Immunization of Woodchucks with Plasmids Expressing Woodchuck Hepatitis Virus (WHV) Core Antigen and Surface Antigen Suppresses WHV Infection

MENGJI LU,[1] GERO HILKEN,[2] JOHANNES KRUPPENBACHER,[1] THEKLA KEMPER,[1] REINHOLD SCHIRMBECK,[3] JOERG REIMANN,[3] AND MICHAEL ROGGENDORF[1]*

Institut für Virologie[1] and Zentraltierlabor,[2] Universitätsklinikum Essen, Essen, and Institut für Medizinische Mikrobiologie und Immunologie, Universität Ulm, Ulm,[3] Germany

DNA vaccination can induce humoral and cellular immune response to viral antigens and confer protection to virus infection. In woodchucks, we tested the protective efficacy of immune response to woodchuck hepatitis core antigen (WHcAg) and surface antigen (WHsAg) of woodchuck hepatitis virus (WHV) elicited by DNA-based vaccination. Plasmids pWHcIm and pWHsIm containing WHV c- or pre-s2/s genes expressed WHcAg and WHsAg in transient transfection assays. Pilot experiments in mice revealed that a single intramuscular injection of 100 μg of plasmid pWHcIm DNA induced an anti-WHcAg titer over 1:300 that was enhanced by boost injections. However, two injections of 100 μg of pWHcIm did not induce detectable anti-WHcAg in woodchucks. With an increase in the dose to 1 mg of pWHcIm per injection, transient anti-WHcAg response and WHcAg-specific proliferation of peripheral mononuclear blood cells (PMBCs) appeared in woodchucks after repeated immunizations. Four woodchucks vaccinated with pWHcIm were challenged with 10^4 or 10^5 of the WHV 50% infective dose. They remained negative for markers of WHV replication (WHV DNA and WHsAg) in peripheral blood and developed anti-WHs in week 5 after challenge. In contrast, woodchucks not immunized or immunized with the control vector pcDNA3 developed acute WHV infection. Two woodchucks immunized with 1 mg of pWHsIm developed WHsAg-specific proliferative response of PBMCs but no measurable anti-WHsAg response. A rapid anti-WHsAg response developed during week 2 after virus challenge. Neither woodchuck developed any signs of WHV infection. These data indicate that DNA-based vaccination with WHcAg and WHsAg can elicit immunity to WHV infection.

Hepatitis B virus (HBV) causes acute self-limiting and chronic infection in humans (24). A chronic HBV infection leads to a high risk for the development of liver cirrhosis and hepatocellular carcinoma (30, 54). The current strategy for preventing HBV infection is vaccination with hepatitis B surface antigen (HBsAg), which induces virus-neutralizing anti-HBsAg antibodies (28). Though HBsAg is a potent immunogen and induces protective immunity in the majority of vaccines, 5 to 10% of persons who receive the HBsAg vaccine failed to develop anti-HBsAg antibodies. In addition, HBV variants carrying mutations within the HBsAg can escape the neutralization of vaccine-induced anti-HBsAg and establish acute or chronic infection (3, 4, 6, 25, 29, 43). Therefore, a new vaccine strategy would be desirable to induce a multiple immune response consisting of HBV-specific T helper (Th), cytotoxic T cells (CTLs), and anti-HBsAg antibodies. HBV-specific Th and CTL responses play a pivotal role for the clearance of virus in a primary HBV infection and may control HBV persisting in unknown reservoirs in patients whose disease is resolved (1, 7, 16, 18, 26, 27, 35, 41, 42, 44, 45). The induction of HBV-specific humoral and cellular immune response by a single vaccine may overcome the nonresponsiveness of individuals to conventional HBsAg vaccines and control immune escape variants of HBV with mutations within HBsAg.

DNA vaccination is a powerful method to induce antigen-specific humoral and cellular immune response (14, 56). DNA-induced immune response provides protective immunity to various viruses in animal models (2, 5, 13, 19, 22, 31, 34, 36, 51, 55, 57). Genetic vaccination to HBsAg, HBV core antigen (HBcAg), and HBV e antigen (HBeAg) was evaluated in different animal models. In mice, a single intramuscular injection of plasmids expressing HBsAg is sufficient to induce a long-lasting humoral response to HBsAg and CTL response (10, 12, 40, 50). A plasmid vaccination of chimpanzees led to the production of low anti-HBsAg antibody titers (11, 47). Recently, Triyatni et al. reported that vaccination of ducks with plasmid expressing duck hepatitis B virus (DHBV) surface antigens (DHBsAg) induced antibodies to DHBsAg (55). Anti-DHBsAg antibodies induced by DNA vaccination were able to neutralize virus in vitro. DHBV was removed more rapidly from the bloodstreams of vaccinated ducks after a challenge. Infection of hepatocytes by DHBV was limited or prevented in vaccinated ducks. Therefore, the genetic vaccination was effective to prime an anti-HBsAg antibody response in this model. The vaccination of mice with HBcAg or HBeAg was also effective for inducing specific CTL responses (33).

The woodchuck (*Marmota monax*) model is useful to study immune response to hepadnavirus and to perform vaccination trials (8, 9, 23, 39, 48, 49, 52). Woodchuck hepatitis virus (WHV) causes acute self-limiting and chronic infection, like HBV in humans (53). The humoral immune responses to woodchuck hepatitis surface antigen (WHsAg) and core antigen (WHcAg) in acute and chronic WHV infection have the same features as those of HBV infection. Anti-WHcAg develops in woodchucks during the early phase of a primary WHV infection and persists lifelong. Anti-WHsAgs, like anti-HBsAgs, increase at the end of the viremic phase and may provide

* Corresponding author. Mailing address: Institut für Virologie, Universitätsklinikum Essen, Hufelandstrasse 55, D-45122 Essen, Germany. Phone: 49-201-723-3550. Fax: 49-201-723-5929. E-mail: roggendorf@uni-essen.de.

