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Therapeutic vaccination of woodchucks against chronic woodchuck hepatitis virus infection

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Background/Aims: **Therapeutic vaccination is a new approach to treat patients with chronic hepatitis B virus infection. We have used the woodchuck model to examine the efficacy and safety of this approach.**
Methods: **Seven woodchucks chronically infected with woodchuck hepatitis virus were immunized with surface antigen from this virus, purified from plasma, in conjunction with a peptide named FIS (encompassing amino acids 106–118: FISEAIIHVLHSR from sperm whale myoglobin), which is recognized by T helper lymphocytes. As controls, two woodchucks chronically infected with woodchuck hepatitis virus were immunized: one with FIS only and the other with surface antigen only.**
Results: **Co-immunization with surface antigen and FIS, but not with FIS or surface antigen alone, induced anti-surface antibodies in 7/7 immunized woodchucks. In the two woodchucks in which the highest titer of anti-surface antibody was elicited, severe liver damage was observed: one died of fulminant hepatitis and the other became seriously ill with hepatic injury and had to be sacrificed.**
Conclusions: **Co-immunization of chronically infected woodchucks with surface antigen and a peptide recognized by T helper cells produces a good anti-surface antibody response. However, this strategy needs to be optimized before its implementation in humans. Although our experiments are not strictly comparable to vaccination of chronically hepatitis B virus-infected patients with recombinant or plasma-derived vaccines, we believe that precautions should be taken to avoid the risk of severe liver injury when immunizing hepatitis B virus carriers.**

Key words: **Antibodies; Hepatitis; Immune therapy; Peptides; T-cell help; Vaccination; Woodchuck; Woodchuck hepatitis virus (WHV); Woodchuck hepatitis virus surface antigen (WHsAg).**

Chronic hepatitis B virus (HBV) infection is characterized by a poor humoral and cellular immune response against HBV antigens. It has been suggested that this lack of response may be associated with a reduced T helper response to antigens of HBV (1–3). At least three groups have attempted to vaccinate chronically HBV-infected patients to induce antibodies against HBV surface antigen (HBsAg) in order to eliminate the chronic infection. The vaccines used were plasma-derived HBsAg (4,5) or recombinant HBsAg containing preS2 sequences (6). In the study by Dienstag et al. (4) there was no evidence that HBV vaccine was effective in eliminating HBV, despite one of 10 HBV "e" antigen-positive carriers losing this antigen and despite decreased ALT activity in half the participants. By contrast, Pol et al. (6) reported that vaccination of chronic carriers with HBsAg containing the preS2 region had the effect of lowering HBV replication (in 28.6% of patients) and decreasing the HBV DNA to undetectable levels (in 21.4% of patients). Wen et al. (5) immunized patients with chronic hepatitis B infection, with hepatitis B vaccine complexed to human hepatitis B immunoglobulin. They found that 9/14 patients became negative for serum HBV DNA after three immunizations with the complex. All groups reported that vaccination appeared to be safe, with no side-effects, and no anti-HBs antibodies were detected after immunization.

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Our laboratory has made attempts to develop strategies to induce humoral (7–9) and cytotoxic (10,11) responses using synthetic peptides. We have shown (9) that SJL/J mice, normally non-responders to HBsAg, can be rendered responders by joint immunization with HBsAg and a T helper cell determinant peptide (TDh) encompassing residues 106–118 from sperm whale myoglobin (from now on designated FIS). This result encouraged us to explore this strategy of joint immunization in the woodchuck model to attempt to eliminate woodchuck hepatitis virus (WHV) chronic infection as a preliminary step to its implementation as therapeutic vaccination in HBV chronic carriers. Woodchucks, when infected with WHV, develop chronic hepatitis that closely resembles chronic HBV infection in humans. Also, infection with WHV, like HBV, carries a significant risk of hepatocellular carcinoma (12,13). Both viruses belong to the hepadnavirus family with a very similar genome organization coding for proteins of high homology (14). Viremia of WHV in the woodchuck reaches high levels (10^9 particles/ml) similar to those observed in HBV-infected patients (15,16).

In this paper we demonstrate that immunization of chronically infected woodchucks with WHV surface antigen (WHsAg) and FIS can induce anti-WHs antibodies and in some instances may be associated with liver injury.

Materials and Methods

Animals

A total of 10 woodchucks were used in the present study. Four 4–5-year-old woodchucks (310, 90-5, 449 and 67) chronically infected with WHV (WHsAg(+), WHV DNA(+), anti-WHs(−)) born in captivity and housed at the animal facilities at the University of Essen (Germany), were used in the first experiment. In a second experiment we used one non-infected (1D) and five chronically infected (1D2I, 845, 848, 079 and 342) woodchucks 2–3 years old; they were born in the wild and were purchased from North Eastern Wildlife (Ithaca, NY, USA). All animals were treated according to the guidelines from our institution (CIFA, Pamplona, Spain).

Peptide synthesis

Peptides were synthesized by the solid-phase method of Merrifield (17) using the Fmoc alternative (18). Peptide $HBs_{120-134}$ (MQWNSTAFHQTLQDP) encompassing residues 120–134 from HBsAg was synthesized using a multiple solid phase peptide synthesizer (19). Peptide FIS (FISEAIIHVLHSR) encompassing residues 106–118 from sperm whale myoglobin was done manually and the ninhydrin test of Kaiser et al. (20) used to monitor every step. FIS was purified by high performance liquid chromatography (HPLC) on a reverse phase C18 column.

Purification of WHsAg

WHsAg was purified according to Gerin et al. (21) with minor modifications. Basically, the antigen was isolated from the serum of a chronically WHV-infected woodchuck by zonal centrifugation on a sucrose gradient followed by isopicnic centrifugation in a gradient of CsCl. The peak of WHsAg was contained in fractions with a density of 1.19–1.22 g/ml and was identified by SDS polyacrylamide gel electrophoresis and silver staining. Those fractions containing the antigen were pooled and dialyzed against 20 mM Tris pH 7.4, 140 mM NaCl, 1 mM EDTA, and quantified using the kit Bio-Rad protein assay (Bio-Rad, Richmond, CA, USA).

