Treatment of Chronic Viral Hepatitis in Woodchucks by Prolonged Intrahepatic Expression of Interleukin-12

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Chronic hepatitis B is a major cause of liver-related death worldwide. Interleukin-12 (IL-12) induction accompanies viral clearance in chronic hepatitis B virus infection. Here, we tested the therapeutic potential of IL-12 gene therapy in woodchucks chronically infected with woodchuck hepatitis virus (WHV), an infection that closely resembles chronic hepatitis B. The woodchucks were treated by intrahepatic injection of a helper-dependent adenoviral vector encoding IL-12 under the control of a liver-specific RU486-responsive promoter. All woodchucks with viral loads below 1010 viral genomes (vg)/ml showed a marked and sustained reduction of viremia that was accompanied by a reduction in hepatic WHV DNA, a loss of e antigen and surface antigen, and improved liver histology. In contrast, none of the woodchucks with higher viremia levels responded to therapy. The antiviral effect was associated with the induction of T-cell immunity against viral antigens and a reduction of hepatic expression of Foxp3 in the responsive animals. Studies were performed in vitro to elucidate the resistance to therapy in highly viremic woodchucks. These studies showed that lymphocytes from healthy woodchucks or from animals with low viremia levels produced gamma interferon (IFN-γ) upon IL-12 stimulation, while lymphocytes from woodchucks with high viremia failed to upregulate IFN-γ in response to IL-12. In conclusion, IL-12-based gene therapy is an efficient approach to treat chronic hepadnavirus infection in woodchucks with viral loads below 1010 vg/ml. Interestingly, this therapy is able to break immunological tolerance to viral antigens in chronic WHV carriers.

Hepatitis B virus (HBV) infection is estimated to cause approximately 1 million deaths per year (http://www.who.int/emc). Current therapies against chronic HBV include pegylated alpha interferon (IFN-α) and nucleoside/nucleotide analogs, such as lamivudine, entecavir, adeefovir, and tenofovir (16). Sustained antiviral responses are achieved in only one-third of the patients treated with pegylated IFN-α (16, 32). Nucleoside/nucleotide analogs are effective, but treatment must be continued for many years, resulting in high costs, the emergence of drug-resistant variants, and frequent relapses after the discontinuation of therapy (32).

Chronic HBV infection is associated with defects in antiviral immunity (3). Patients with an acute self-limiting HBV infection develop neutralizing anti-HBs antibodies and multispecific CD4+ and CD8+ T-cell responses with a type 1 cytokine profile (3). In contrast, patients with chronic HBV infection show no protective humoral immunity and a weak or undetectable virus-specific T-cell response (5). The precise mechanism responsible for this immunotolerance is still unknown. Recently, several studies have indicated that regulatory T cells (Tregs), immunosuppressive cytokines, and inhibitory receptor-ligand interactions, such as PD1-PDL1, contribute to the impairment of virus-specific T-cell responses in chronic HBV infection (1, 17, 23, 26, 28).

Interleukin-12 (IL-12) is a cytokine produced by antigen-presenting cells that is essential for the induction of effective cell-mediated immunity against viruses and other pathogens (30). IL-12 promotes Th1-type responses, enhances cytotoxic T-cell activity, and stimulates T lymphocytes and NK cells to produce IFN-γ (30). The administration of recombinant IL-12 (rIL-12) to HBV-transgenic mice resulted in the inhibition of HBV replication in the liver (8). In vitro studies demonstrated that IL-12 was able to enhance HBV-specific T-cell responses in chronic HBV carriers (15, 22, 31). Moreover, the upregulation of IL-12 production has been shown to be associated with HBs seroconversion, indicating an important role for this cytokine in the control of HBV infection (22). In two studies, rIL-12 administered to patients with chronic HBV infection once per week as monotherapy (7) or twice weekly in combination with lamivudine (21) increased virus-specific T-cell reactivity and exerted significant antiviral activity. However, in both studies, a rebound of viremia occurred following drug
withdrawal. The antiviral effects of rIL-12 were dose dependent, but the therapy was limited by severe toxicity when high doses of the cytokine were used (7, 21).

Gene therapy can significantly increase cytokine expression in the target organ without excessively elevating systemic cytokine levels, which leads to an increased efficacy/toxicity ratio. In the present study, we tested the antiviral potential of IL-12-mediated gene therapy using a high-capacity adenovirus (HC-Ad) encoding murine IL-12 (mIL-12) under the control of a liver-specific inducible promoter responsive to the progestosterone antagonist RU486 (30). HC-Ad is a nonintegrating vector characterized by strong hepatotropism, low toxicity, long-term transgene expression, and high cloning capacity (13). All these properties make HC-Ad a useful tool for therapeutic applications in human liver diseases.

As an animal model of chronic HBV infection, we used woodchucks that were chronically infected with woodchuck hepatitis virus (WHV). WHV is a hepadnavirus with a genomic organization, biological properties, and a replicative strategy that are essentially identical to those of HBV. When WHV infects woodchucks in the perinatal period of life, the infection causes chronic hepatitis in most animals. This condition resembles the pathological features and natural history of chronic hepatitis B (18). Here, we demonstrate that prolonged intrahepatic expression of IL-12 overcomes immunological tolerance for WHV antigens and induces sustained antiviral effects in woodchucks with chronic WHV infection and viral loads below 10^{10} viral genomes (vg)/ml. These observations indicate that IL-12 gene therapy is an alternative approach for the treatment of chronic HBV infection.

