The Effect of an Immunogenic Complex Containing WHV Viral Particles and Non-Neutralizing Anti-HBs Antibodies on the Outcome of WHV Infection in Woodchucks

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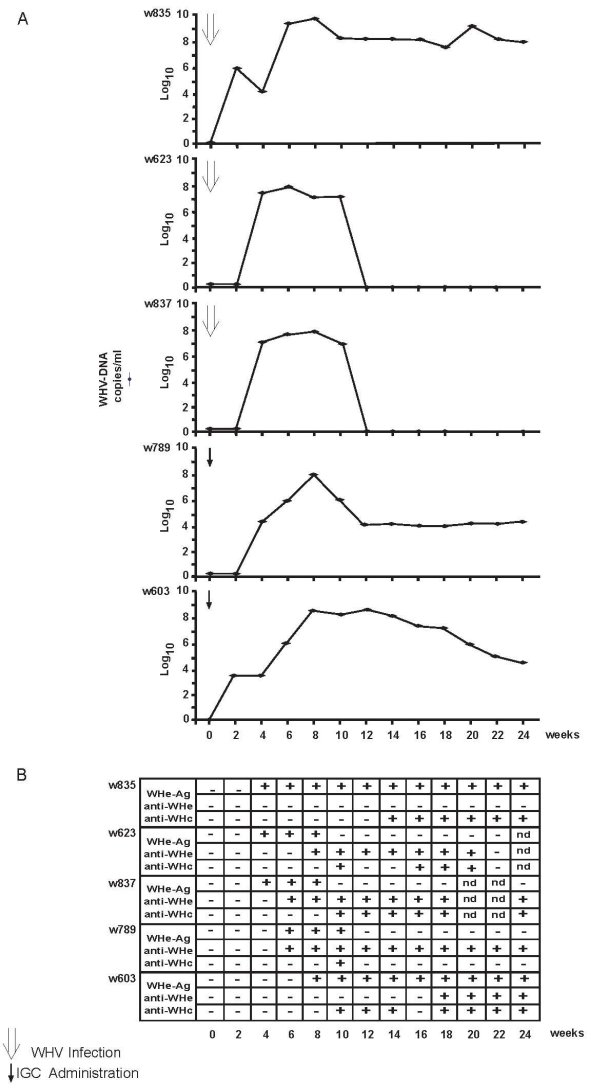
Keywords:	immune complex, WHV-DNA, HBV infection

TABLE I. IGC Administration in WHV-Negative Woodchucks and in WHV Chronic Carriers

	Woodchucks	Quantity of anti-HBs (mIU) in IGC	No. of IGC doses* and route of administration
Control woodchucks	w835	-	1/iv
	w623	-	1/iv
	w837	-	1/iv
WHV-negative woodchucks	w789	2.5	1/iv
	w603	32.5	1/iv
WHV chronic woodchucks	w3967	2.5	1/iv+ 2/id
	w3981	2.5	1/iv + 4/id
	w3953	2.5	1/iv
	w840	2.5	1/iv+ 1/id

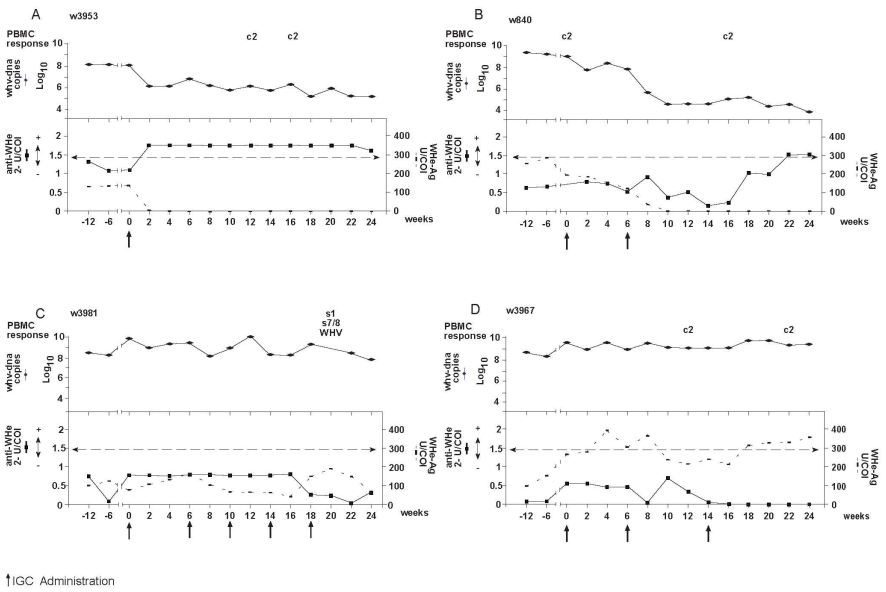
* IGC is composed of anti-HBs at different concentrations and a fixed quantity of WHV-DNA copies (10^7). In the control animal (w835), only viral particles containing 10^7 WHV-DNA copies were administered. iv = intravenous. id = intradermic

Fig. 1 - The effect of an immunogenic complex (IGC) containing WHV viral particles and non-neutralizing anti-HBs antibodies on the outcome of WHV infection in woodchucks - Emilio D'Ugo et al.