Preparation of antibodies to WHsAg

Anti-WHs antibodies were induced by intradermal immunization of New Zealand white male rabbits with 100 μg of WHsAg emulsified in complete Freund's adjuvant. Rabbits were boosted subcutaneously with the same dose of antigen in incomplete Freund's adjuvant at day 30 after the first immunization. Immunoglobulins containing anti-WHs antibodies were isolated from the sera at day 45 by chromatography on a column of protein A superose HR 10/2 (Pharmacia, Uppsala, Sweden). The column was equilibrated by washing with 8 ml of distilled water followed by 3.2 ml of 0.1 M Tris pH 8.0. One ml of serum, with an anti-WHs antibody titer of 1:10 500 was centrifuged at 10 000 *g* for 10 min and the supernatant passed through a 0.22 μm filter (Micron Separations Inc., Westboro, MA, USA). The filtrate (0.9 ml) was mixed with 0.1 ml of 1 M Tris pH 8.0 and injected into the column. The column was then washed with 16 ml of 0.1 M Tris pH 8.0 at a flow rate of 0.5 ml/min for 4 min and at 2 ml/min for 7 min, followed by another wash with 16 ml of 0.01 M Tris pH 8.0 at 2 ml/min. The IgGs were eluted with 16 ml of 0.1 M glycine-HCl pH 3.0 (2 ml at 0.5 ml/min followed by 14 ml at 2 ml/min). The peak of IgGs was collected, quickly neutralized with 1 M Tris pH 8.0 and dialyzed against 0.1 M carbonate/bicarbonate buffer pH 9.8. This afforded 7.7 mg of IgG at a concentration of 3.8 mg/ml and was concentrated to 8.5 mg/ml by centrifugation at 10 000 *g* for 60 min using a centrifuge tube with a 10 K filter (Filtron Technology Corp., Northborough, MA, USA).

Labeling of anti-WHs antibodies with peroxidase

Labeling of anti-WHs antibodies was carried out by reacting IgGs in 0.1 M carbonate/bicarbonate buffer

pH 9.8, with a 5 molar excess of activated horseradish peroxidase (HRPO) according to manufacturer's instructions (Boehringer Mannheim GmbH, Mannheim, Germany). Briefly, 300 μl of IgGs (8.5 mg/ml) were reacted with 100 μl of HRPO (16 mg/ml) for 2 h at 25°C and 12 h at 4°C. The reaction was stopped with 40 μl of 2 M triethanolamine solution (pH 8.0). Freshly made 0.2 M solution of sodium borohydrate (50 μl) was added and incubated for 30 min at 4°C. Then, 25 μl of 2 M triethanolamine (pH 8.0) was added and incubated for a further 2 h. To stabilize the conjugate, 10 μl of 1 M glycine-NaOH solution pH 7.0 were added and dialyzed against saline phosphate pH 7.4 containing 0.01 M glycine. After 16 h dialysis at 4°C, bovine serum albumin and Kathon CG (Rohm & Haas, Frankfurt/Main, Germany) were added to a final concentration of 10 mg/ml and 1 mg/ml, respectively. The final solution was kept in small aliquots, which were quickly frozen in liquid nitrogen and stored at −80°C for further use.

Detection of WHsAg in sera

Wells from ELISA plates (Nunc Maxisorp, Roskilde, Denmark) were coated at 4°C overnight, with 50 μl of 1/1500 dilution of a stock serum of anti-WHs antibodies (3.8 mg/ml) in 0.1 M sodium carbonate pH 10.5. After three washes with phosphate buffered saline containing 1% Tween 20 (PBST), the plates were incubated at 37°C for 1 h with PBSMT (PBST with 1% powdered milk) supplemented with 2% serum from woodchucks negative for markers of WHV infection. Following removal of this solution, 50 μl per well of serum dilutions in PBSMT were added and incubated at 37°C for 1 h. The wells were then washed three times with PBST, and incubated at 37°C for another hour with 50 μl of a 1/1000 dilution of a 4.25 mg/ml stock solution of anti-WHs-HRPO in PBSMT, containing 2% serum from a non-infected woodchuck (PBSMTW). This PBSMTW dilution had been previously incubated for 1 h at 37°C to block antibodies against woodchuck serum proteins that might contaminate the anti-WHs-HRPO preparation. After three washes with PBST, the color reaction was started by adding 100 μl/well of a solution prepared by mixing 10 ml 0.6% acetic acid (pH 4.7), 7.5 μl of 33% (w/v) hydrogen peroxide and 100 μl of 45 mM aqueous solution of ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid). After 1 h, the OD at 405 nm was read. The amount of WHsAg in the sera (in μg/ml) was calculated by comparison against a standard curve obtained with known concentrations of WHsAg. In this assay, the determined standard deviations of the means were less than 10% (range 8–11%).

Immunization experiments

Woodchucks (non-infected, as well as chronically infected with WHV) were immunized intradermally in 10 different sites with 100 μl of immunization mixture per site. This immunization mixture was obtained by sonication of 0.5 ml of complete Freund's adjuvant, and 0.5 ml of 0.1 M sodium carbonate solution containing the TDh peptide and WHsAg isolated from the serum of a chronically infected woodchuck (see above). In a first experiment (Fig. 1), this emulsion was made with 600 μg of either FIS or $HBs_{120-134}$, and 200 μg or 100 μg of WHsAg. In the following experiments (Fig. 2) we used 200 μg of FIS and 50 μg of WHsAg. As control, two chronically infected animals were immunized respectively with FIS (200 μg) or with WHsAg (50 μg) only. Three booster injections with the same dose of antigens, in incomplete Freund's adjuvant, were given as indicated in Fig. 1 and 2. The first boost was given by subcutaneous injection and the remaining two by intramuscular injection. Woodchucks were bled from the femoral vein under sedation (25 mg/kg Imalgene 500 (Rhone Mérieux, Lyon, France) and 2.5 mg/kg Rompun (Bayer, Leverkusen, Germany)). Blood was allowed to clot at 37°C for 1 h, and sera were collected by centrifugation at 8000 *g* for 2 min and stored at −80°C for further use.