TABLE 1. Peptides of WHsAg for stimulation of
woodchuck PBMC

Peptide[a]	Amino acid sequence
S1-15	MGNNIKVTFNPDKIA
S16-36	AWWPAVGTYYTTTYPQNQSVF
S37-44	QPGIYQTT
S45-59	SLINPKNQQELDSVL
S60-81	INRYKQIDWNTWQGFPVDQKF
S82-101	SLVSRDPPPKPYINQSAQTF
S102-121	EIKPGPIIVPGIRDIPRGLV
S122-141	PPQTPTNRDQGRKPTPPTPP
S142-161	LRDTHPHLTMKNQTFHLQGF
S162-181	VDGLRDLTTTERQHNAYRDP
S182-203	FTTLSPAVPTVSTILSPPSTTG
S204-226	DPALSPEMSPSSLLGLLAGLQVV
S227-234	YFLWTKIL
S235-243	TIAQNLDWW
S244-261	CTSLSFPGGIPECTGQNS
S262-281	QFQTCKHLPTSCPPTCNGFR
S282-291	WMYLRRFIIY
S302-321	LLVLLDWKGLIPVCPLQPTT
S322-341	ETTVNCRQCTISAQDMYTPP
S342-361	YCCCLKPTAGNCTCWPIPSS
S362-371	WALGNYLWEW
S372-381	ALARLSWLNL
S382-401	LVPLLQWLGGISLIAWFLLI
S402-411	WMIWFWGPAL
S412-432	LSILPPFIPIFVLFFLIWVYI

[a] Peptides were synthesized according to the deduced amino acid sequence of the large surface antigen (LWHsAg) of WHV8. Peptides are designated according to their position within LWHsAg. The peptides have different lengths because of the limitations of the synthesis procedure.

immunity to a secondary WHV infection. Recently, T-cell response to WHsAg and WHcAg in woodchucks during acute and chronic WHV infection was investigated by an in vitro assay to measure the antigen-specific proliferation of peripheral blood mononuclear cells (PBMCs) (8, 32, 38, 39). Multispecific Th response to WHcAg and WHsAg was present during acute WHV infection but absent in woodchucks with chronic WHV infection (39). Thus, the Th response to WHV in woodchucks closely resembles the HBV-specific Th response in humans (52).

The woodchuck model is informative in the study of immune response induced by vaccines and virus challenge. Immunization of woodchucks with WHsAg-induced anti-WHsAg antibodies provided protection against a subsequent challenge with WHV (9). Interestingly, woodchucks immunized with WHcAg were protected against WHV challenge even though
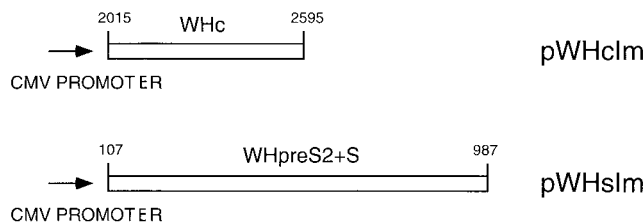

FIG. 1. Construction of pWHcIm and pWHsIm. Both pWHcIm and pWHsIm were constructed on the basis of pcDNA3 (Invitrogen). The fragments from the WHV genome comprising the complete reading frame of WHcAg and WHsAg (including pre-S2) were cloned into pcDNA3 as described in Materials and Methods. The expression of WHcAg and WHsAg is under the control of the CMV promoter of the vector. The cloned fragments of the WHV genome are numbered according to Girones et al. (21).
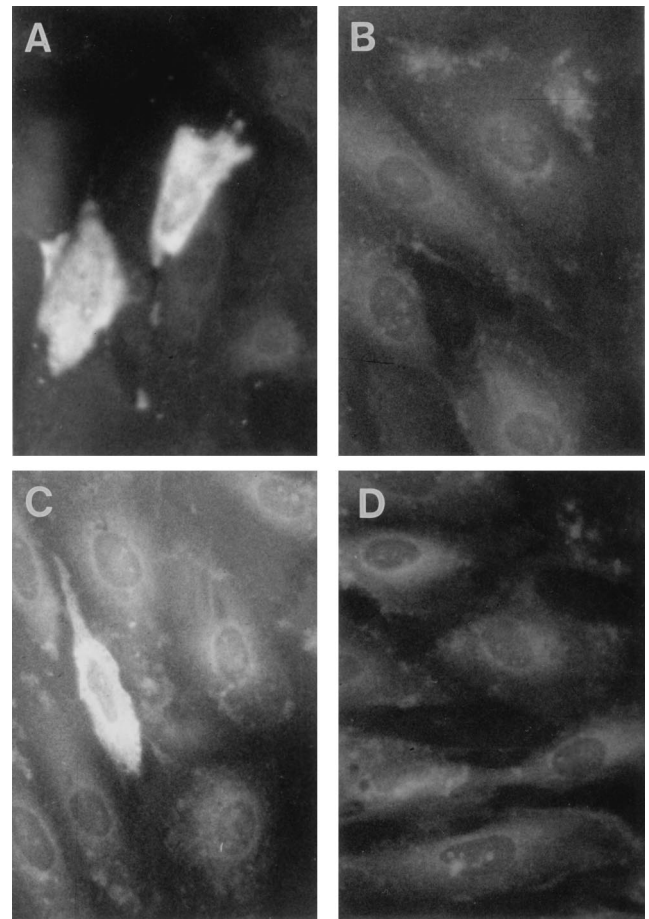

FIG. 2. Expression of WHsAg and WHcAg in woodchuck liver cells (WH12/6) after transfection of pWHsIm and pWHcIm, respectively. WH12/6 cells were transfected with 4 μg of plasmids. After 48 h, transfected cells were fixed with acetone-methanol (1:1). The expressed WHcAg and WHsAg were detected by immunofluorescence staining with rabbit antisera to respective WHV antigens. (A) Transfection with pWHcIm and staining with anti-WHcAg antibody. (B) Transfection with pcDNA3 and staining with anti-WHcAg antibody. (C) Transfection with pWHsIm and staining with anti-WHsAg antibody. (D) Transfection with pcDNA3 and staining with anti-WHsAg antibody.

anti-WHcAg antibodies do not possess the ability to neutralize WHV (49, 52). Apparently, WHcAg induced a specific T-cell response which conferred protective immunity (39). We demonstrated that immunization with a peptide containing a T-cell epitope derived from WHcAg leads to the protection of woodchucks against WHV infection. These results emphasize the significance of T-cell response to the core antigen for control of hepadnavirus infection (16, 18).