**TABLE 1. Nucleotide sequences of primers and probes used in the present study to quantify DNA or mRNA of target sequences by qPCR**

<table>
<thead>
<tr>
<th>Target sequence</th>
<th>Probe</th>
<th>Sense</th>
<th>Primer</th>
<th>Antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>WHV (core Ag)</td>
<td>5'-AGAAGATCCTGAGGCCTCGGA-3'</td>
<td>5'-AGAAGATCCTGAGGCCTCGGA-3'</td>
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<td></td>
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<td>HBV</td>
<td>5'-CCTGAGCAATCCTCGCAATCTT-3'</td>
<td>5'-GTCGCTTCTGGCAATTTTG-3'</td>
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<td></td>
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<tr>
<td>IRES (rIL-12)</td>
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<td>5'-TCATCGTCTTACGCTGATTCGGT-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wIFN-γ</td>
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<td>5'-TGGCAGATGGAGATTGAGAGC-3'</td>
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<tr>
<td>wIFN-β actin</td>
<td>5'-GAGTGCACTTGCAATTGACTGT-3'</td>
<td>5'-GAGTGCACTTGCAATTGACTGT-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wFoxP3</td>
<td>NA (a)</td>
<td>5'-GCCGACACGAGGAGGGACTACG-3'</td>
<td>5'-GCCGACACGAGGAGGGACTACG-3'</td>
<td></td>
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<tr>
<td>wCD4</td>
<td>NA (a)</td>
<td>5'-TGAAAGACGCACTCCCTCTCCT-3'</td>
<td>5'-TGAAAGACGCACTCCCTCTCCT-3'</td>
<td></td>
</tr>
<tr>
<td>WHVcccDNA</td>
<td>5'-ATGAGAGATGAGATGAGATGAG-3'</td>
<td>5'-GCAGGGAGGAGGAGGGAC-3'</td>
<td></td>
<td></td>
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<tr>
<td>WHV t-DNA</td>
<td>5'-TCATCGTCTTACGCTGATTCGGT-3'</td>
<td>5'-TCATCGTCTTACGCTGATTCGGT-3'</td>
<td></td>
<td></td>
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</tbody>
</table>

(a) IRES, internal ribosome entry site; w, woodchuck.

(b) NA, not applicable.

**MATERIALS AND METHODS**

HC-Ads. HC-Ads expressing green fluorescent protein (GFP) under the control of the cytomegalovirus (CMV) promoter (HC-Ad-CMV–GFP) (14) or mIL-12 under the control of a liver-specific inducible promoter responsive to mifepristone (RU486) (HC-Ad/RUmIL-12) were constructed and produced as described previously (13, 30).

Animals. Adult woodchucks (Marmota monax) chronically infected with WHV were trapped in the state of Delaware and purchased from North Eastern Wildlife (Ithaca, NY). The manipulation of animals, including ultrasound-guided biopsies and virus injection, was performed under general anesthesia. A dose of 2 × 10^{10} infectious units (i.u.) of HC-Ad/RUmIL-12 or HC-Ad-CMV–GFP was injected into the liver following medial laparotomy (9). After laparotomy, the liver was exposed, the needle was introduced into the liver parenchyma, and the virus was injected very slowly in a volume of 200 to 500 μl, depending on the purpose of the study. In the first study, which was carried out to determine D2 transduction efficiency of HC-Ad and the biological activity of mIL-12, the virus was injected into the major liver lobe (the L3 lobe) in a volume of 500 μl. In the second study, which was performed to test the antiviral potency of the vector, the virus was injected into three different lobes of the liver in a volume of 200 μl per injection site. IL-12 expression was induced intraperitoneally with 10 μg of RU486/kg body weight (30). Blood was obtained from the saphena vein in isoflurane-anesthetized woodchucks. The study was performed in accordance with the guidelines of the local ethical commission for animal experiments.

Organ sample processing. After sacrifice or the natural death of the animal, the liver, lung, spleen, heart, kidney, pancreas, intestine, stomach, muscle, brain, and ovaries or testes were harvested. The liver was divided into different parts that we named L1, L2, L3, L4, and L5. Three different samples were obtained from every organ and liver region, and each sample was processed individually.

**Determination of serum and tissue mIL-12 levels.** Serum mIL-12 (p70) was quantified using OptEIA ELISA kits (PharMingen, San Diego, CA) according to the manufacturer's instructions. Frozen tissues were homogenized in cytokine buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, and a protease inhibitor cocktail) and centrifuged for 5 min at 9,300 × g. The supernatants were used for cytokine level analysis. The total protein concentration was determined by using the Bradford assay.

**Histological studies.** Immunohistochemical staining was performed using the EnVision + System (Dako, Glostrup, Denmark) and rabbit anti-human CD3 epsilon and peroxidase-labeled goat anti-rabbit antibodies according to the manufacturer's recommendations. The reactions were visualized by incubating the sections with DAB+ chromogen (Dako). The stained sections were counterstained with hematoxylin, dehydrated, and mounted in DPX. Positively stained cells were enumerated with the motorized Axioplan 2ie microscope. At least 50 fields were examined, and the number of CD3+ cells per 100 hepatocytes was determined. Liver biopsy specimens were evaluated by experienced liver pathologists, and histological changes were scored according to the method developed by Knodell.