437x617mm (96 x 96 DPI)

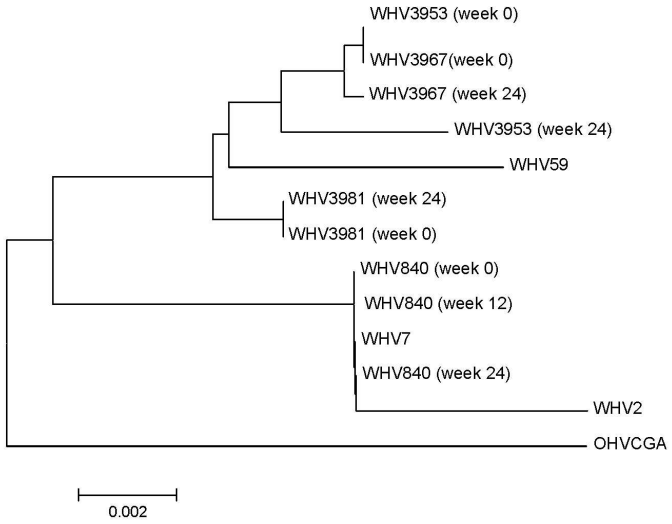
FIGURE 2 Administration of immunogenic complex (IGC) in chronic WHV-infected woodchucks



†IGC Administration

296x209mm (200 x 200 DPI)

Fig. 3 - The effect of an immunogenic complex (IGC) containing WHV viral particles and non-neutralizing anti-HBs antibodies on the outcome of WHV infection in woodchucks - Emilio D'Ugo et al.



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The Effect of an Immunogenic Complex Containing WHV Viral Particles and Non-Neutralizing Anti-HBs Antibodies on the Outcome of WHV Infection in Woodchucks

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Running Head: Immune Complex Administration in WHV Infection

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ABSTRACT

The Eastern woodchuck (*Marmota monax*) is a useful experimental model for evaluating antiviral therapy against chronic HBV infection. In the present study, an immunogenic complex (IGC) composed of immune sera containing PreS/S heterologous antibodies (anti-HBs) and serum-derived WHV particles containing 10^7 WHV-DNA copies/50 μl was developed. The IGC was administered to WHV-negative woodchucks and natural chronic WHV carriers, with the final aim of evaluating the outcome of WHV infection in both groups. A control group of three animals, infected experimentally with viral particles only, was also evaluated. Following IGC administration, two WHV-negative woodchucks exhibited persistent infection, with WHV-DNA levels 3-6 logs lower than the WHV-DNA levels of the controls that developed persistent infection. WHeAg seroconversion to anti-WHe was observed in these two woodchucks and in two control woodchucks which developed self-limited infection. In two of the four chronic carriers, the WHV-DNA level decreased significantly (by 4-6 logs) following IGC administration, with no rebound in viral load during follow-up. WHeAg seroconversion to anti-WHe was observed also in these animals. Analyses of the sequences derived from envelope proteins confirmed that IGC did not induce the emergence of resistant viral variants. The results of this study indicate that the IGC could be useful for breaking the tolerance in hepadnaviral infection and for boosting the host's innate and adoptive immune response.

KEY WORDS: immune complex; WHV-DNA; HBV infection

INTRODUCTION

The approach to therapy for chronic HBV infection has changed significantly in recent years, largely because of new antiviral treatments and more sensitive laboratory assays. The use of interferon alpha, also in its pegylated form, and of various nucleotide analogues has provided good results in terms of achieving viral suppression and improving chronic HBV-induced liver injury. However, interferon is not the best treatment for all patients with chronic HBV infection, in that the non-response and relapse rates limit its use. Regarding nucleotide analogues, with prolonged use, the long-term control of viral replication is hindered by the selection of the polymerase gene mutants of the virus, which results in reduced susceptibility to the nucleoside analogues.

Immunomodulatory strategies to boost or broaden the virus-specific T-cell response have also been proposed to control chronic HBV infection. The use of conventional HBV vaccines could induce a specific immune response to HBV, yet conflicting results have been reported [Gérolami et al., 2007; Vandepapelière, 2002; Vandepapelière et al., 2007]. Other immunomodulatory approaches, such as the use of interleukines or antigen pulsed dendritic cells, have shown promising results in terms of decreasing the viral load and HBsAg seroconversion, though these approaches need to be evaluated further [Akba et al., 2006; Rigopoulou et al., 2005]. More recently, a promising new approach has been described, in which an antigen is combined with antibodies to produce immunomodulatory active complexes [Xu et al., 2008].