IL-2 production assay

Peripheral blood mononuclear cells (PBMC) were isolated from fresh EDTA-treated blood (4–5 ml) by Ficoll-Hypaque (Nycomed Pharma AS, Oslo, Norway), washed three times, counted, and resuspended at 4×10^6 cells/ml in complete medium (RPMI-1640, 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2×10^{-5} M β-mercaptoethanol). If necessary, remaining red cells were lysed by incubation at room temperature for 5 min with 5 ml of erythrocyte lysis buffer (0.15 M ammonium chloride, 1 mM potassium bicarbonate, 0.1 mM EDTA (pH 7.2–7.4)). Then, 15 ml of 0.9% NaCl aqueous solution was added to the incubation mixture and washed twice with the same solution. PBMC were taken after the third (woodchuck 848) or fourth immunization (woodchucks 1D, 1D2I and 845).

Cells (4×10^5 cells/ well in 0.1 ml) were added in triplicate to a 96-well flat-bottomed plate containing 100 μl of antigen solution and cultured at 37°C and 5% CO_2 for 24 h (WHsAg was tested at 2, 10 and 100 μg/ml, and FIS was tested at 10 and 50 μg/ml). Concanavalin A (20 μg/ml) was used as a positive control. Supernatants (50 μl) were removed and tested for their IL-2 content using the CTL-L bioassay as already described (22). Briefly, CTL-L cells were resuspended in RPMI-

1640 with 10% fetal calf serum, antibiotics and 2-mercaptoethanol (5×10^{-5} M), and were plated (8×10^3 cells/well) in a 96-well flat-bottomed plate with the supernatant to be assayed (25% v/v). After 24 h of culture, the stimulated CTL-L cells were pulsed with 1 μCi/well of [^{3}H]thymidine for 18 h and thymidine incorporation was determined by liquid scintillation. Results are expressed as stimulation index (SI), which is the ratio of the mean counts $\cdot$ min^{-1} incorporated in the presence of antigen to the mean counts $\cdot$ min^{-1} obtained in the absence of antigen.

Determination of anti-WHs and anti-peptide antibodies
Antibodies were titrated by ELISA. Microtiter wells were coated by overnight incubation at 4°C with either 50 μl of peptide solutions (20 μg/ml) or WHsAg (2 μg/ml) in 0.1 M sodium carbonate buffer. Wells were then washed three times with a solution of PBST. To block non-specific antibody binding, the wells were incubated at room temperature for 1 h with 400 μl/well of the above buffer, but containing 1% powdered milk and 0.1% Tween 20 (PBSMT). After removing the PBSMT, 50 μl of different serum dilutions in PBSMT were added and incubated at 37°C for 1 h. Wells were washed three times with PBST and then incubated at 37°C for 1 h with 50 μl of a solution in PBSMT of Protein A-peroxidase (1 μg/ml) (Prot A-HRPO, Sigma Chemical Co., St. Louis, MO, USA). To measure anti-WHs antibody titers in rabbit sera, the plates were incubated with 50 μl/well of a 1:12 800 dilution of anti-rabbit IgG labeled with HRPO (Amersham International plc., Amersham, UK). After washing three times with PBST, the color reaction was carried out as described for the detection of WHsAg in the sera. Antibody titers correspond to the highest serum dilution to yield three times the absorbance of a negative serum and were expressed as the reciprocal value of the dilution. In the determination of anti-WHs antibody titers, the determined standard deviations of the means were less than 18% (range 13–22%). In the determination of anti-FIS antibody titers, the determined standard deviations of the means were less than 13% (range 12–14%).

Levels of γ-GTP and bilirubin
γ-GTP and bilirubin were measured as previously reported (23,24) but using the autoanalyzer Hitachi 717 (Hitachi Ltd., Tokyo, Japan).

Determination of WHV DNA levels
By dot blot. The level of WHV DNA in the sera was measured by dot blot hybridization, using a plasmid (pBr325WHS) containing the entire WHV genome (WC8 clone) as the DNA probe (American Type Culture Collection). Dot blot and DNA probe labeling were performed following standard protocols (25). Basically, the sera were diluted several times in H_2O and 5 μl from the undiluted sera, or from each dilution, was spotted onto a positively charged nylon membrane (Boehringer Mannheim GmbH, Mannheim, Germany). Denaturation of WHV DNA was performed by treating the membrane with a solution containing 1 M NaCl and 0.1 M NaOH and then with another solution composed of 1 M NaCl and 0.1 M Tris-HCl (pH 7.5). Finally, the DNA was fixed to the membrane by UV crosslinking. Before hybridization, the membrane was incubated at 42°C with a hybridization solution composed of formamide (50%) (v/v), 5×SSC (0.75 M NaCl, 0.085 M Na-citrate, pH 7.0); N-lauroylsarcosine (0.1%) (w/v), SDS (0.02%) (w/v), sperm salmon DNA (0.5 mg/ml) and blocking reagent (5%) (Boehringer Mannheim GmbH, Mannheim, Germany). After 4 h, 50 ng/membrane of labeled DNA probe were added to the hybridization solution and the membrane was reincubated for another 15 h at 42°C. The DNA probe was previously labeled with digoxigenin (Dig-DNA probe) by random primer labeling. To eliminate non-specific binding of Dig-DNA probe, the membrane was washed 2×15 min at 42°C with 2×SSC, SDS (0.1%) (w/v) and 2×15 min at 68°C with 0.1×SSC, SDS (0.1%) (w/v). After hybridization, the membrane was blocked with a solution composed of 0.15 M NaCl, 0.1 M Tris (pH 7.5) and 0.5% blocking reagent from Boehringer Mannheim. The hybrids formed by WHV DNA and Dig-DNA probe were detected according to Bronstein (26) with minor modifications, using an anti-digoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim GmbH, Mannheim, Germany) and Lumigen PPD (Lumigen, Inc., Detroit, MI, USA) as chemiluminescent substrate. The light emitted by decomposition of the substrate was documented on a Kodak X-OMAT film. The density of the spot was measured by Scan Analysis (Apple Computer Inc., Cupertino, CA, USA). The amount of WHV DNA in each spot was expressed in ng/ml by comparison against a calibration curve obtained using solutions of WHV DNA of known concentration. In the determination of WHV DNA levels, the determined standard deviations of the means were less than 18%.