**Virological assays.** PCR primers for the amplification of total DNA (t-DNA) were designed to detect all forms of WHV DNA, including replication-competent DNA, intermediate forms (replicative intermediate DNA [RI-DNA]), and covalent closed circular DNA (cccDNA). The detection limit of the assay was 10 vg per μl of serum (2). t-DNA and the cccDNA-enriched fraction were extracted from the liver as previously described (33). For the extraction of intracellular viral DNAs, liver biopsy samples were disrupted in 1.5 ml of 0.01 M Tris-HCl (pH 7.5) and 0.01 M EDTA. Half of the homogenate was used for the extraction of RI-DNA, and half was used for cccDNA preparation. For the extraction of RI-DNA, the homogenate was adjusted to a total volume of 6 ml with 0.025 M Tris-HCl (pH 7.4), 0.01 M EDTA, 0.25% (wt/vol) sodium dodecyl sulfate, 0.05 M NaCl, and pronase (2 mg/ml). After a 1-h incubation at 37°C, nucleic acids were extracted with a 1:1 mixture of phenol-chloroform and collected by ethanol precipitation. For cccDNA extraction, the volume of the remainder of the homogenate was adjusted to 3 ml with 0.01 M Tris-HCl (pH 7.5) and 0.01 M EDTA; 200 μl of 10% (wt/vol) sodium
dodecyl sulfate was then added, the mixture was briefly vortexed, and 1 ml of 2.5 M KCl was added. The mixture was incubated at room temperature for 20 min, and potassium dodecyl sulfate-protein complexes were then collected by centrifugation at 10,000 rpm for 20 min at 4°C. The supernatant was subjected to phenol-chloroform extraction, and the DNA was precipitated with ethanol overnight at room temperature (33). WHV DNA was analyzed by Southern blotting as previously described. WHV RI-DNA, cccDNA, and woodchuck β-actin copy numbers were quantified by qPCR. Primers and probes are shown in Table 1. The cccDNA primers were designed using an approach verified for HBV by others (25). The sense primer (1701 to 1918) is located upstream of the DR3 region, within the single-stranded region of WHV genomic DNA. The antisense primer is located downstream of the DR3 sequence (1972 to 1953), and the probe corresponds to the 1846-to-1868 sequence. The numbers denote the position of the sequence in the WHV genome according to GenBank accession number AY628100. Using these primers, no amplification product could be obtained from WHV genomic DNA. To confirm the specificity of the PCR assay for cccDNA, the PCR product was sequenced after being cloned into the Topo-TA system (data not shown). In addition, the cccDNA quantification was repeated after digestion with Plasmid-Safe ATP-dependent DNase, an enzyme that digests linear double-stranded DNA, as well as closed and single-stranded DNAs, but not closed double-stranded circular DNA (data not shown). The plasmid DNA construct pWHVs containing a monomer of the WHVs genome, provided by M. Roggendorf, was used as a positive control. This plasmid was also used as a standard to determine total WHV-DNA and cccDNA copy numbers. A good linear range was observed from 10^2 to 10^7 copies of plasmid in real time with both sets of primers (data not shown). The lowest detectable cccDNA copy number was assigned as the limit of detection of the assay.

The presence of serum WHV surface antigen (WHsAg) levels was determined by enzyme-linked immunosorbent assay (ELISA) (2, 19). The WHV e antigen (WHeAg) concentration was determined using a commercial ELISA for HBVe antigen that recognizes woodchuck antigen (DiaSorin S.p.A., Saluggia, Italy).

**Anti-WHV specific cellular immunity.** The T-cell proliferative response to WHeAg stimulation was analyzed using freshly isolated peripheral blood mononuclear cells (PBMCs) as previously described (2). Briefly, freshly isolated PBMCs were cultured in 96-well U-bottom plates at a density of 2 x 10^5 cells/well in complete RPMI medium. WHeAg was added in triplicate at a final concentration of 1 μg/ml. Forty-eight hours later, the supernatants were harvested and the IL-2 concentration was determined using a CTL-L2 assay (2). Stimulation index (SI) values of ≥3.1 were considered positive for a specific PBMC response.

**Activation of woodchuck PBMCs with plate-bound anti-CD3 antibody.** Woodchuck peripheral blood lymphocytes (PBLs) were stimulated with immobilized anti-human, anti-mouse, and anti-rat CD3 (clone UCHT1, 145-2C11, and G4.18, respectively; BD Biosciences) at a concentration of 2 μg/ml. Only the anti-rat CD3 antibody was able to induce IFN-γ production from woodchuck PBLs (data not shown; I. Otano, J. Prieto, S. Henne, and G. G. Aseguinolaza, respectively; BD Biosciences) at a concentration of 2 μg/ml. The proliferation induced by plate-bound anti-rat CD3 was performed using a flow cytometry assay with 5- and 6-carboxyfluorescein diacetate succinimidyl ester (CFSE) ( Molecular Probes), as described elsewhere, with minor modifications (12). Briefly, woodchuck PBMCs were labeled with 5 μM of CFSE and were activated with plate-bound anti-CD3 antibody at a concentration of 2 μg/ml. The plates were incubated for 3 days. The cells were harvested and analyzed on a FACSCalibur flow cytometer (Becton Dickinson). The lymphocyte population was gated, and the diluted CFSE fluorescence in dividing cells was determined using FlowJo software (Becton Dickinson). The SI was defined by the percentage of dividing cells.