In this regard, the woodchuck hepatitis virus (WHV) and its natural host, the Eastern woodchuck (*Marmota monax*), constitute a useful model for evaluating antiviral therapy against chronic HBV infection [Cote and Gerin, 1996; Menne and Cote, 2007; Roggendorf and Tolle, 1995]. In a previous study, this model was used to study a heterologous vaccine containing HBV surface proteins ($PreS_2$, S_1, S), which induced a humoral response and

provided protection after WHV challenge [Argentini et al., 2005]. This vaccine was also combined with Lamivudine, which suppressed WHV-DNA, yet only temporarily [D'Ugo et al., 2007]. To continue the evaluation of immunomodulatory strategies, in the present study an immunogenic complex (IGC) composed of immune sera containing PreS/S heterologous antibodies (anti-HBs) and serum-derived WHV particles was developed. The complex was administered to WHV-negative woodchucks and to woodchucks that were chronic WHV carriers infected naturally, to evaluate the outcome in terms of a decrease in viral load and WHeAg seroconversion.

MATERIALS AND METHODS

Ethic Statement

The study was conducted on nine adult woodchucks, trapped in the mid-Atlantic region of the United States and purchased from International Animal Exchange Inc. (Ferndale, MI, USA). The animals received humane care in compliance with the guidelines of the Italian Ministry of Health and were fed with aflatoxin-free laboratory rabbit chow until death.

Woodchucks, Immunogenic Complex (IGC) Composition

Of the nine woodchucks, five (w789♂, w623♂, w837♀, w603♂, w835♀) were negative for WHV infection (i.e., negative for antibodies, antigens and WHV-DNA in serum and liver) and four (w3953♂, w840♀, w3981♂ and w3967♀) were naturally infected chronic WHV carriers. The serum samples that were used to obtain the anti-HBs necessary for IGC production were collected from two woodchucks, each of which was vaccinated with four 50-µg doses of CHO-HBsAg (PreS/S region) vaccine, without adjuvants. This vaccine, kindly provided by Dr. R. Glück (Berna Biotech, Bern, Switzerland), was administered intramuscularly at weeks 0, 4, 12 and 16. Anti-HBs were measured every two weeks during the vaccination period, using the Ausab EIA (Abbott Laboratory Diagnostics, IL, USA). Sera from the two vaccinated animals were pooled, quantified and diluted to obtain aliquots containing different quantities of anti-HBs (2.5, 7.5 and 32.5 mIU) in a final volume of 350 µl.

A previous WHV preparation, derived from the experimental infection of a woodchuck (w197) was used to compose the IGC. This preparation was characterized by sequence

analysis of the PreS/S region of WHV-DNA and by the study of the evolution of the experimental infection [Argentini et al., 1999]. Following the dilution of pooled sera from w197, the viral load was quantified by Real-Time PCR, and aliquots containing 10^7 WHV-DNA copies and 15-20 μg of WHsAg in a final volume of 50 μl were prepared. To compose the IGC, the aliquots containing WHV particles (10^7 WHV-DNA copies), anti-HBs and WHV particles were mixed, incubated at 1200 rpm in a thermomixer for 1 h at 37°C and stored at 4°C until administration. Aliquots containing only WHV particles, without adding anti-HBs, were used as a control preparation for the evaluation of antigen-antibody binding.

To verify the IGC formation, the IGC was centrifuged at 13,000 *g* for 40 min at 4°C; the pellets and supernatants were separated and analyzed for viral DNA and anti-HBs content. In each pellet and supernatant, WHVDNA was extracted by robot with EZ1 Virus Mini Kit 2.0 (QIAGEN), and anti-HBs was analyzed by AUSAB EIA (ABBOTT Laboratory Diagnostics, IL, USA). Furthermore, different anti-HBs concentrations (2.5, 7.5, and 32.5 mIU) were added during the incubation period, and the amount of anti-HBS was evaluated in each pellet (Table 1). Each pellet contained a constant value of 1.5-2 mIU of anti-HBs, independently of the concentration of anti-HBs added, whereas the excess of anti-HBs remained in the supernatants. Anti-HBs antibodies were not detected in the pellets that did not contain WHV particles (data not shown).The amount of WHV-DNA was evaluated by Real-Time PCR in each pellet that contained IGC. Each pellet that contained IGC was also evaluated by spectrophotometer analysis to evaluate the amount of WHsAg. In order to evaluate the specificity of WHsAg in the pellet, a12% polyacrylamide gel electrophoresis (SDS-PAGE), and Western blot analysis, were performed as described previously [Glebe et al., 2009]. The mean WHV-DNA copies contained in the IGC was 2 x 10^6, which corresponded to 4-5 μg of WHsAg.