By competitive PCR. Construction of the competitive template: Plasmid pBR325WHS containing the WHV whole genome was digested with AvrII and XbaI enzymes and ligated to generate the plasmid pBR325WHSdel. This plasmid has a deletion of 69 bp between positions 312 and 381 of WHV genome.

Serum DNA extraction: 50 μl of woodchuck serum

were incubated with SDS and protein kinase at 65°C for 1 h. DNA was isolated by fenol:chloroform extraction followed by ethanol precipitation. The resultant DNA pellet was dissolved in 40 μl of distilled water.

Competitive PCR: The method used is similar to the one previously reported for the absolute quantitation of viremia in human immunodeficiency virus infection (27). Four reaction mixtures were analyzed: each mixture contained a constant amount of extracted serum DNA (5 μl) but increasing copy numbers of DNA competitor molecules (1.55×10^8, 1.55×10^7, 1.55×10^6 and 1.55×10^5 copies/tube, respectively). These numbers of competitor molecules were chosen because they cover the range of viral DNA concentrations normally found in the sera of infected woodchucks. Competitive PCR was done in 40 μl (final volume) containing the following: 5 pmol of WJL1 (TAAGCCCTGTGGTTCCTACT) and 5 pmol of WJL2 (ACCTACATAGACGCCGCAAA) primers, 0.2 mM deoxynucleotides, 2.5 mM magnesium chloride, 20 mM Tris-HCl (pH 8.55), 16 mM $(NH_4)_2SO_4$ and 1.5 units of Taq polymerase (Biotaq, Bioprobe System, Paris, France). This solution was subjected to 35 cycles of 15 s at 95°C, 12 s at 52°C and 20 s at 72°C with an extension of 1 min at 72°C. A Perkin Elmer GeneAmp PCR System 9600 (Norwalk, CT, USA) was used. Amplified cDNA was electrophoresed through 2.5% agarose and visualized by UV after ethidium bromide staining. Gels were scanned with a video documentation system (GelDoc 1000, Bio-Rad Laboratories, Hercules, CA, USA) by positive fluorescent emission on the transilluminator using the Molecular Analyst Software (Bio-Rad Laboratories, Hercules, CA, USA). Thus, the area of the peak corresponding to the wild type band sequence (WA) and that corresponding to the deleted competitor (DA) were used to quantify the viremia of the serum samples. DA was corrected for its lower molar ethidium bromide incorporation as follows: $DAc=DA\times1.277$, where 1.277 is the ratio between the number of bp from the wild type band sequence and the number of bp from the deleted competitor. The DAc/WA ratio was calculated for each sample and plotted on the Y axis against the copy number/ml of the deleted competitor. The copy number/ml of the wild type template was calculated from the curve expression $DAc/WA=1$ and converted to ng/ml by dividing by the factor 1.55×10^7 (number of copies contained in 1 ng of WHV DNA).

Liver biopsies

Liver biopsies were taken by puncture with a biopsy needle Tru-Cut 18 Ga UTW×7.6 cm (Baxter Healthcare Corporation, Valencia, CA, USA) at times indicated in the *Results* section. Biopsies were fixed in neutral buffered formaldehyde, embedded in paraffin and sectioned at 6 μm thickness. Tissue sections were stained with hematoxylin and eosin, Masson's Trichrome and Periodic Acid Schiff methods. Sections were also examined by immunohistochemistry using a polyclonal rabbit antibody to recombinant WHV core antigen. The WHV core antigen was provided by Dr. Paul Cole, Georgetown University School of Medicine. Primary antibody was used at a 1:400 dilution, secondary antibody was biotin-conjugated goat anti-rabbit (Biogenics, San Ramon, CA, USA) used at 1:200 dilution. Peroxidase conjugated with avidin and diaminobenzidine were used as the chromogen.

Results

In the initial experiment (Fig. 1) we tested our hypothesis by immunizing woodchucks with WHsAg in conjunction with FIS, a TDh peptide from sperm whale myoglobin well recognized by several class II restrictions (7–9,28,29) or with $HBs_{120-134}$, another TDh peptide also described as being recognized by several class II molecules (30). Woodchuck 310 (Fig. 1A) and woodchuck 90-5 (Fig. 1B) were immunized with 100 μg of WHsAg in conjunction with 600 μg of FIS, whereas woodchucks 449 and 67 (Fig. 1C and D, respectively) were immunized with a mixture of 100 μg of WHsAg and 600 μg of $HBs_{120-134}$. Fig. 1 shows that only woodchuck 310, immunized with WHsAg in conjunction with FIS, elicited relevant levels of anti-WHs as well as of anti-FIS antibodies. The highest level of WHsAg in the serum was observed in this animal approximately 3 weeks after the second immunization, whereas that of WHV DNA in the serum was observed after 3 weeks from the first immunization. These levels decreased later below the levels of the 2nd week.