**Purification and analysis of woodchuck regulatory T cells.** To purify woodchuck regulatory T cells, we first tested antibodies against CD25 that were antibody at a concentration of 2 μg/ml. The plates were incubated for 3 days. The proliferation induced by plate-bound anti-CD3 antibody was compared using Student’s t test, and the SI was determined.

**Analysis of the immunostimulatory activity of mIL-12.** Freshly isolated PBMCs were cultured in 96-well U-bottom plates at a density of 1 x 10^6 cells/well in complete RPMI medium. mIL-12 was added in triplicate at a concentration of 1 ng/ml. Forty-eight hours later, the cells were harvested and RNA was extracted. Woodchuck IFN-γ and β-actin mRNA levels were quantified using reverse transcription (RT)-qPCR, as described below.

**Analysis of mRNA expression by qPCR.** Total RNA was extracted using Trizol, and the total RNA (2 μg) was treated with DNase (Gibco-BRL, Paisley, United Kingdom) prior to RT with Moloney murine leukemia virus reverse transcriptase (Gibco-BRL) in the presence of RNaseOut (Gibco-BRL). Transgenic mIL-12 (internal ribosome entry site sequence), woodchuck transfection growth factor β (TGFB), WHV pregenomic RNA (pgRNA), and woodchuck β-actin were measured by real-time qPCR using a LightCycler (Roche Diagnostic, Mannheim, Germany), primers, and Taqman probes (Applied Biosystems). Woodchuck CD4 and Foxp3 expression levels were measured using a LightCycler or a TaqMan ABI Prism 7000 instrument (Applied Biosystems) with the LC-DCNA Master SYBR green mix. Primers and probes were designed using Primer-Express software (Table 1). The number of copies of mIL-12, woodchuck IFN-γ, woodchuck TGFB, WHV pgRNA, and woodchuck β-actin were determined by interpolation using external DNA standards. Woodchuck CD4 and Foxp3 final amplification products were analyzed by using melting curves. The relation between the two transcripts was expressed by the following formula: 2^ΔΔCT = 2^(-Ct(pgRNA) - Ct(TGFB)) with C being the threshold cycle, the point at which the fluorescence rises significantly above background.

**Biochemistry.** Alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin, and lactate dehydrogenase (LDH) levels were determined by using a Boehringer Mannheim Hitachi (Mannheim, Germany) 911 automatic analyzer. In our laboratory, the normal range for these biochemical markers in woodchucks chronically infected with WHV are as follows: ALT, 0 to 20 IU/liter; AST, 0 to 40 IU/liter; bilirubin, 0.10 ≤ 0.50 μg/dl; and LDH, 400 to 700 IU/liter.

**Hematology analysis.** Whole blood was collected in EDTA-containing tubes, and complete blood counts were determined using a semiautomatic electronic blood cell counter (Sysmex F-800). Normal blood cell counts found in woodchucks chronically infected with WHV are as follows: platelets, 500 x 10^10 to 800 x 10^10/liter; leukocytes, 13 x 10^9 to 16 x 10^9/liter; lymphocytes, 4 x 10^9 to 8 x 10^9/liter; and neutrophils, 5.5 x 10^9 to 9 x 10^9/liter.

**Statistical analysis.** All statistical calculations were performed with SPSS 11.0 software (SPSS Inc., Chicago, IL). To establish significance between woodchuck groups, statistical analysis was performed using the Kruskal-Wallis test followed by a comparison of the control group with the experimental groups that used the Mann-Whitney test with Bonferroni’s correction. The liver biopsy data was evaluated by the Knodell score and was placed in a contingency table for comparison with a Somer’s D test. Hepatic expression of Foxp3 between woodchuck groups was compared using Student’s t test after logarithmic transformation of the data.

**Nucleotide sequence accession number.** The Woodchuck Foxp3 cDNA sequence GenBank accession number is EU168437.

**RESULTS**

**RU486 administration induced IFN-γ expression and lymphocyte infiltration in the livers of animals treated with HC-Ad/RuMIL-12.** The homologies of the woodchuck IL-12 p35 and IL-12 p40 proteins to the corresponding known subunits of the murine forms of these molecules were 49% and 63%, respectively. In vitro tests showed that mIL-12 was active on woodchuck PBMCs by efficiently inducing IFN-γ mRNA expression in these cells (Fig. 1). In addition, preliminary experiments were performed to study the biodistribution of HC-Ad/RuMIL-12 and the vector’s ability to transduce the woodchuck liver and to induce IL-12 expression following RU486 administration. Two woodchucks with chronic WHV infection and viral loads of 2.52 x 10^11 vg/ml (woodchuck no. 350) and 1.19 x 10^11 vg/ml (woodchuck no. 813) received a single dose of 2 x 10^11 i.u. of HC-Ad/RuMIL-12 by direct intrahepatic injection into the largest liver lobe (9). Two weeks later, transgene expression was induced for 5 days by daily intraperitoneal