In the present study, we wanted to determine whether vaccination of woodchucks with plasmids expressing WHV proteins can induce a protective immune response to WHV. We vaccinated mice and woodchucks with plasmids expressing WHcAg and WHsAg and investigated the humoral and cellular immune response to WHcAg and WHsAg in woodchucks. The protective efficacy of plasmid vaccination was demonstrated in woodchucks in subsequent challenge experiments.

MATERIALS AND METHODS

Woodchucks. Adult WHV-negative woodchucks trapped in the state of New York were purchased from North Eastern Wildlife (Ithaca, N.Y.). Previous exposure to WHV of these woodchucks was excluded by testing for anti-WHcAg, anti-WHsAg, and WHsAg.
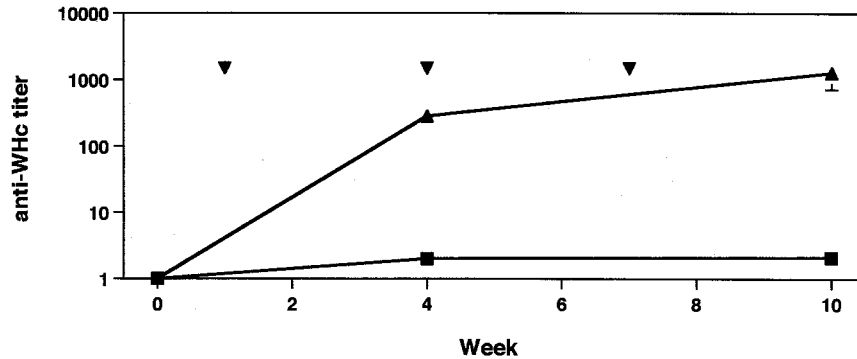
FIG. 3. Anti-WHcAg response in pWHcIm-immunized mice. Mice were immunized thrice with 100 μg of plasmid pWHcIm (▲) or control (■) at 3-week intervals. Vaccinations with plasmid are indicated by ▼. Anti-WHcAgs were tested with sera from mice collected at weeks 0, 3, and 12. The mean titer of anti-WHcAg is shown.

Construction of plasmids pWHcIm and pWHsIm for DNA vaccination. The core gene of WHV8 was amplified by PCR with primers wc1 (nucleotides [nt] 2015 to 2038, 5′-TGGGGCCATGGACATAGATCCTTA-3′) and wc2 (nt 2595 to 2570, 5′-CATTGAATTCAGCAGTTGGCAGATGG-3′) according to the sequence described by Girones et al. (21). The PCR products were cloned into pCRII vectors (Invitrogen, San Diego, Calif.) according to the manufacturer's instructions. A clone, pWHc, was selected by sequencing to verify the correct nucleotide sequence of the PCR product. The fragment containing the WHV core gene was isolated by digestion with *Eco*RI and inserted into the *Eco*RI site of the pcDNA3 vector (Invitrogen). A generated plasmid, pWHcIm, contains the WHV core gene under the control of the cytomegalovirus (CMV) promoter (Fig. 1). The pre-S2-S region of WHV8 (nt 107 to 987) was amplified by PCR with primers whpres2 (nt 107 to 129, 5′-CACTTAACTATGAAAAATCAGAC-3′) and whs2 (nt 987 to 968, 5′-CCACCATTTTGTTTTATTAA-3′). This PCR fragment was cloned into pCRII vector and recloned into the *Eco*RI site of pcDNA3 to generate plasmid pWHsIm by a procedure similar to that described above (Fig. 1). The integrity of the clones was verified by sequencing.

Purification of plasmids for immunization. Plasmids pWHcIm and pWHsIm for immunization were prepared by using the Giga plasmid purification kit (Qiagen, Hilden, Germany). Plasmids were dissolved in phosphate-buffered saline (PBS) at a concentration of 1 mg/ml. The amount of bacterial protein contaminants in these preparations was in the range of 11 ng/ml, as determined by using microbicinchoninic acid (BCA) protein assay reagent (Pierce, Oud Beijerland, The Netherlands).

Transient expression of WHcAg and WHsAg by transfection of pWHcIm and pWHsIm into a baby hamster kidney (BHK) cell line and a woodchuck liver cell line. A BHK cell line and a woodchuck liver cell line WH12/6 (kindly provided by P. Banasch, Deutsche Krebsforschungszentrum, Heidelberg, Germany) were used for transfection experiments. Transfection of liver cells was performed with Lipofectamine (Gibco BRL, Eggenstein-Leopoldshafen, Germany). Plasmid (4 μg) was incubated with 10 μg of lipofectamine in 100 μl of media for 45 min and incubated further with cells in 1 ml of Opti-Media (Gibco BRL) for 6 h at 37°C, 5% CO$_2$. Transfected cells were maintained for 48 h at 37°C in 5% CO$_2$ and fixed with acetone-methanol (1:1). The expressed WHcAg and WHsAg were detected by indirect immunofluorescence staining with rabbit antisera to respective WHV proteins.

Immunization of mice and woodchucks by intramuscular injection of pWHcIm and pWHsIm. Immunization of mice was performed by the procedure described by Schirmbeck et al. (50). Briefly, mice were pretreated by intramuscular injection of 50 μl of cardiotoxin (10 μM) into musculus tibialis anterior. After a week, 50 μg of plasmid (1 mg/ml) was injected into each site in the same muscle. The plasmid injection was repeated twice at 3-week intervals. Mice were sacrificed 3 weeks after the last immunization. This immunization protocol was modified for woodchucks. A week prior to the injection of plasmids, 500 μl of cardiotoxin (10 μM in PBS) was injected into the M. tibialis cranialis of woodchucks. Woodchucks were vaccinated three times by intramuscular injection of 50 or 500 μl of plasmid (1 mg/ml in PBS) into each M. tibialis cranialis at 4- or 5-week intervals. Four weeks after the last vaccination, woodchucks were challenged with an inoculum containing 10^4 or 10^5 WHV genome equivalents.