In woodchucks 449, 90-5 and 67 only low levels of anti-WHs antibodies (titers below 70) were elicited. For this reason, woodchucks 449 and 67, which had been immunized with WHsAg in conjunction with peptide $HBs_{120-134}$, were re-immunized at week 25, but this time with WHsAg and FIS emulsified in incomplete Freund's adjuvant. As before, low levels of anti-WHs antibodies were observed (titers below 50). Moreover, re-immunization of woodchuck 90-5 with WHsAg and FIS did not enhance the low levels of anti-WHs antibodies previously induced. After immunization of woodchucks 449, 90-5 and 67 with WHsAg in conjunction with FIS, the levels of anti-FIS antibodies were unmeasurable in woodchuck 67 and low in woodchuck 90-5. Woodchuck 449 showed moderate levels of anti-FIS antibodies but only after 11 weeks following joint immunization with FIS and WHsAg. The level of WHV DNA in the serum of woodchucks 449, 90-5 and

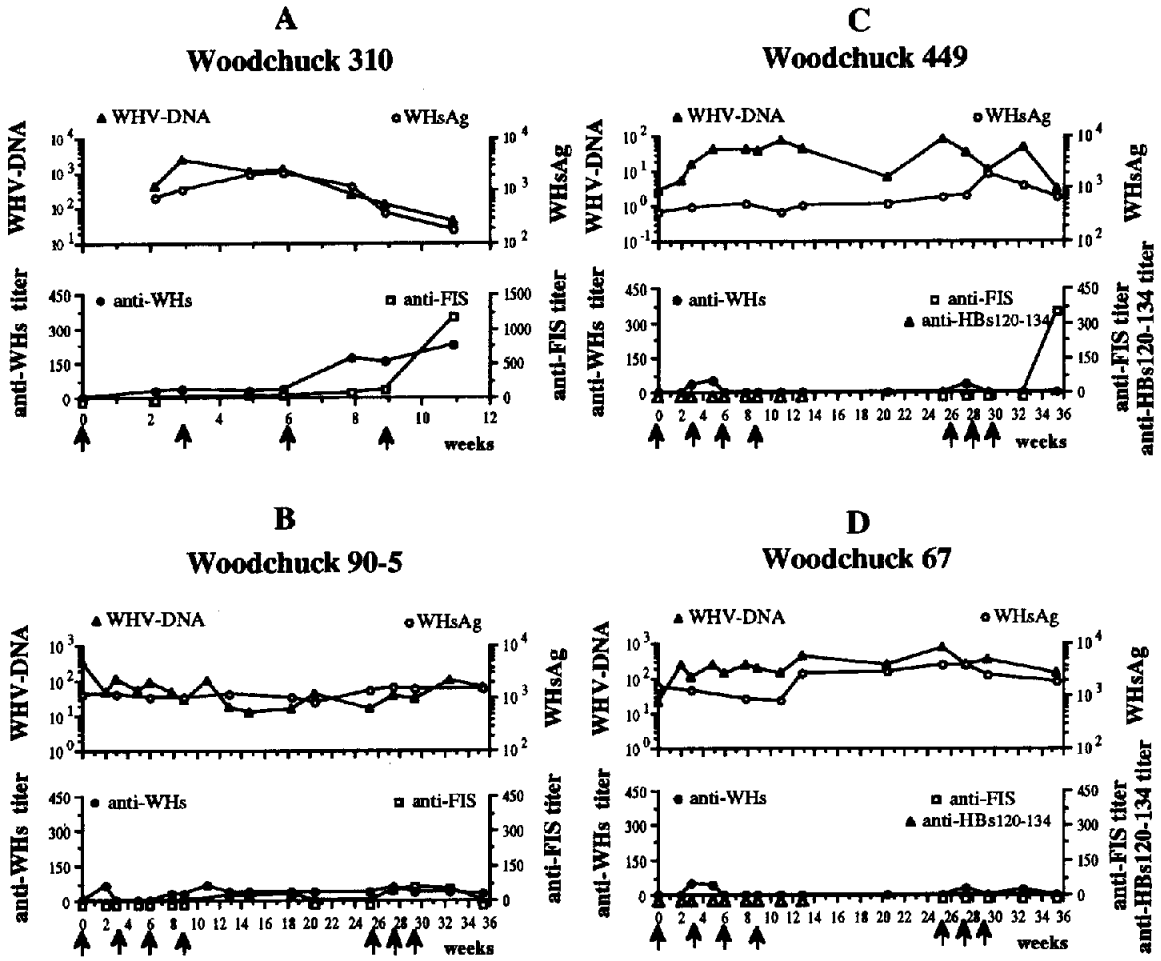

Fig. 1. Immunization of four chronically infected woodchucks with WHsAg in conjunction with a TDh peptide. Woodchucks 310 and 90-5 were immunized at weeks 0, 5, 6 and 9 (indicated by arrows) with 100 μg of WHsAg in conjunction with 600 μg of FIS. At weeks 25, 27 and 29, woodchuck 90-5 was re-immunized with 200 μg of WHsAg and 600 μg of FIS. Woodchucks 449 and 67 were first immunized with 100 μg of WHsAg in conjunction with 600 μg of $HBs_{120-134}$ and at a later stage with 100 μg of WHsAg and 600 μg of FIS. The levels of WHV DNA (ng/ml) and WHsAg (μg/ml) in the sera, as well as the titers of anti-WHs, anti-FIS and anti-$HBs_{120-134}$ antibodies, are shown. In all animals, the levels of WHV DNA were measured by dot blot. For woodchuck 310 the sample from day 0 is not shown because it was accidentally lost. This animal became seriously ill and was sacrificed at week 11 after immunization.