Evaluation of Natural Immune Complexes

To evaluate the presence of natural WHV-immune-complexes, at week 0 WHV chronically woodchucks sera were incubated at 1200 rpm in a thermomixer (Eppendorf) for 2 h at 37℃ and were centrifuged at 13,000 *g* for 40 min at 4℃; the pellets and supernatants were separated, extracted as described above, and analysed with Real-Time PCR. Sera containing anti-WHV (obtained by vaccination of a woodchuck and a rabbit with inactivated WHV particles) were incubated with plasma derived WHV particles inactivated previously for 96h at 37℃ in a final formalin concentration of 1:2000, and the presence of the immune complex was evaluated; these sera were used as positive controls. The sera from WHV-negative woodchucks were also incubated with WHV inactivated particles, and the pellet and the supernatant obtained were evaluated as negative controls.

Of the five WHV-negative animals, three (w835, w623 and w837) were infected experimentally with w197 inoculum (10^7 copies/ml) and considered as infection control animals, whereas the other two received one intravenous dose of IGC containing various anti-HBs concentrations. The four woodchucks infected chronically received from one to five doses of IGC containing 2.5 mIU of anti-HBs, the first of which was administered intravenously. If the animal did not seroconvert to anti-WHe after the first dose, additional doses were administered intradermically. Time 0 was considered to be the time immediately prior to the first IGC administration; the duration of follow-up was 24 weeks. Sera from 15 WHV-positive animals (10 sera for each animal) were evaluated for 6 months to evaluate fluctuations in WHV-DNA titres.

Enzyme Immunoassays

Electro-Chemo-Luminescence Immuno-Assays (Elecsys anti-HBc, Elecsys anti-HBe, Elecsys HBeAg, Roche Molecular Diagnostics, Mannheim, Germany) were used to detect antibodies to WHV core antigen (anti-WHc), antibodies to WHV “e” antigen (anti-WHe), and WHV “e” antigen (WHeAg). Cut-off levels were calculated for each of the three markers as previously described [D’Ugo et al., 2004]. Regarding the assay for detecting anti-WHe, given that the antibodies included in the kit compete with the serum antibodies and the maximal cut-off index (COI) value was 1.5, the individual results were expressed as 2 minus the COI value, for the sake of convenience. An automatic chemical analyzer (DACOS, Coulter Corporation) was used to measure serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma glutamyl transferase (GGT).

WHV TaqMan Real-Time PCR

DNA was extracted from 200 µl of plasma using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. WHV genome copies were determined using a TaqMan Real-Time PCR [Xu et al., 2008]. Probe and primers that amplify a region of 56 bp within the X region of the WHV genome (accession number M11082) were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA). The region was cloned in pCR 2.1 vector (TA cloning Kit, Invitrogen, Paisley, UK) and used as standard. The TaqMan Probe, 5'-CTGTGCAGACTTGC-3' (nt 1724-1737), is an MGB probe marked at the 5' with a reporter fluorochrome FAM (6-carboxyfluorescein). The primers used were: forward, 5'-TCCGTGTTGCTTGGTCTTCA-3' (nt 1703-1722), and reverse, 5'-TCACGGTGGAATCCATGGTT-3' (nt 1758-1739). The samples were analyzed in a total volume of 25 µl of mixture containing 5 µl of extracted DNA, 12.5 µl PCR Master Mix (Applied Biosystems), 900 nM of each primer and 180 nM of probe. The thermal cycling conditions were: 2 min at 50°C and 10 min at 95°C for 1 cycle, followed by 15 sec at 95°C and 1 min at 60°C for 50 cycles. Amplification and analysis were performed using the ABI Prism 7000 sequence detection system (Applied Biosystems). The sensitivity of the Real-Time PCR was 50 copies per millilitre (copies/ml).

Peripheral Blood Mononuclear Cells (PBMCs) Lymphoproliferative Assay

PBMC responses were analyzed monthly, as reported previously [D'Ugo et al., 2004; Menne et al., 1997]. Briefly, PBMCs were separated by Ficoll-Hypaque gradient centrifugation, washed twice in Phosphate Buffer Saline, and resuspended in AIM-V medium (Gibco, Invitrogen, Paisley, UK), supplemented with 2 µM of l-glutamine (Sigma Aldrich, St. Louis, MO, USA), 1% non-essential amino acids (Sigma Aldrich), 1 µM sodium