Serology and detection of WHV DNA. Anti-WHcAg, anti-WHsAg, and WHsAg were determined by enzyme-linked immunosorbent assay (ELISA) as described previously (49, 52). The sensitivity of ELISA was determined by tests of serially diluted positive sera of woodchucks experimentally infected with WHV. The ELISA was able to detect anti-WHcAg in woodchuck sera at a dilution of 10^{-3} to 10^{-6}. Anti-WHsAg titers of post-acute phase sera were positive, ranging between 10^{-3} and 10^{-4}. The ELISA for WHsAg detected WHsAg in sera of chronic WHV-infected woodchucks in dilutions of up to 10^{-4}. The dot blot technique was routinely performed to detect WHV DNA in woodchuck sera. For PCR detection of WHV DNA in woodchuck sera, nucleic acids were isolated from sera by proteinase K digestion and phenol extraction. PCR for amplification of the WHV core gene was run with primers wc1 (nt 2015 to 2038, 5′-TGGGGCCATGGACATAGATCCTTA-3′) and wc2 (nt 2595 to 2570, 5′-CATTGAATTCAGCAGTTGGCAGATGG-3′). In testing serial dilutions of a cloned WHV core fragment, 10 copies of specific templates were sufficient to give a positive result by PCR. Therefore, a virus DNA titer of 500 copies per ml of serum could be detected.

Measurement of WHV antigen-specific proliferation of woodchuck PBMC. Antigen-specific proliferation of woodchuck PBMCs was determined by 2[^{3}H]adenine assay described previously (32). Briefly, woodchuck PBMCs were separated by Ficoll-Paque (Pharmacia, Freiburg, Germany) density gradient centrifugation and suspended in 0.9% NaCl. Triplicates of 5×10^4 PBMCs were cultured in flat-bottom 96-well microtiter plates (Falcon, Becton Dickinson, N.J.)
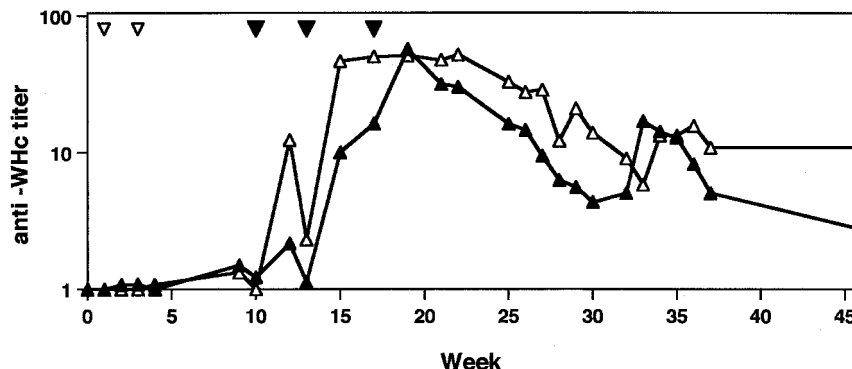

FIG. 4. Anti-WHcAg response in woodchucks WH282 and WH564 after immunization with pWHcIm. Vaccinations with plasmids are indicated with ▽ (100 μg) and ▼ (1 mg). Sera from woodchucks were used for titration of anti-WHcAg (WH282 [▲]; WH564 [△]) by serial dilution.
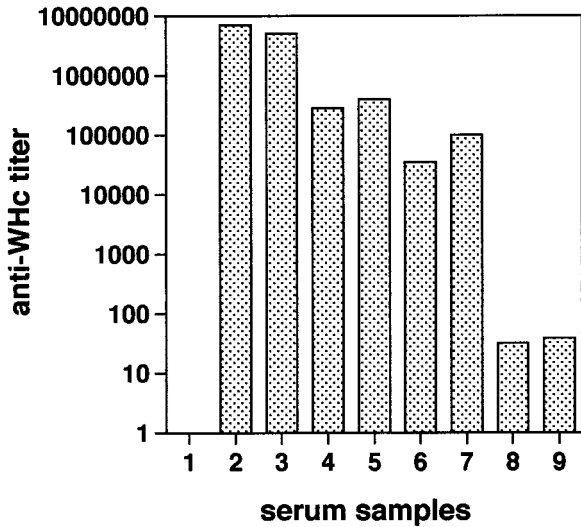
FIG. 5. Comparison of anti-WHc titer in sera of pWHcIm-immunized, WHV-infected and recombinant WHcAg-immunized woodchucks. Sera from seven woodchucks of different status were tested for anti-WHcAg. Samples: 1, woodchuck without WHV infection; 2 and 3, two woodchucks with chronic WHV infection; 4 and 5, two woodchucks after three immunizations with 50 μg of recombinant WHcAg; 6 and 7, two woodchucks whose WHV infection was resolved. Samples 4 to 7 were taken from woodchucks included in experiments described by Menne et al. (39). Samples 8 and 9, WH282 and WH564 after the last immunization with pWHcIm.

at 37°C in a humidified atmosphere containing 5% CO_2. AIM-V medium (200 μl; Gibco BRL) supplemented with 2% 0.2 M L-glutamine (Sigma), 1% 0.125 M gentamicin sulfate (Sigma), and 10% fetal calf serum (Gibco BRL) was added to each well. PBMC proliferation in response to WHcAg, WHsAg, or peptides was measured at an antigen concentration of 1 μg/ml. Peptides of the WHcAg were described previously by Menne et al. (39). Nonoverlapping peptides of WHsAg, including the pre-S1 region as listed in Table 1, were purchased from Genosys (Cambridge, United Kingdom). After a 5-day incubation, cells were labeled with

TABLE 2. DNA immunization of woodchucks with plasmids pcDNA3, pWHcIm, and pWHsIm

Woodchuck	Immunogen[a]	Interval (no. of weeks)	WHV ID_{50} used for challenge
WH282	pWHcIm		
WH564	pWHcIm		
WH880	None		10^5
WH882	None		10^5
WH281	pcDNA3	4	10^4
WH574	pcDNA3	4	10^4
WH290	pWHcIm	4	10^4
WH291	pWHcIm	4	10^4
WH8902	pWHcIm	3	10^5
WH8904	pWHcIm	3	10^5
WH8899	pWHsIm	3	10^5
WH8897	pWHsIm	3	10^5

[a] WH282 and WH564 were immunized twice with 100 μg of pWHcIm and then three times with 1 mg of pWHcIm but not challenged (see text).