**Downloaded from** http://jvi.asm.org/ on October 16, 2017 by guest
administration of 500 µg of RU486/kg body weight. Ten hours after the first and fifth dose of RU486, blood samples were obtained to determine mIL-12 levels in serum by ELISA. As shown in Fig. 2A, RU486 induced a significant rise in serum mIL-12 levels in both animals. Following the last dose of RU486 on day 5, the woodchucks were sacrificed for analysis of mIL-12 levels in different organs. The presence of mIL-12 was detected by ELISA in different areas of the liver, but it was also found in the kidney (Fig. 2B). The IL-12 concentration was highest in the area surrounding the injection site (L3), followed by the neighboring liver regions (L2 and L4) (Fig. 2B). mIL-12 mRNA was also detected in L3, as well as in the other liver lobes, but it was found only at very low levels in other organs, such as the spleen, lung, heart, and kidney (Fig. 2C). The analysis of virus biodistribution in these animals was performed by qPCR and showed that the virus was mainly detected in the liver (1.3 \times 10^4 \pm 0.25 \times 10^4 and 1.1 \times 10^4 \pm 0.32 \times 10^4 vg of HC-Ad/RUmIL-12 per 10^6 woodchuck β-actin copies were detected in woodchuck no. 350 and woodchuck no. 813, respectively) and at very low levels in the spleen, lung, heart, and kidney (data not shown). This indicated that no significant leakage of virus occurred after intrahepatic injection, as we had previously described for mice (9).

Because IFN-γ is induced by IL-12, the expression of this cytokine in different tissues was also determined. The highest values for IFN-γ mRNA were seen in the injected liver lobe, but increased mRNA expression was also detected to a lesser extent in other organs (Fig. 2D). Hematoxilin-eosin staining (Fig. 2E) and anti-CD3 immunohistochemistry in liver biopsies (Fig. 2F) performed before and after mIL-12 induction revealed lymphocytic infiltration not only in the injected lobe, but also in other areas of the liver (data not shown).

**FIG. 1.** Analysis of the immunostimulatory activity of mIL-12 in woodchuck PBMCs. freshly isolated PBMCs from non-WHV-infected woodchucks were cultured in 96-well U-bottom plates at a density of 1 \times 10^5 cells/well in complete RPMI medium. mIL-12 was added in triplicate at different concentrations: 0, 0.01, 0.1, 1, 10, and 100 ng/ml. Forty-eight hours later, cells were harvested, RNA was extracted, and woodchuck IFN-γ and woodchuck β-actin mRNA levels were estimated by RT-qPCR. The means and standard deviations are indicated.

**TABLE 1.** IFN-γ and β-actin mRNA levels determined by RT-qPCR in woodchucks with chronic WHV infection treated with a single dose of 2 × 10^10 i.u. of HC-Ad/RUmIL-12. The virus was administered at laparotomy by intrahepatic injection in three different liver areas. Two weeks after vector injection, mIL-12 expression was induced for 42 days by daily intraperitoneal administration of 500 µg of RU486/kg body weight. Two control groups were included in the study; one group (n = 2) received a single dose of 2 × 10^10 i.u. of HC-Ad–CMV–GFP intrahepatically, and the second group (n = 3) received only intraperitoneal injections of RU486 daily for 42 days.

Serum samples were obtained before vector injection and then every 2 weeks during the induction period and follow-up (for 200 days after the injection of the vector). Liver biopsy specimens were obtained at days −14, 35, and 130. One woodchuck in the treatment group died after being biopsied on day 35 because of intestinal perforation (woodchuck no. 796), and a second woodchuck died on day 90 because of pneumonia (woodchuck no. 622). The organs from these animals were still harvested and used for analysis.

In woodchucks treated with HC-Ad/RUmIL-12 plus RU486, a reduction in the viral load of no greater than 2 log units was determined to be a lack of response to the therapy. Remarkably, all animals with basal viremia levels below 10^10 vg/ml exhibited an intense and sustained decrease of serum WHV DNA from pretreatment levels upon IL-12 induction (Fig. 3A). In contrast, mIL-12 induction did not produce comparable effects on WHV replication in woodchucks with pretreatment viremia levels above 10^10 vg/ml (Fig. 3B). None of the woodchucks included from the control groups showed changes in viremia during the study period (Fig. 3C).

Following RU486 administration, mIL-12 expression levels in serum varied widely among animals but were comparable between the two groups. Further, mIL-12 concentrations became undetectable by the end of the RU486 administration period (Fig. 3D and E). No detectable mIL-12 was present in the sera of control animals during the study (data not shown).

Enough liver tissue could be harvested from all the treated woodchucks except for one animal (woodchuck no. 156) for WHV DNA determination by Southern blotting. A marked reduction of WHV DNA in the liver was found in responder woodchucks no. 796, no. 173, and no. 622, whereas woodchucks from the high-viremia group showed no changes in the levels of hepatic WHV DNA (Fig. 4A and B). Levels of WHV t-DNA and cccDNA in the liver were determined by qPCR on t-DNA- and cccDNA-enriched fractions, respectively, and were normalized using β-actin copy numbers (33). As shown in Fig. 3A and B, the percentages of the initial levels of t-DNA and cccDNA remaining at the end of treatment were closely correlated with the decrease in viremia. Moreover, analysis of WHV pgRNA by RT-qPCR following RNA extraction from liver tissue and normalization to β-actin mRNA levels demonstrated a marked reduction of this transcript in woodchucks with low viremia levels, whereas comparable reductions were not observed in woodchucks with high viremia levels (Fig. 4C).

Correlating with these results, woodchucks from the responder group that were WHeAg and WHsAg positive prior to the treatment cleared WHeAg and WHsAg levels in the serum during the IL-12 induction period or shortly thereafter. Animals that failed to respond to the treatment remained positive for WHeAg and WHsAg throughout the study (Fig. 4D and E).