67 was much lower than in woodchuck 310. No clear changes in the level of WHsAg were seen in woodchuck 90-5 during the 36 weeks after the first immunization. By contrast, in woodchucks 449 and 67 a small peak of WHsAg was observed, reaching the highest level at weeks 30 and 27, respectively, after the first immunization. These levels of WHsAg were similar to that observed 6 weeks after the first immunization for woodchuck 310, but in woodchuck 310, WHsAg decreased more rapidly and to lower values. Woodchuck 310 became seriously ill and was sacrificed at week 11, as anti-WHs antibody levels were increasing.

In the second set of experimental vaccinations (Fig. 2) we tested the effect of a lower immunization dose of FIS and WHsAg on the outcome of immunization. This was decided on because in a similar experiment carried out in SJL mice (non-responders to HBsAg), we observed that lower doses of FIS elicited equal or better humoral responses against HBsAg (data not shown). Three chronically WHV-infected woodchucks (1D2I, 845 and 848) as well as one non-infected woodchuck (1D) were immunized with WHsAg (50 μg) in conjunction with FIS (200 μg), emulsified in complete Freund's adjuvant as specified in *Methods*. To test the effects of FIS and WHsAg alone, we immunized chronically WHV-infected woodchuck 079 with 200 μg of FIS only and woodchuck 342 with 50 μg of WHsAg only.

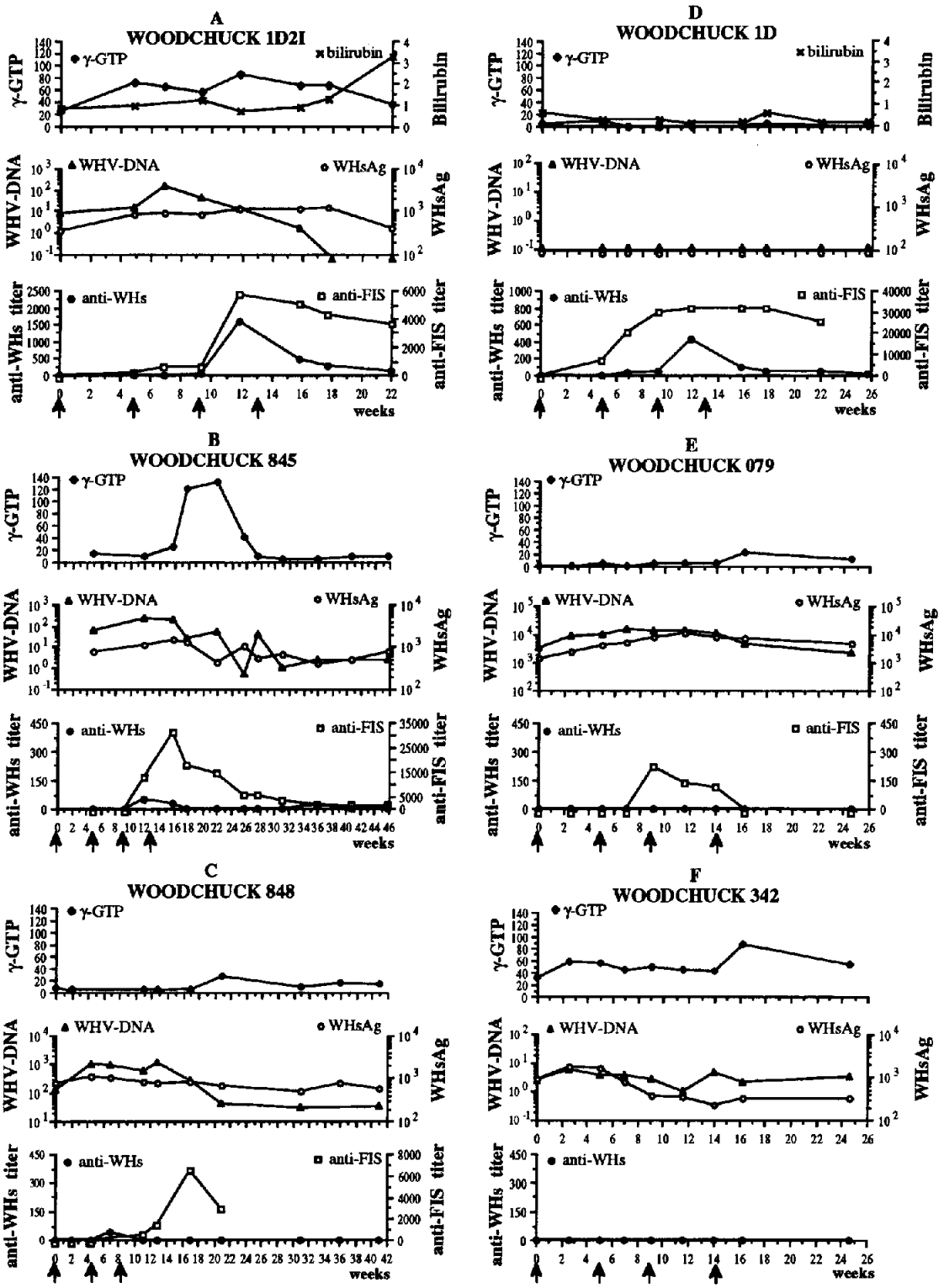

Fig. 2. Immunization of woodchucks with WHsAg (50 μg) in conjunction with FIS (200 μg) (panels A, B, C and D). Immunization with FIS only (200 μg) (panel E) or with WHsAg only (50 μg) (panel F). Immunizations were carried out at the weeks indicated by arrows. Woodchucks 1D2I, 845, 848, 079 and 342 were chronically infected with WHV, whereas 1D was uninfected. The levels of WHV DNA (ng/ml), WHsAg (μg/ml), γ-GTP (IU/l) and bilirubin (mg/dl) in the sera, as well as anti-WHs and anti-FIS antibody titers, are shown. However, for woodchuck 845 the sample from day 0 is not shown because it was accidentally lost. In all animals, the levels of WHV DNA were measured by dot blot, except in woodchuck 342 where competitive PCR had to be used due to the low levels of DNA in this animal. Woodchuck 1D2I died at week 24 of fulminant hepatitis (see text). Levels of 2 IU/l of γ-GTP and 0.5 mg/dl of bilirubin are considered normal.