1 μCi of 2[³H]adenine (Amersham, Braunschweig, Germany) for 20 h and collected with a cell harvester (Skatron).

Results for triplicate cultures are presented as mean stimulation index (SI [mean total absorption for stimulated PBMCs divided by the mean total absorption for control]). The standard deviations of the means were less than 30% of the mean (range, 15 to 50%). An SI of ≥3.1 was considered significant, to distinguish the specific stimulation and possible variation within an assay as described previously (39).

RESULTS

Construction of plasmids expressing WHV core and pre-S2-S protein. The WHV core region in pWHcIm and the pre-S2-S region in pWHsIm were cloned into pcDNA3 and placed under the control of the CMV promoter (Fig. 1). To test the expression of WHV proteins by the plasmids, BHK cells and woodchuck liver cells (line WH12/6) were transiently
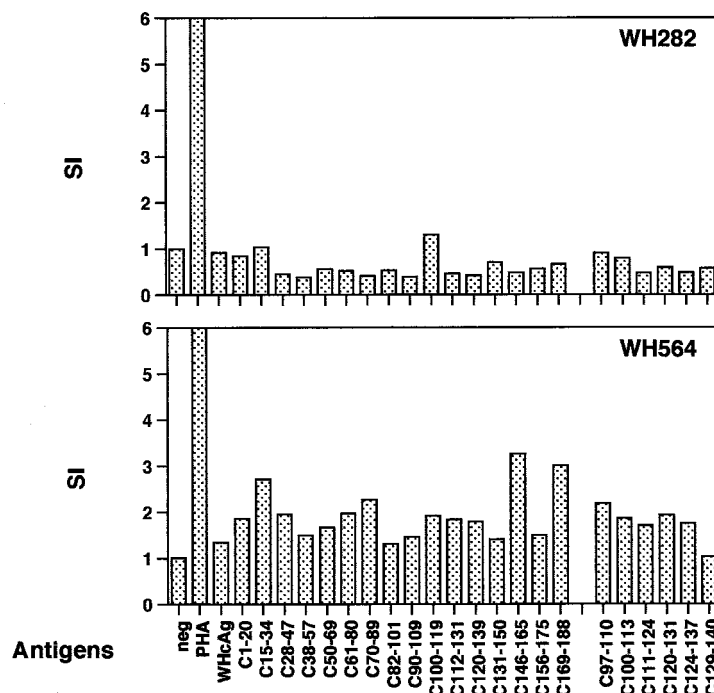
FIG. 6. The proliferative response to recombinant WHcAg and peptides of WHcAg of PBMC from woodchucks WH282 and WH564 after immunizations with pWHcIm at week 21. neg, no WHV antigen was added; PHA, phytohemagglutinin.
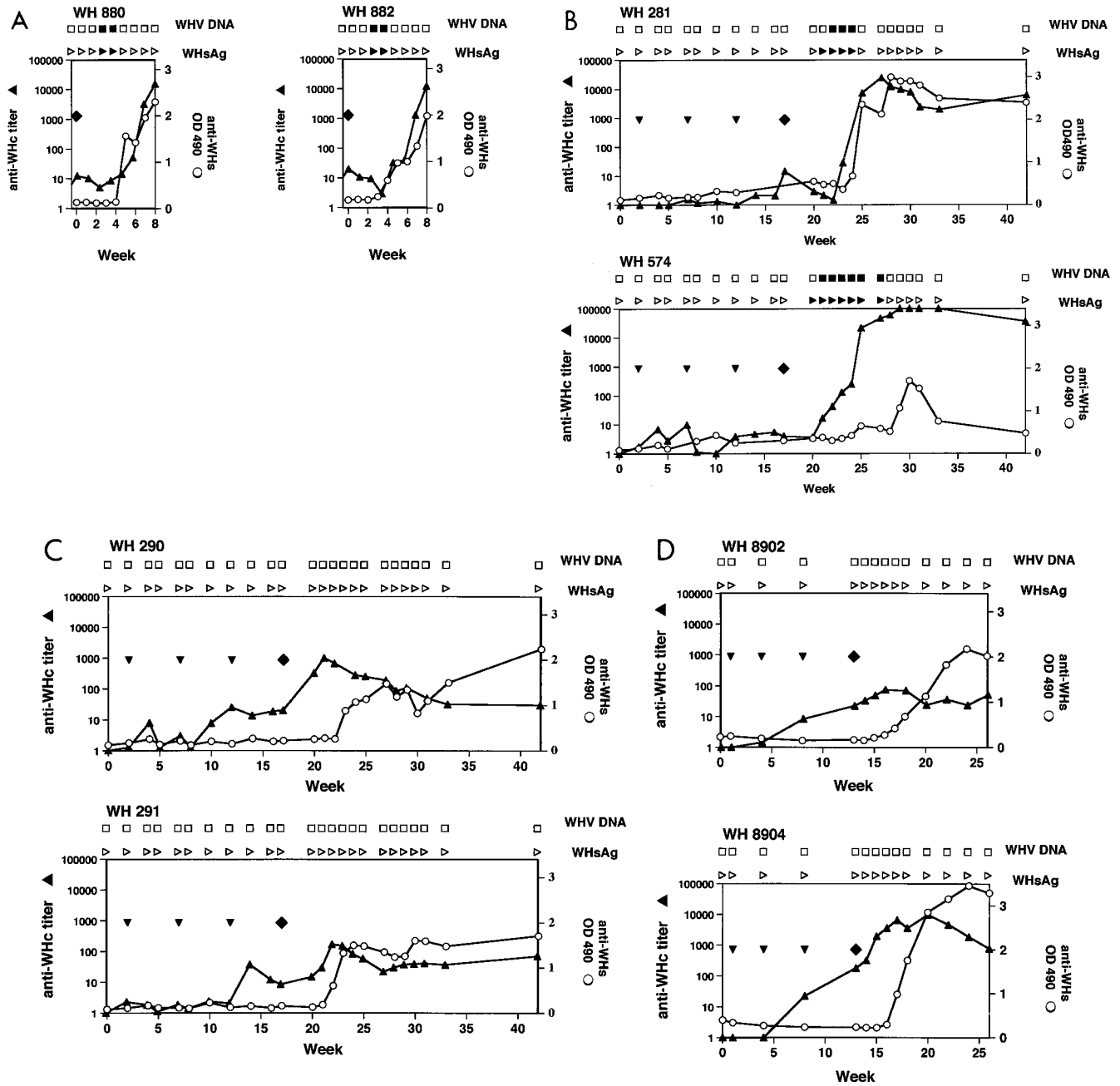
FIG. 7. Immunization of woodchucks with pWHcIm and challenge with WHV. (A) Unimmunized control animals WH880 and WH882. (B) Immunization with pcDNA3. (C) Immunization with pWHcIm. Vaccinations with plasmids are indicated with ▼. ◆, challenge with WHV; ▲, anti-WHcAg; ○, anti-WHs; □, WHV DNA negative; ■, WHV DNA positive; ▷, WHsAg negative; ▶, WHsAg positive. PCR was used to prove the absence of WHV DNA in sera from WH290, WH291, WH8902, and WH8904. OD, optical density.