**FIG. 2.** Hematoxilin-eosin staining and immunohistochemistry in liver biopsies performed before and after mIL-12 induction revealed lymphocytic infiltration not only in the injected lobe, but also in other areas of the liver (data not shown). Because IFN-γ is induced by IL-12, the expression of this cytokine in different tissues was also determined. The highest values for IFN-γ mRNA were seen in the injected liver lobe, but increased mRNA expression was also detected to a lesser extent in other organs (Fig. 2D). Hematoxilin-eosin staining (Fig. 2E) and anti-CD3 immunohistochemistry in liver biopsies (Fig. 2F) performed before and after mIL-12 induction revealed lymphocytic infiltration not only in the injected lobe, but also in other areas of the liver (data not shown).
FIG. 2. Liver transduction by HC-Ad/RUmIL-12 in WHV-infected woodchucks and analysis of IL-12’s immunostimulatory activity. Two woodchucks with chronic WHV infection received HC-Ad/RUmIL-12 by injection into the major liver lobe (L3). Two weeks later, mIL-12 was induced for five consecutive days. (A) Serum mIL-12 levels were measured by ELISA before induction (day 0) and at days 1 and 5 following RU486 administration. (B) The woodchucks were sacrificed 10 h after the last RU486 administration, and different organs were dissected from the two animals as detailed in Materials and Methods. Three samples from each organ were obtained, and each sample was processed individually. Tissue homogenates were prepared from different liver lobules (L1, L2, L3, L4, and L5) and various organs (S, spleen; Lu, lung; H, heart; K, kidney). The mIL-12 concentration was determined by ELISA, and the total protein concentration was determined using the Bradford assay. Samples in both assays were evaluated in triplicate. The values represent the means ± standard deviations (SD) of the data obtained from the three samples of each tissue from woodchucks no. 350 (w350) and 813 (w813). (C and D) Total RNA was extracted from the different samples, and the levels of mIL-12 (C), woodchuck IFN-γ (D), and woodchuck (w) β-actin transcripts were quantified by RT-qPCR in triplicate. The values represent the means ± SD of the data obtained from the three samples of each tissue from woodchucks no. 350 (w350) and 813 (w813). (C and D) Total RNA was extracted from the different samples, and the levels of mIL-12 (C), woodchuck IFN-γ (D), and woodchuck (w) β-actin transcripts were quantified by RT-qPCR in triplicate. The values represent the means ± SD of the data obtained from the three samples of each tissue from woodchucks no. 350 (w350) and 813 (w813). (C and D) Total RNA was extracted from the different samples, and the levels of mIL-12 (C), woodchuck IFN-γ (D), and woodchuck (w) β-actin transcripts were quantified by RT-qPCR in triplicate. The values represent the means ± SD of the data obtained from the three samples of each tissue from woodchucks no. 350 (w350) and 813 (w813). (E and F) Liver sections were stained with hematoxylin-eosin (E) or for CD3 (F) before treatment and after mIL-12 induction. The numbers of CD3-positive T cells per 100 hepatocytes ± SD are shown.
IL-12 treatment reduces liver inflammation in responder animals. Liver biopsy samples from treated woodchucks were analyzed in a blinded manner by an experienced pathologist. The responder group showed an improvement in the Knodell score (P value < 0.063), while nonresponding animals with high viremia did not (Fig. 5).

Intrahepatic expression of IL-12 activates the antiviral immune response. The T-cell response to WHcAg was tested monthly by measuring the production of woodchuck IL-2 from PBMCs stimulated with WHcAg (2). None of the animals showed an antigen-specific T-cell response prior to treatment (Fig. 6A and B). Following induction of mIL-12 expression, all woodchucks with low viremia developed a positive T-cell response to WHV core antigen either during the mIL-12 induction period or shortly thereafter. Importantly, this development of a T-cell response paralleled the drop in viremia levels. In contrast, only one of the woodchucks from the high-viremia group gave a positive T-cell response to WHV core antigen after the IL-12 induction period, and after cessation of RU486 administration.

Characterization of woodchuck regulatory T cells. Antiviral T-cell responses are inhibited by poorly understood mechanisms in chronic HBV carriers. Tregs have been shown to be expanded in chronic HBV carriers and can inhibit specific antiviral T-cell responses (26, 27). In mice, the transcription factor Foxp3 is a key marker for regulatory T cells (10). However, in humans, Foxp3 can also be expressed by activated CD4 and CD8 T cells (23). Thus, we tested whether woodchuck T cells expressing Foxp3 have regulatory activity. For this purpose, we purified CD25+ cells from woodchuck PBMCs by magnetic separation. As shown in Fig. 7A, 60% of woodchuck CD25+ cells expressed Foxp3. Furthermore, after α-CD3 stimulation, the Foxp3+ enriched T-cell fraction produced TGF-β, but not IFN-γ, while PBMCs expressed both cytokines (Fig. 6B). Next, we analyzed the regulatory activity of the Foxp3+ enriched T-cell fraction. For this purpose, CFSE-labeled CD25+ cells were stimulated with plate-bound α-CD3 in the presence or in the absence of CD25+ cells. As shown in Fig. 7C and D, the presence of CD25+ cells inhibits the proliferation of α-CD3-stimulated CD25+ cells.