pyruvate (Sigma Aldrich), 100 U/ml penicillin (Sigma Aldrich), 100 µg/ml streptomycin (Sigma Aldrich), and 10% fetal calf serum (FCS) (Gibco). The proliferation assay was performed in triplicate by seeding 1 x 10^5 cells/well in 96-well flat-bottomed plates (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA). PBMCs stimulations were performed with 10 µg/ml of PreS/S peptides (**S1** MGNNIKVTFNPDKIA **1-15**, **S7/8** GRKPTPPTPPLRDTHPHLTM **132-150, S18** YCCCLKPTAGNCTCWPIPSS **341-360)**, 10 µg/ml of Core peptide (**C2** KVRQSLWFHLSCLTF **96-110**) and 10 µg/ml of inactivated WHV particles. As a positive control, 10 µg/ml of Phytohemoagglutinin or Concanavalin A was used (Gibco). After five days of incubation at 37°C in a humidified atmosphere containing 5% CO_2, 1 µCi/well of [2-^{3}H] adenine (Amersham, Aylesbury, UK) was added; after an additional 12 h, the cells were harvested, and nucleic acid-incorporated radioactivity was measured by liquid scintillation counting. The proliferative response was expressed as a Stimulation Index (SI), calculated as the ratio of the count per minute (cpm) obtained in the presence of antigen to the cpm obtained without antigen. The immunoproliferative response was considered as positive when the SI was ≥2.1.

Sequence Analysis

Sequence analysis was performed in the chronically infected animals (w3953, w840, w3967, w3981). DNA was extracted from 200 µl sera obtained at baseline (prior to IGC administration) and at week 24, using the QIAamp DNA Mini kit, following the manufacturer's protocol. The primers for amplification and sequencing were synthesized using the WHV genome (M19183) as reference. The PreS/S region was amplified using two sets of primers: WHV-F1 5'-AATTCGGGACATACCACGTG-3' (position 1-20)/WHV-R3 5'-ATGTAAATGATTGCCCCACC-3' (position 1002-983) and WHV-F10 5'-TGGACTTTATGGGAAGCAGG-3' (position 2886-2905)/WHV-R1 5'-

AGGCTTTCTCCCTTGATCTC-3' (position 70-51). The core region was amplified using the following primers: WHV-CoreF1 5'-GGCAGCATTGATCCTAG-3' (position 1857-1873)/WHV-CoreR1 5'-ATTGAACTGAGCAGCTTGG-3' (position 2654-2636). The PCR reaction mixture consisted of 1x PCR buffer II (Perkin Elmer, Waltham, MA, USA), 2.5 mM $MgCl_2$ (Perkin Elmer), 0.5 µM of each primer, 0.2 mM dNTPs (Promega, Madison, WI, USA), and 2.5 U Taq Polymerase (Perkin Elmer). A thermal cycler (Gene Amp PCR System 9600 Perkin Elmer) was used with the following conditions: 95°C for 10 min and 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min; the last three steps were performed for 35 cycles. PCR products were analyzed by agarose gel electrophoresis and then purified using spin columns (Microcon, Amicon, Millipore, Billerica, MA, USA), following the manufacturer's instructions. Sequencing was carried out with the ABI prism Dye-Terminator Sequencing Kit (version 1.1) in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems), using the same primers used for amplification and two internal primers for each region: for PreS/S WHV-F2 5'-AGTGCACTGGCCAAAATTCG-3' (position 429-448) and WHV-R2 5'-GTGGACAGGAGGTTGGCAAG-3'(position 488-469) and for the Core region WHV-F8 5'-GATAGCTTGGATGAGCTC-3' (position 2224-2241) and WHV-CoreR2 5'-TGAAACCATAAACTTTGTCTCACC-3' (position 2331-2308). The PreS/S and Core sequences were aligned as described previously [La Sorsa et al., 2002] and analyzed in MEGA3 package for phylogenesis (neighbour joining method with a bootstrap of 1000). The predicted protein sequences were aligned and the alignment was analyzed. All of the comparisons were conducted with the WHV59, WHV7, WHV2, and OHVCGA virus prototypes.

RESULTS

Immune Precipitation of WHV Particles (IGC composition)

The immunogenic complex (IGC) was composed of immune sera containing PreS/S heterologous antibodies (anti-HBs) and serum-derived WHV particles containing 10^7 WHV-DNA copies/50 μl. The WHV-DNA level, as determined by Real-Time PCR in each pellet, was between $10^{5.8}$ and $10^{6.3}$ copies, indicating that these heterologous antibodies were able to bind stable amounts of WHV particles in the IGC, independently of the concentration of anti-HBs added to the mixtures.

IGC was administered to four WHV-negative woodchucks and four natural chronic WHV carriers, in order to evaluate the outcome of WHV infection in both groups.

IGC Administration in the WHV-Negative Woodchucks

No alterations in ALT or evident adverse effects (e.g., weight loss or local or general cutaneous reactions) were observed in any of the animals after the intravenous and intradermic administration of IGC. In Figure 1 (panels A-B), WHV virological and serological markers are reported for three animals in which WHV infection was induced only by viral particles (w835, w623, w837) and for two animals that were WHV-negative at the beginning of the study (w789, w603) and in which IGC was administered.

