494, which had higher initial serum WHsAg concentrations. WH17497 had a relatively low serum WHsAg concentration at 250 μg/ml and the lowest level at 37.7 μg/ml. However, no anti-WHs antibodies were detected in this animal.

Lymphoproliferative responses in vaccinated woodchucks. WHV-specific lymphoproliferative responses were not detectable in the majority of chronically WHV-infected woodchucks in this study, even in woodchucks that were immunized with IC/DNA vaccines and exhibited virological responses (data not

TABLE 2. Histological examination of liver tissues of treated woodchucks

Group	Woodchuck	Score[a]		
		Portal inflammation	Lobular inflammation	Sum
Control	17496	2	1	3
	17498	0	2	2
DNA	17490	2	1	3
	17491	3	2	5
	17495	2	1	3
	17502	1	2	3
IC and DNA	17492	3	2	5
	17493	0	1	1
	17494	2	1	3
	17497	3	1	4

[a] The histological evaluation was done according to the Ishak scoring method (15). The grade 0 for portal inflammation indicates a minimal portal infiltrate.

shown). Significant lymphoproliferative responses to WHV antigens were measured only in woodchuck WH17502 after three vaccinations with associations of significant reductions of WHV markers (Table 1). These results were largely consistent with previous results (23). The time points of sampling are unlikely to be the reason for the negative results, since assays were performed every 2 weeks. These results may indicate the inability of vaccines to induce specific cellular responses. However, it is also possible that specific T cells were sequestered to the liver and therefore not detectable in peripheral blood.

Liver histology in immunized woodchucks. The liver biopsy specimens taken after the completion of the treatment at week 25 revealed mild or moderate hepatitis in all animals, according to the Ishak score (15) (Table 2), similar to untreated carrier woodchucks (5, 6). The levels of severity of portal inflammation varied in different animals, from a minimal portal infiltrate (grade 0) to a mild periportal infiltrate to a maximal periportal infiltrate with mild piecemeal necrosis (grade 3). The lobular inflammation also varied in different animals from grade 1 to grade 2 (mild inflammation but no necrosis or with focal necrosis), resulting in a range of the total score from minimal 1 to maximal 5. The two control animals showed a score of 2 and 3. The scores were between 3 and 5 or 1 and 5 for animals vaccinated with DNA and IC/DNA, respectively. In summary, immunizations with either of the protocols did not result in severe liver disease in woodchucks.

Taken together, the findings demonstrated that a combination of lamivudine treatment and immunizations with a vaccine containing IC and plasmid DNA was able to reduce the viral load up to 2.9 log and the serum WHsAg load up to 92% and induced specific anti-WHs antibodies in chronic carrier woodchucks. The lamivudine contributed to the suppression of viral loads despite its low effectiveness in woodchucks (28), as a viral rebound uniformly occurred immediately after the discontinuation of lamivudine treatment.

DNA immunization is considered to be a powerful method of inducing cellular immune responses to pathogens. However, the DNA vaccinations in our study did not clearly show any additional therapeutic effect compared to the lamivudine treatment alone. Similarly, DNA vaccination failed to induce viral clearance in chronically infected ducks (8, 19, 36). A DNA vaccine expressing HBsAg has been tested in patients for immunotherapy and appeared to enhance T-cell responses (27). Further studies on DNA vaccines are needed to improve their effectiveness for immunotherapies.

The present protocol with the lamivudine treatment and IC/DNA immunizations led to only a transient response in woodchucks. More potent antivirals, like entecavir (4, 9), and multiple vaccinations of more than three injections could further enhance specific immune responses and yield better results.

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