Intrahepatic expression of IL-12 was associated with a reduction of Foxp3 levels in the livers of the chronic WHV carriers that responded to therapy. After confirming the regulatory activity of the Foxp3+ enriched T-cell fraction, we analyzed the expression of Foxp3 in the livers of 10 healthy woodchucks, 10 woodchucks with chronic WHV infection, and 5 woodchucks that had resolved acute WHV infection between 3 and 5 years before the study. Foxp3 values were significantly increased in woodchucks with chronic WHV infection compared to uninfected woodchucks and compared to animals that cleared the virus after acute infection (Fig. 7E). We observed a tendency for hepatic Foxp3 values to decrease during the IL-12 induction period with respect to pretreatment levels in
all animals subjected to IL-12 gene therapy. Interestingly, Foxp3 expression continued to decrease after the discontinuation of IL-12 induction in woodchucks that responded to treatment, while there was a marked increase of Foxp3 expression in animals that failed to respond to therapy (Fig. 7F).

In vitro responsiveness of PBMCs to rIL-12 depends upon the viral load. Most of the immunostimulatory effects of IL-12 are mediated by IFN-γ (29). Thus, to further characterize the mechanisms underlying the lack of a response to mIL-12 in woodchucks with high viremia, we tested the effect of this cytokine on IFN-γ induction in PBMCs from healthy and WHV-infected woodchucks. PBMCs obtained from healthy woodchucks (n = 3), woodchucks with viremia levels below $10^{10}$ vg/ml (n = 3), and woodchucks with viremia levels above $10^{10}$ vg/ml (n = 6) were incubated with mIL-12 at a concentration of 1 ng/ml. After 24 h, the cells were harvested, total RNA was extracted, and IFN-γ expression was analyzed by RT-qPCR. Interestingly, we found that while IFN-γ was clearly induced upon mIL-12 stimulation in PBMCs from healthy woodchucks, there was no IFN-γ response to mIL-12 in animals with viremia levels above $10^{10}$ vg/ml (Fig. 8). These data reveal that the blockade of IFN-γ induction may be an important factor in the resistance to IL-12-based therapy.

Toxicity of HC-Ad/RUmIL-12-based therapy. To evaluate the possible toxicity associated with mIL-12 induction, blood samples from woodchucks treated with HC-Ad/RUmIL-12 were obtained weekly between days 24 and 100 of the study. Slight increases of serum bilirubin, transaminases, and LDH shortly after vector injection were observed, followed by a...
and LDH levels after the end of the IL-12 induction period (Fig. 9A and B). All parameters returned to normal values by day 100. In addition to the changes in liver enzymes, we also observed a moderate increase of platelets and leukocytes (both neutrophils and lymphocytes) during the mIL-12 induction period; these values returned to pretreatment levels by day 100 (Fig. 9C and D). Changes in serum transaminases, bilirubin, LDH, and blood cell counts were similar in both responders and nonresponders to therapy.

**DISCUSSION**

Ideally, a therapy for chronic HBV infection would activate antiviral immunity in order to promote seroconversion and induce efficient and long-lasting control of viral replication. In vitro, IL-12 has been reported to restore the hyporesponsiveness of T cells to viral antigens from patients with chronic hepatitis B (15, 22, 31). It has also been shown that an increase of IL-12 precedes seroconversion to anti-HBe, and therapy with rIL-12 can reduce the viral load in chronic HBV infection (7, 21, 22). However, the use of rIL-12 is limited by IFN-γ-mediated toxicity when IL-12 is given intravenously (7, 21).

The induction of IL-12 expression within the liver is an attractive therapeutic strategy because it makes it possible to achieve higher concentrations of the cytokine in the diseased organ rather than in the systemic circulation, thus increasing the therapeutic window (30). In order to transduce liver cells, we employed HC-Ad because these vectors allow long-term transgene expression, have low toxicity, and possess a high cloning capacity that permits the introduction of regulatory sequences to control transgene expression (13). We used mIL-12 as a transgene, since this murine cytokine induced IFN-γ expression in woodchuck lymphocytes similarly to woodchuck IL-12, and it has the advantage that it can be detected and quantified using commercial tools (data not shown). In our study, intrahepatic administration of HC-AdRU/mIL-12 was well tolerated and allowed higher levels of both IL-12 and IFN-γ to be expressed in the liver than in other organs. In previous work, we showed that intrahepatic injection of adenoviral vectors caused fewer inflammatory reactions than the intravascular route of injection. This is likely because a higher proportion of the vector dose gains direct access to hepatocytes, thus avoiding Kupffer cell filtering (9).

The present work demonstrates that liver-directed mIL-12-based gene therapy in chronic WHV infection induces a marked antiviral effect in the group of animals with pretreatment viremia levels below $10^{10}$ vg/ml, but not in animals with viremia levels above $10^{10}$ vg/ml. The difference in the efficacy of the treatment between the two groups of woodchucks did not depend on the level of transgene expression because the serum mIL-12 concentrations were similar for both groups, although individual variation was noticed (20). Despite the induction of antiviral effects, the serum mIL-12 levels were almost undetectable in some of the treated animals. Thus, intrahepatic mIL-12 expression levels do not parallel circulating levels of the cytokine. In fact, in the two animals depicted in Fig. 1 (woodchucks no. 350 and 813), which were sacrificed during the induction of mIL-12, we observed considerable differences in serum mIL-12 levels while the intrahepatic IL-12 concentrations were similar in the two animals. These findings are reminiscent of observations in patients with renal carcinoma who were treated with rIL-12 (20). In these patients, high variability in serum IL-12 concentrations was detected among subjects receiving similar doses of the cytokine. It was suggested that this variability might be related to individual variation in the expression of IL-12 receptor. This might also explain the variability of serum IL-12 levels in woodchucks that received similar doses of vector and inducer. This hypothesis should be tested in further studies.