WHV infection in w835 was characterised by persistently elevated viremia levels (WHV-DNA titres between 10^7 and 10^{10}), as generally found in chronic carrier woodchucks. WHV infection in w623 and w837 was characterised by peak viremia levels of 10^8 copies/ml; which decreased to undetectable levels at week 12, as generally found in

self-limited viral infection. In all animals, WHV-DNA and WHeAg positivity were observed at week 4, whereas anti-WHc appeared at week 14.

Following IGC administration, WHV-DNA appeared at week 2 in w789 and week 4 in w603 and persisted for the remainder of the follow-up period. During follow-up, the WHV-DNA titres of these animals were up to two logs lower (at week 8) than the titres observed in woodchuck w835, which was characterised by persistently elevated viremia levels (Figure 1). In w789 and w603, WHeAg seroconversion to anti-WHe was observed (at week 6 for w789 and week 18 for w603). Anti-WHc disappeared at week 12 in w789 and remained negative until the end of follow-up.

IGC Administration in Woodchucks infected Chronically with WHV

The evaluation of WHV-DNA titres in sera of 15 chronic carrier woodchucks infected naturally (10 sera for each animal) revealed a level of titres higher than 10^8 copies/ml in 99% of the analysed sera, with mean fluctuations of 1-2 logs at individual points in the six-month follow-up (data not shown). The kinetics of WHV-DNA, WHeAg, anti-WHe, and anti-WHc and the PBMC response following IGC administration are shown in Figure 2. WHV-DNA was nearly stable in the 6-12 weeks prior to the IGC administration in the four woodchucks infected chronically.

The effects of IGC in chronically infected animals showed two different patterns. The first pattern was observed for w3953 and w840, in which there was a rapid decrease in WHV-DNA viremia. In w3953, WHV-DNA viremia decreased rapidly immediately after IGC administration (from $10^{8.2}$ to 10^6 copies/ml) and then continued to decrease at a slower rate, with slight fluctuations which reached a maximum of $10^{5.2}$ copies/ml (Figure 2A). In w840, the decrease in viremia was slower (beginning after 6 weeks) yet steeper (from 10^8 to 10^4 copies/ml) (Figure 2B). In these two animals, following IGC administration, a

sustained WHeAg seroconversion to anti-WHe was observed, beginning at week 2 in w3953 and at week 22 in w840. The second pattern was observed for w3981 and w3967. In these animals, there was no decrease in WHV-DNA viremia following IGC administration. In fact, the range of detected viremia was very similar to that which was observed before IGC administration, with values in the range10^{8}-$10^{10.5}$ copies/ml in w3981 (Figure 2C) and $10^{8.8}$-$10^{9.5}$ copies/ml in w3967 (Figure 2D). The serological markers also showed no change, with WHeAg and anti-WHc remaining positive throughout follow-up.

Evaluation of Natural WHV Immunocomplexes in Woodchucks Infected Chronically

Prior to IGC administration to animals infected chronically, the ratio of WHV in pellet (natural WHV immuno-complex) to that in supernatant (free WHV particles) was evaluated. In the two animals (w3953 and w840) in which a decline of WHV viral load was observed, the pellet: supernatant ratio of WHV-DNA was 1:1. This indicates that the amount of WHV particles complexed with antibodies in pellets was the same as that of free WHV particles in supernatants. In the remaining two animals (w3967 and w3981), the ratio was 5:1 and 10:1, respectively, indicating that there was a higher amount of WHV particles complexed with antibodies than free WHV particles.

Lymphoproliferative Responses

A slight PBMC response against the C2 peptide was observed in w840 at baseline (prior to the first IGC administration) and at week 16, in w3953 at weeks 12 and 16, and in w3967 at weeks 12 and 22. (Figure 2A). In w3981, a slight PBMC response against the inactivated WHV particles and S1 and S7/8 peptides was also observed at week 22. No

specific response (SI <2.1) against peptides or WHV particles was observed at any other time during follow-up.

Sequence Analysis

To evaluate virus variability following IGC administration, sequence analysis was performed in chronically infected animals. Amplified products from the core region showed homogeneity of the isolates obtained at baseline and at week 24. No significant mutations were observed within the same isolate of each animal or among the isolates of the four animals.

The phylogenetic characterization of the PreS/S region is shown in Figure 3. The w3953, w3981 and w3967 isolates correlated with the WHV59 prototype, whereas the w840 isolates were more closely related to the WHV7 and WHV2 prototypes. In w840 and w3981, the analysis of the genetic variability showed sequence homogeneity between isolates obtained at baseline and those obtained at week 24, whereas in w3967 and w3953, the isolates varied by, respectively, 0.1% and 0.5%. In w3953, the polypeptidic analysis showed the following amino-acid mutations: I85Q, Q65K and I194T.