transfected with pWHcIm and pWHsIm. WHcAg and WHsAg expressed in transfected WH12/6 cells were detected by indirect immunofluorescence staining with polyclonal rabbit anti-WHsAg antibody or mouse anti-WHcAg antibody, respectively (Fig. 2). The expression of WHV proteins did not show an obvious difference in BHK cells (data not shown). No specific immunofluorescence staining was seen in cells transfected with control plasmid pcDNA3. WHsAgs produced in transfected BHK cells were released into medium and detectable by ELISA.

Induction of WHcAg-specific immune response by vaccination of mice and woodchucks with pWHcIm. Eight mice were

immunized with pWHcIm to test its ability to induce an WHcAg-specific immune response. Immunized mice developed an anti-WHcAg titer of about 1:300 after one injection of 100 μg of pWHcIm (Fig. 3). The titer of anti-WHcAg in these mice increased to 1:500 and 1:1,800 after three immunizations with pWHcIm. Immunizations with control plasmids which do not express WHcAg did not lead to production of anti-WHcAg in mice.

Though a single injection with 100 μg of pWHcIm induced an anti-WHc response in mice, no anti-WHcAg was measurable in woodchuck WH282 or woodchuck WH564 after two vaccinations at weeks 1 and 3 with the same dose of pWHcIm.

FIG. 8. Immunization with pWHsIm. Vaccinations with plasmids are indicated with ▼. ◆, challenges with WHV; ▲ anti-WHcAg; ○, anti-WHs; □, WHV DNA negative; ▷, WHsAg negative; PCR was used to prove the absence of WHV DNA in sera from WH8897 and WH8899.

Both woodchucks remained negative for anti-WHcAg until week 10 (Fig. 4). The dose of 100 μg of plasmids was not sufficient to induce anti-WHcAg in woodchucks. Therefore, both woodchucks received three additional immunizations with 1 mg of pWHcIm at weeks 10, 13, and 17. Woodchucks WH282 and WH564 developed anti-WHcAg after two additional vaccinations at 10 and 13 weeks. The titer of anti-WHcAg increased transiently and dropped gradually from week 20 to week 46 (Fig. 4). In WH282, the anti-WHcAg titer even dropped under 1:10. Control animals either unvaccinated or vaccinated with control plasmid pcDNA3 remained anti-WHcAg negative (see below).
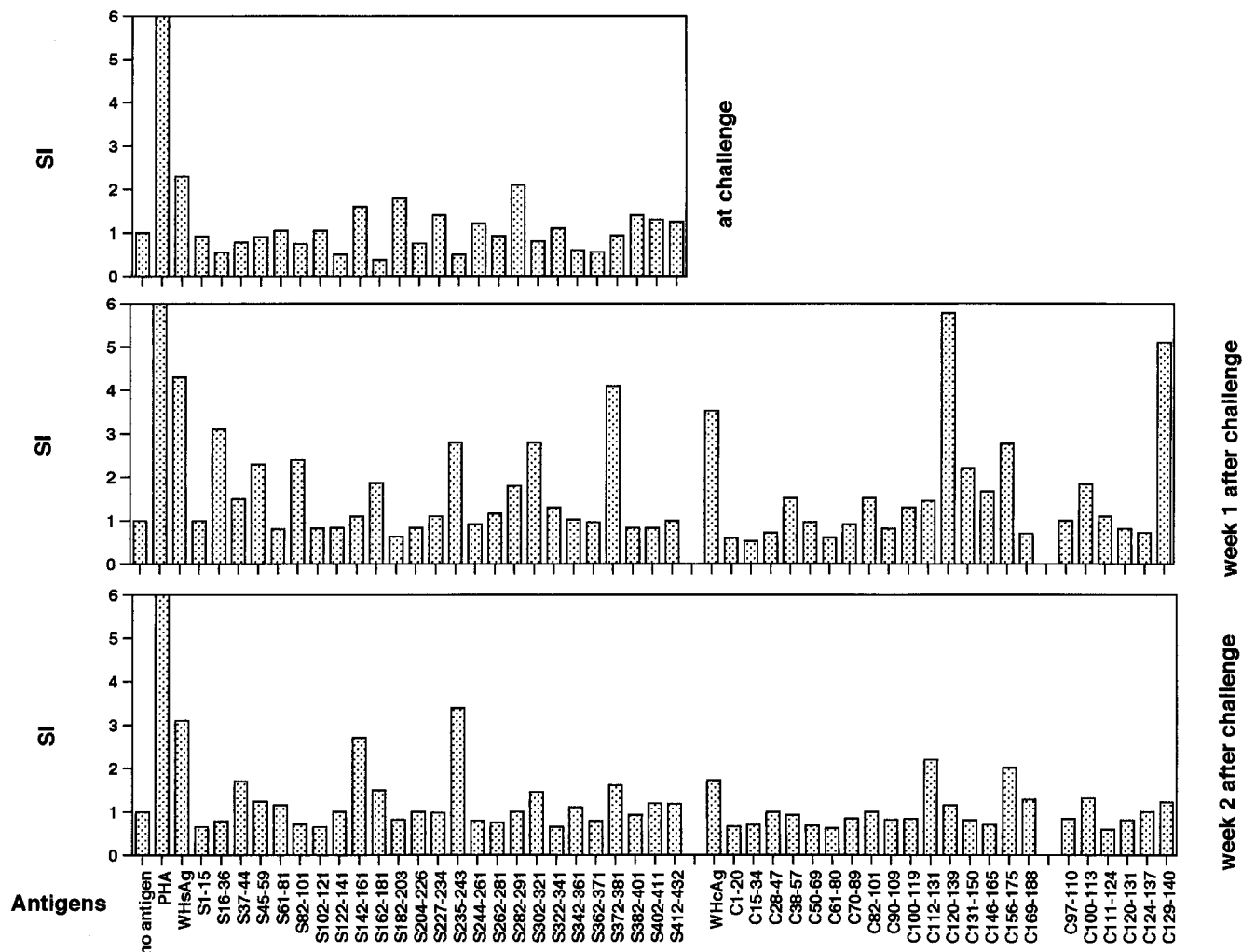
Titers of anti-WHcAg in sera from pWHcIm-vaccinated woodchucks were compared with antibody titers in woodchucks with acute or chronic WHV infection and in woodchucks immunized with recombinant WHcAg (Fig. 5). Titers of anti-WHcAg in sera from WHV-infected woodchucks and WHcAg-vaccinated woodchucks ranged between 1:10[4] and 1:10[6]. In comparison, woodchucks WH282 and WH564 developed a rather low anti-WHcAg titer under 1:100 after immunization with pWHcIm.