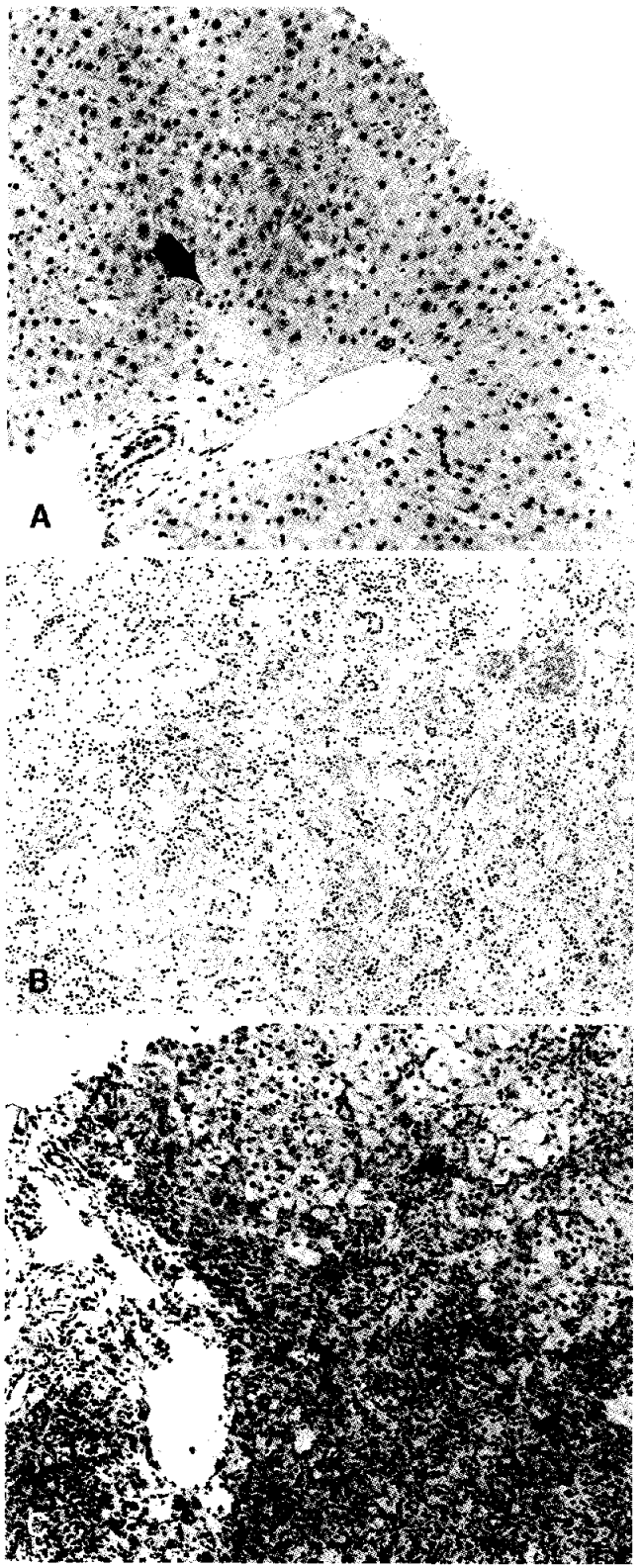

Fig. 3. (A) Liver from woodchuck 1D2I obtained before treatment. The histologic appearance of the liver is essentially normal. There are a few mononuclear inflammatory cells in the portal tract (Arrow). Hepatocytes are normal. (B) Liver from woodchuck 1D2I, which died following treatment. The liver is characterized by massive necrosis of hepatocytes with areas of collapse of parenchyma, focal hemorrhage, moderate biliary hyperplasia and fine strands of collagen deposition extending from portal areas and central veins. The few remaining hepatocytes are distended by lipid vacuoles. These histologic changes are compatible with fulminant hepatic failure. (C) Liver from woodchuck 845 after treatment. This liver is characterized by moderate mononuclear, mostly lymphocytic, infiltrates of the portal tracts that extend into the adjacent parenchyma. Small numbers of neutrophils are also present. Marked biliary proliferation is also evident with cholangioles extending into the parenchyma. Moderate parenchymal collapse with little fibrosis was evident in reticulum and trichrome stains. Periportal hepatocytes are distended by microvesicular and macrovesicular vacuoles that contain glycogen and lipid. Centrilobular hepatocytes are less vacuolated.

Immunization with WHsAg in conjunction with FIS, but not with FIS or WHsAg alone, induced detectable levels of anti-WHs antibodies (Fig. 2). The peak of anti-WHs antibodies was low in woodchucks 845 and 848 (titers below 50). By contrast, woodchucks 1D (non-infected) and 1D2I (chronically infected) showed substantially higher anti-WHs antibody titers. In all animals immunized with WHsAg in conjunction with FIS (Fig. 2A, B, C and D), or with FIS alone (Fig. 2E) anti-FIS antibodies were elicited.

After immunization of chronically WHV-infected woodchucks with WHsAg in conjunction with FIS (Fig. 2A, B, C) or with FIS alone (Fig. 2E) a transient peak of WHV DNA in serum was observed. However, in woodchuck 342 (Fig. 2F) the levels of WHV DNA in the serum could not be reliably measured by dot blot and for this reason they were studied by competitive PCR. No important changes in the levels of WHV DNA were observed. The levels of WHsAg in the sera in all chronically infected woodchucks, with the exception of woodchuck 848 (Fig. 2C), were also affected by immunization (Fig. 2A, B, E and F). These changes were independent of the antigen used in these immunization protocols.