DISCUSSION

Immune complexes composed of antigen and antibodies have been used in animal models and in humans to induce an antiviral response against viral infections [Xu et al., 2008; Lu et al., 2008; Randall et al., 1988; Wen et al., 1994; Wen et al., 1995]. In the present study, an immune complex composed of anti-HBs and WHV viral particles was used to evaluate its effect on acute and chronic WHV infection. The decision to use anti-HBs antibodies was based on the results of a previous study, in which the administration of a human vaccine in woodchucks was followed by anti-HBs production and abortive viral replication after the WHV challenge. This indicated an antiviral effect of the anti-HBs, though not a neutralizing effect, probably because of the heterologous nature of antibodies [Argentini et al., 2005].

In this study, the animals that were infected with WHV viral particles showed the appearance of markers of infection, with the characteristic pattern of persistent infection in one animal (w835) and of self-limited infection in two animals (w623 and w837). In the two WHV-DNA negative woodchucks in which IGC was administered (w789 and w603), although WHV-DNA persisted, WHV-DNA levels were lower than in w835 (animal with persistent WHV-DNA). Moreover, the disappearance of anti-WHc was observed, as was sustained seroconversion of WHeAg to anti-WHe, similarly to that which was found in w623 and w837 (animals with self-limited infection). The viral persistence was probably due to the only partial ability of the IGC (composed of WHV particles and antibodies) to protect the animals. Nonetheless, the observed virological and serological patterns could indicate that IGC induced an immune response able to control viremia in part, though not completely. Regarding chronic WHV infection, according to the serological follow-up study performed on 15 naturally infected chronic WHV carrier woodchucks, the WHV-DNA titres were almost always at levels higher than 10^8 copies/ml, with fluctuations generally in the

range of 10^8-10^{10} copies/ml. In this study, WHV viremia was stable prior to IGC administration, and in two of the four WHV chronically infected woodchucks the WHV-DNA level decreased greatly (4-6 logs) after IGC administration. Moreover, in these two animals, no rebound in viral load was observed during follow-up, and anti-WHe seroconversion was observed in both animals. According to the other studies [Roggendorf and Tolle, 1995] and based on our own experience, WHeAg seroconversion does not seem to occur spontaneously during the natural course of chronic WHV infection. The results of the present study suggest that IGC administration has an effect on breaking the immune tolerance, as indicated by the anti-WHe seroconversion and by the decrease in WHV-DNA levels. The reduction of WHV-DNA and the seroconversion to anti-WHeAg seem to be directly related to the administration of these specific IGC; however, an antiviral effect due to an overall stimulation of the immune system resulting from the IGC administration cannot be ruled out.

The results of this study are in accordance with data from studies conducted in patients with chronic HBV infection [Xu et al., 2008; Wen et al., 1995; Yao et al., 2007]. In one study [Xu et al., 2008; Yao et al., 2007], the use of an immune complex was followed by a significant reduction in HBV-DNA levels in 9 of the 14 patients, whereas in a recent randomized controlled trial [Xu et al., 2008], the use of an immune complex ("YIC") induced the break of immune tolerance against HBsAg, which was followed by a decrease in HBV-DNA and by HBeAg seroconversion in some of the patients. The results of this study are also consistent with data on woodchucks in which the use of a combined approach [Lamivudine and an immune complex (WHsAb-WHsAg) and DNA vaccination] led to an anti-WHs response and to a significant reduction in WHV-DNA [Lu et al., 2008].

The finding that ALT and AST did not increase significantly with the decrease in WHV-DNA probably indicates that non-cytotoxic immune mechanisms are responsible for the decrease in WHV replication [Glebe et al., 2009] However, as suggested previously,

the woodchuck liver has a high potential to regenerate, which may compensate the severity of liver damage. This could explain the apparent lack of a correlation between the decrease in WHV-DNA, which indicates the death of WHV infected cells, and altered transaminase levels.

Circulating immune complexes have been reported to play a critical role in the early elimination of viral particles during acute WHV infection, as part of the humoral immune response against WHsAg [Glebe et al., 2009]. The role of these immune complexes in woodchucks chronically infected with WHV is not clear. Interestingly, in two animals in which a decrease in WHV-DNA was observed following IGC administration, the ratio of complexed WHV particles and free WHV particles was lower than that in the other 2 animals. Although the small number of animals does not allow conclusions to be drawn regarding the role of these circulating immune complexes in the antiviral response, in the animals in which a decrease in WHV-DNA viremia was observed, small amounts of circulating immunocomplexes may have influenced the magnitude of the effect of the IGC in breaking the immune tolerance against chronic WHV infection. The antiviral response also may have been related to a better recognition of the IGC viral particles by the antigen presenting cells (APC). Specifically, the antigen-antibody binding was presumed to be low because of the use of heterologous anti-HBs antibodies, and this could have improved the uptake of viral particles by the APC [Burnet et al., 1937; Burton, 2002; Dimmock, 1995]. The potentially preferential cross-linking of IGC on the activating Fc receptors (FcR) of adaptive immune response cells, such as dendritic cells (DC), could allow for better presentation of viral antigens to the memory cells. The proportion of activating DC receptors (Fc-IIIγ), with respect to inactivating DC receptors (Fc-IIB), could have played a role in the break in immune tolerance and led to different individual antiviral responses, triggering the antiviral response in w3953 and w840 but not in w3967 and w3981 [Nimmerjahn and Ravetch, 2007; Ochoa-Callejero et al., 2007; Savina and Amigorena,