The proliferative response to WHcAg of PBMCs from WH282 and WH564 was determined at each blood drawing indicated in Fig. 4 by in vitro assay. Proliferation of PBMCs from both animals to WHcAg and WHcAg-derived peptides by in vitro assays was low. Figure 6 shows the PBMC proliferation to WHcAg measured at week 21 after three immunizations with 1 mg of pWHcIm. A PBMC proliferation with an SI of ≥3 was seen in only WH574 in response to two WHcAg-derived

peptides, C156-175 and C169-188. No stimulation of PBMC proliferation by WHcAg or peptides was measurable in WH282.

Challenge of pWHcIm-vaccinated woodchucks with WHV. To test whether DNA immunization confers protection against WHV infection, four woodchucks, WH290, WH291, WH8902, and WH8904, were challenged with WHV after three immunizations with 1 mg of pWHcIm. As a control, two untreated woodchucks, WH880 and WH882, and two woodchucks which received plasmid pcDNA3, WH281 and WH574, were challenged with WHV (Table 2).

Two naive woodchucks, WH880 and WH882, received 10[5] WHV at a 50% infective dose (ID$_{50}$). They were viremic during weeks 3 and 4 week postinfection (p.i.) and positive for anti-WHs at 5 weeks p.i. (Fig. 7A). The peak level of WHV titers ranged from 10[6] to 10[8], as estimated by dot blot hybridization. WH281 and WH576, which received three injections of pcDNA3, did not show antibody response or PBMC proliferation in response to WHV proteins. After the challenge with 10[4] WHV ID$_{50}$, both woodchucks were positive for WHsAg and WHV DNA at 5 and 4 weeks p.i., respectively (Fig. 7B) and had a maximum WHV titer of 10[6] to 10[8]. Immediately after the viremic phase, anti-WHsAg developed in WH281 and WH574 at weeks 8 and 11 p.i., respectively.

In all four woodchucks receiving pWHcIm, a seroconversion to anti-WHc occurred after three injections (Fig. 7C and D). Like WH282 and WH564, these animals developed only a low anti-WHcAg titer. The anti-WHcAg in WH290, WH291, and WH8902 ranged between 1:10 and 1:100 after three immunizations. WH8904 showed an anti-WHc titer of 1:179 at week 13 after challenge. Two pWHcIm-vaccinated woodchucks, WH290 and WH291, were challenged with 10[4] WHV ID$_{50}$ at week 5 after the last DNA injection (Fig. 7C). Two woodchucks, WH8902 and WH8904, were vaccinated with 1 mg of pWHcIm three times at 4-week intervals and challenged with 10[5] WHV ID$_{50}$ at week 4 after the last DNA injection (Fig. 7D). All sera from these four animals were negative for WHsAg and WHV DNA in a follow-up period of 14 weeks. Anti-WHcAg titer was transiently increased in all woodchucks, presumably due to limited viral replication and production of viral proteins. In WH8904, anti-WHcAg titer reached nearly 1:10,000 at the peak level at week 17 but decreased continuously thereafter. Unlike in control animals, anti-WHsAg developed independently on the virus titer of challenge at 5 weeks p.i. Thus, transient WHV replication could take place in pWHcIm-vaccinated woodchucks and was sufficient to induce an anti-WHsAg response.

Similar to previous experiments, PBMCs from woodchucks showed no or weak WHcAg- or peptide-specific proliferation after immunization with pWHcIm. After challenge, there was no significant increase in PBMC proliferation to WHV core and surface proteins in these animals (data not shown). These results indicate that T-cell response to WHV proteins, if present, was local and did not spread, consistent with the previous observation that T-cell response to WHV proteins was measurable in the periphery only during the acute viremic phase of WHV infection (37).

Immunization of woodchucks with pWHsIm and challenge. Two woodchucks, WH8897 and WH8899, received three intramuscular injections of 1 mg of pWHsIm each, in parallel to other immunizations described above (Table 2 and Fig. 8). Anti-WHsAg antibody was not detectable after three immunizations but developed 2 weeks after challenge with 10[5] WHV ID$_{50}$. Thus, anti-WHsAg antibody response of pWHsIm-vaccinated woodchucks resembled a rather anamnestic response. WHsAg and WHV DNA were not detected in peripheral

FIG. 9. The proliferative response to WHsAg and WHcAg of PBMCs from WH8899. WH8899 was vaccinated with pWHsIm three times and challenged with 10^5 WHV ID_{50} at week 5 after the last vaccination. The proliferation of PBMCs in response to stimulation with WHsAg, recombinant WHcAg and peptides at week 13, 14, and 15 is shown. PHA, phytohemagglutinin.

blood in the follow-up period of 14 weeks. Anti-WHcAg was only transiently detectable in WH8899, mainly due to carryover of antibodies in the inoculum. WH8897 developed a low titer of anti-WHcAg (1:26) at week 20.