Importantly, woodchucks that responded to the therapy and were WHeAg and WHsAg positive prior to IL-12 induction cleared both antigens from the serum and developed specific T-cell responses against WHcAg. In contrast, WHeAg and WHsAg levels persisted in the sera of all nonresponder animals, and a WHcAg-specific antiviral T-cell response was observed in only one woodchuck from this group. These findings indicate that IL-12 gene transfer to the liver is a therapeutic strategy that is capable of breaking the immunotolerance to viral antigens that develops in animals infected during the neonatal period of life.
The relationship between the antiviral effect of IL-12 gene therapy and the level of viremia is in agreement with observations in patients with chronic hepatitis B. Boni et al. have demonstrated that in HBV-infected patients with low viremia, the reservoir of CD8+ cells present in the peripheral circulation was able to expand after specific virus recognition, whereas such expansion did not occur in highly viremic patients (4, 6). Similar data have emerged from other studies showing that woodchucks with chronic WHV infection were able to mount a strong antigen-specific immune response following treatment with HC-Ad/RU486 plus RU486.
lowing vaccination only after prolonged viremia reduction with potent antiviral drugs given for several months (19).

Interestingly, we found that PBMCs from woodchucks with high viremia levels showed an impairment in the induction of IFN-γ following stimulation with IL-12, while this alteration was not observed in animals with low viremia levels. Although the molecular mechanisms responsible for this defect clearly deserve further investigation, it must be noted that this alteration has also been detected in patients with chronic hepatitis B. The presence of large amounts of HBV DNA in serum is associated with suppressed costimulatory effects of IL-12 on HBV-induced immune responses (24). The elucidation of the mechanism responsible for the IL-12 unresponsiveness will be of great value for the development of more efficient therapies based on the gene delivery of immunostimulatory cytokines.

Recently, chronic HBV infection has been shown to be associated with an expansion of Tregs in the peripheral blood (7, 26, 27). This cell population mediates peripheral immunotolerance and can inhibit specific antiviral immune responses to HBV (26). Here, we have shown in woodchucks that the

FIG. 8. Activation of woodchuck PBMCs by rmIL-12. Freshly isolated PBMCs from healthy woodchucks (n = 3) and from woodchucks with low (n = 3) and high (n = 5) viremia were incubated with or without mL-12 (1 ng/ml) for 48 h in triplicate. Then, woodchuck IFN-γ and β-actin mRNA levels were quantified by RT-qPCR. The mean values of triplicates are indicated. The analysis was repeated at least three times with similar results.

FIG. 9. Biochemical and hematology data from HC-Ad/RUmIL-12-treated animals. AST, ALT, LDH, and bilirubin were measured before and after HC-Ad/RUmIL-12 injection and during and after IL-12 induction. (A) We observed a small increase in serum transaminases after virus injection and during the IL-12 expression period. These levels returned to normal after cessation of IL-12 expression. (B) Bilirubin elevation was observed after virus injection and during the IL-12 induction period, while LDH increased only after virus injection. (C and D) Platelet (C) and leukocyte, lymphocyte, and neutrophil (D) numbers were measured in all woodchucks treated with HC-Ad/RUmIL-12 plus RU486. Day zero is the day of HC-Ad/RUmIL-12 injection. IL-12 expression induction was initiated at day 14 during 42 days as described in the text. The error bars represent standard deviations. V.i., virus injection; Ind., Induction.
Foxp3⁺-enriched T-cell fraction has immunosuppressive functions, produces large amounts of TGF-β, and inhibits the proliferation of anti-CD3 activated T cells (Fig. 5B to D). Furthermore, as previously described in humans, we demonstrated that chronic WHV infection is characterized by high levels of Foxp3 expression in the liver (Fig. 5E). Interestingly, we observed a tendency for Foxp3 values to decrease during the induction of IL-12 in all animals. After interruption of the therapy, hepatic Foxp3 expression almost disappeared in responder animals, while Foxp3 was markedly upregulated in nonresponder woodchucks. This result suggests a reexpansion of Tregs in the liver in this group of animals. Our data do not allow us to discriminate whether the decrease of Foxp3 expression observed in the livers of responder woodchucks is merely a consequence of the reduction in viral load or is due to a specific effect of IL-12 on the T reg population.

The antiviral effect mediated by IL-12 gene therapy was associated with only a modest elevation of liver enzymes, indicating that the control of WHV infection is mainly carried out by a noncytolytic, cytokine-mediated mechanism, as previously demonstrated in HBV-transgenic mice and HBV-infected chimpanzees (11). Such a mechanism appears to be capable of reducing or eliminating viremia and clearing WHV from the livers of the responder animals. Interestingly, we observed an improvement of liver histology in those animals that showed a positive antiviral response to therapy.

Chronic WHV infection is associated with a greater viral load than chronic HBV infection. In chronic hepatitis B, patients with high viremia have HBV DNA concentrations that range between 10⁷ and 10⁸ vg/ml. It is noteworthy that all WHV loads were lower than chronic HBV infection. In chronic hepatitis B, patients who showed a positive antiviral response to therapy.

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ERRATUM

Treatment of Chronic Viral Hepatitis in Woodchucks by Prolonged Intrahepatic Expression of Interleukin-12

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