2007]. Furthermore, whether or not live WHV particles (used in IGC) can enter (via FcRs) and replicate in the APCs, charging viral epitopes onto MHC-I molecules and triggering the $CD8^+$ antiviral response, could be evaluated in specifically designed studies with an IGC including inactivated WHV particles [Welsh and Fujinami, 2007].

Regarding the immune cellular response, PBMC responses against the core peptide (C2) were observed occasionally, and in one animal they coincided with the decrease in WHV-DNA. In one woodchuck, the PBMC response against the C2 peptide was also observed prior to IGC administration and may thus have constituted a host immune response against this strong immunogenic core peptide (C2: 96-110), rather than an immune response stimulated by the IGC [Frank et al., 2007]. In accordance with other studies, no correlation was found between the cellular response and the viral replication outcome during chronic infection. However, this does not mean necessarily that no correlation exists, and it could be speculated that the specific T-cells were sequestered into the liver and therefore were not detectable in the peripheral blood [Lu et al., 2008].

The sequence analysis of the PreS/S region did not show significant mutations in any of the woodchucks that were infected chronically with WHV. The homogeneity of viral isolates indicates that the IGC does not play an important role in inducing variants that could be resistant to this potential therapeutic approach. Nonetheless, a longer follow-up would be needed to define better the effect of the immune response induced by IGC on the possible selection of escape viral variants.

In conclusion, the results of this study provide new information on the effect of an immune complex containing viral particles and non-neutralizing antibodies on the outcome of WHV infection. The use of the IGC could improve the outcome of acute and chronic WHV-infection, in terms of viral load and WHeAg seroconversion. These data indicate that strategies based on immune complexes could contribute to breaking the immune tolerance in hepadnaviral infection and triggering an effective antiviral response.

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Potential conflicts of interest: None.

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FIGURE LEGENDS

Figure 1. Administration of immunogenic complex (IGC) in WHV-negative woodchucks. Kinetics of virological markers (WHV-DNA in Panel A and WHeAg, anti-WHc and anti-WHe in Panel B) in the control animals w835, w623 and w837 (infected with WHV alone) and in animals to which IGC was administered, w789 and w603. Quantifiable levels of serum WHV-DNA (by Real-Time PCR) are expressed as copies/ml and indicated as —◆— (Panel A). WHeAg, anti-WHe and anti-WHc are expressed as negative (-) or positive (+) (Panel B). WHV infection is indicated with a white arrow (⇓) and IGC administration is indicated with a bold arrow (🡻). Week 0 indicates the time immediately prior to the first IGC administration.

Figure 2. Administration of immunogenic complex (IGC) in chronic WHV-infected woodchucks. Kinetics of the virological markers (WHV-DNA, WHeAg and anti-WHe) and the immunological response (PBMCs proliferation) in chronic WHV-infected woodchucks following IGC administration: w3967, w3981, w3953 and w840. Quantifiable levels of serum WHV-DNA (by Real-Time PCR) are expressed as copies/ml and indicated as —◆—. WHeAg and anti-WHe are expressed as —□— and —■—, respectively. IGC administration is indicated with a bold arrow (🡻). PBMCs response is indicated with the name of the peptide and corresponds to the time in which S.I. was ≥2.1. Week 0 indicates the time immediately prior to the first IGC administration.

Figure 3. Phylogenetic tree of the PreS/S region of the WHV isolates in WHV chronically infected woodchucks. The phylogenetic tree obtained by neighbour joining method is shown. The virus isolate and the sample (in parenthesis) are indicated. The accession numbers of the WHV sequences analyzed are as follows: strain WHV 3953

(week 0): FM212012, strain WHV 3967 (week 0): FM212015, strain WHV 3967 (week 24): FM212014, strain WHV 3953 (week 24): FM212013, strain WHV 3981 (week 0): FM212016, strain WHV 3981 (week 24): FM212017, strain WHV 840 (week 0): FM212010, strain WHV 840 (week 12): FM212011, strain WHV 840 (week 24): FM212009. The accession numbers of the WHV prototypes used are as follows: WHV7: M18752, WHV2: AY628096, WHV59: M19183, and OHVCGA: J02442.