Vaccination with pWHsIm did not induce a significant proliferative response to WHsAg and WHcAg of PBMCs from WH8897 and WH8899. One week after challenge, WHsAg- and WHcAg-specific PBMC proliferation were detected in WH8899 (Fig. 9). PBMC proliferation with an SI of >3 was measured to WHsAg-derived peptides S16-36, S235-243, and S372-381 and to two overlapping WHcAg-derived peptides, C120-139 and C129-140. In the second week, PBMCs from WH8899 responded to WHsAg-derived peptides S142-161 and S235-243. The proliferative response of PBMCs to WHcAg and peptides decreased to an SI of under 2. No significant increase of PBMC proliferation to WHsAg and WHcAg was seen in WH8897.

DISCUSSION

In the present study, we have demonstrated that vaccination of woodchucks with plasmids expressing WHcAg and WHsAg

induced immune response and controlled a subsequent WHV infection.

The level of antibody response induced by plasmid vaccination appears to be dependent on the relation between the doses of DNA vaccines and the body weight of the animals. After immunization with 100 μg of pWHcIm, an anti-WHcAg titer of 1:300 developed in mice. However, the same dose of plasmids was not effective to induce a measurable anti-WHcAg response in woodchucks. Woodchucks transiently developed anti-WHcAg of low titer after receiving 10-fold doses of plasmids. Considering that woodchucks weigh 4 kg on average, a dose of 1 mg of plasmids per injection is rather low compared with doses used for mice (100 μg of plasmids for 20 g of body weight). Our finding is concordant with the published results of Davis et al. that the effect of plasmid immunization of chimpanzees with an HBsAg-expressing plasmid was also dependent on the amount of plasmids (11).

The challenge experiments showed that a WHV infection could be controlled by vaccination with pWHcIm. These results are consistent with previous experiments showing that immunization with WHcAg confers protection against WHV infection (17, 28, 49). Anti-WHcAg antibody does not possess

the activity to neutralize infectious virions of WHV. Therefore, a minimal WHV infection of hepatocytes obviously took place in vaccinated woodchucks and induced a transient increase of anti-WHcAg titer and in the production of anti-WHsAg. The release of WHsAg and WHV virions into the periphery was apparently limited by the cellular branch of the immune system which was primed by vaccination. WHcAg-specific PBMC proliferation was at least measurable in some vaccinated woodchucks. It appears that the plasmid vaccination primed a localized immune response, and the number of WHV antigen-specific T cells in peripheral blood was rather low. These results are very similar to a previous immunization experiment with peptides containing a T-cell epitope. Immunization of woodchucks with C91-110 of WHcAg, though it did not induce measurable PBMC proliferation, protected woodchucks from WHV infection (39). An increase of peptide-specific PBMC proliferation occurred after a subsequent challenge. Anti-WHsAgs appeared 5 weeks after challenge and may contribute to virus clearance.

Three vaccinations of woodchucks with 1 mg of pWHsIm each did not induce a measurable anti-WHsAg response in woodchucks; this may be explained by the following reasons. Whereas WHcAg is a potent immunogen and leads to a high level of anti-WHc antibody in WHV-infected or WHcAg-vaccinated woodchucks, the anti-WHsAg antibody response is usually lower and can be absent in WHsAg-vaccinated woodchucks (nonresponders). The difference between anti-WHcAg and anti-WHsAg may also be partly biased by ELISAs used for follow-up of WHV infection. Since plasmid vaccination in woodchucks induced only a low humoral response, as shown for anti-WHcAg, the anti-WHsAg response induced by pWHsIm might be below the detection limit of the ELISA. Nevertheless, the rapid appearance of anti-WHsAg 2 weeks after challenge demonstrated clearly that priming of WHsAg-specific B-cell response took place as a result of plasmid vaccination. Similar to results for the vaccination with pWHcIm, WHsAg, and WHV DNA, the periphery remained below the detection limit in both woodchucks. Our results are in concordance with results for vaccination of chimpanzees with plasmids expressing HBsAg (11, 47). In general, a transient low-level anti-HBsAg response could be measured in vaccinated chimpanzees. Upon an additional vaccination with HBsAg (11) or a challenge with HBV (47), an anamnestic anti-HBsAg developed in these chimpanzees. The primed anti-WHsAg B-cell response seems unable to block the infection of hepatocytes by input virus. A minimal anti-WHc response was measured in WH8897. A WHcAg-specific PBMC proliferation was detected in WH8899 in the first 2 weeks after challenge before the appearance of anti-WHsAg. These facts indicate that WHcAg was synthesized at a minimal level in pWHsIm-vaccinated woodchucks. The protection conferred by the plasmid vaccination may be improved by different WHsAg expression vectors or by coadministration of cytokine-expressing plasmids (55). In ducks, two DHBsAg-expressing plasmids showed very different protection efficacies for preventing infection of hepatocytes, though both induced high titers of anti-DHBs (55).

Successful genetic vaccination may depend on an appropriate delivery of plasmid DNA. In an early experiment, three woodchucks were vaccinated three times intramuscularly with 1 mg of WHsAg-expressing plasmid at many randomly chosen sites. Two woodchucks developed viremia after challenge, and only one woodchuck remained negative for viral marker (13a). Therefore, M. tibialis cranialis in woodchuck was chosen for DNA vaccination because of its small size and easy location. Pretreatment with cardiotoxin may induce a local inflammatory response and thereby enhance the antigen-specific immune response. Experiments are under way to compare different delivery protocols for their efficacy for inducing immunity to subsequent WHV infections. Intradermal application of plasmids by gene gun was reported to be especially effective for inducing Th2-dominant response and would be useful for achieving an enhanced humoral immune response to surface antigens (15, 46). DNA vaccination against hepadnavirus infection provides new opportunities for the immunotherapy of chronic hepatitis B. Unlike conventional vaccines, DNA vaccines may be modified and enhanced by adding other relevant genes or applied through different routes (15, 20, 46). With an understanding of the mechanisms of viral persistence, effective therapeutic vaccines may be developed to overcome these mechanisms, leading to unresponsiveness of the immune system to hepatitis B proteins in chronically infected patients.

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