Fluctuations in the levels of γ-GTP were observed after immunization of all chronically infected woodchucks in the second experiment (Fig. 2). However, only woodchuck 845 showed a clear peak of γ-GTP after the fourth immunization (Fig. 2 B). Woodchuck 1D2I developed jaundice. Quantification of bilirubin in the sera of this animal showed slightly increased levels of this marker from the beginning of the experiment, reaching a level of around seven times the normal value at week 22 (Fig. 2 A). This animal died at week 24 of fulminant hepatitis (see below and Fig. 3).

Livers from four chronically WHV-infected animals

(1D2I, 310, 845 and 848) and one non-infected (1D), all of them immunized with WHsAg in conjunction with FIS, were examined histologically. For woodchuck 1D2I, biopsies were obtained 4 months before immunization and 22 weeks after immunization. The initial biopsy was characterized by a light lymphocyte infiltrate of the tracts and occasional lipid-containing vacuoles in hepatocytes (Fig. 3A). WHV core antigen was present, but in low amounts in hepatocyte cytoplasm. The liver obtained at death, following treatment of this animal, was characterized by a variety of lesions. The portal tracts were indistinct because of moderate biliary hyperplasia, mild fibrosis and a moderate lymphocyte infiltrate that bridged portal tracts and central veins (Fig. 3B). Significant hepatocyte necrosis was evident and remaining hepatocytes contained microvesicular and macrovesicular lipid vacuoles. Widespread hemorrhage was present within the parenchyma. Intracanalicular bile plugs were prominent. These changes were compatible with a diagnosis of acute fulminant hepatitis. WHV core antigen was not detected. The liver from woodchuck 310, sacrificed at week 11 due to poor condition, was characterized by widespread panlobular macrovesicular lipid vacuolization of hepatocytes. Lymphocytes and occasional plasma cells and neutrophils were scattered throughout the parenchyma and aggregates of lymphocytes were associated with isolated areas of spotty hepatocyte necrosis. The portal areas were moderately infiltrated with lymphocytes and occasional plasma cells and neutrophils. The infiltrate expanded the portal tract and extended along the terminal branches of the portal vessels. The inflammatory infiltrates penetrated the limiting plate in multiple sites, but extended only a short distance into the parenchyma. Bile ducts were moderately hyperplastic. Kupffer cells appeared prominent. Other sections of liver were characterized by localized areas of infarction due to the presence of large thrombus in a sublobular vein. The liver biopsy from animal 845 obtained at week 24 after immunization showed prominent biliary hyperplasia and contained a prominent inflammatory cell infiltrate of lymphocytes, neutrophils and smaller number of plasma cells. These infiltrates bridged portal tracts (Fig. 3C). The majority of hepatocytes contained microvesicular lipids. Occasionally, small aggregates of sinusoidal lining cells were found around necrotic hepatocytes. WHV core antigen was found in about 40% of hepatocytes. The liver from animal 848 at week 20 after immunization was similar, but less seriously affected. Liver from uninfected animal 1D, obtained 5 months before and 24 weeks after immunization was essentially normal, characterized by light lymphocyte portal infiltrates and occasional vacuolated hepatocytes in both biopsies (data not shown).

TABLE 1

In vitro IL-2 production by peripheral blood mononuclear cells following stimulation with WHsAg or FIS

Woodchuck*	Immunized with	Stimulation index (SI)	
		In vitro stimulation with FIS (10 μg/ml)	*In vitro* stimulation with WHsAg (100 μg/ml)
ID^{ni}	WHsAg+FIS	5.6	1.1
$1D2I^{ci}$	WHsAg+FIS	8.9	0.8
845^{ci}	WHsAg+FIS	15.0	1.7
848^{ci}	WHsAg+FIS	4.7	1.1
079^{ci}	FIS only	109.7	NT
342^{ci}	WHsAg only	NT	1.0

* Non-infected (ni) and chronically infected (ci) woodchucks were immunized with the antigens shown. Production of IL-2 was measured using the CTL-L bioassay. PBMC from these animals were re-stimulated with antigens as shown. Results are expressed as stimulation index (SI), which is the ratio of the mean (three determinations) counts · min^{-1} incorporated in the presence of antigen to the mean (three determinations) counts · min^{-1} obtained in the absence of antigen. Only SI≥3 was considered significant.

To identify the antigen responsible for providing T cell help for antibody production, we measured IL-2 production after *in vitro* stimulation of peripheral blood mononuclear cells (PBMC) from immunized woodchucks 1D, 848, 1D2I and 845, with WHsAg (100 μg/ml) or peptide FIS (10 μg/ml). We also studied IL-2 production in two control animals, immunized with FIS alone (woodchuck 079) or with WHsAg alone (woodchuck 342). Table 1 shows that re-stimulation with FIS, but not with WHsAg, induced significant levels of IL-2 (SI≥3) for woodchucks 1D, 848, 1D2I and 845. Equivalent results were obtained after *in vitro* stimulation with 2 and 10 μg/ml of WHsAg or with 50 μg/ml of FIS (data not shown). The PBMC from woodchuck 079 produced high levels of IL-2 after re-stimulation with FIS (SI=119.7), whereas woodchuck 342 did not respond to re-stimulation with WHsAg. These results support the hypothesis that FIS behaves as a TDh peptide in these woodchucks, and suggests that this peptide, but not WHsAg, is responsible for providing T cell help for antibody production.

Discussion

As found in human HBV carriers (1–3), insufficient T cell help provided by viral antigens may be responsible for the lack of humoral response against WHsAg in chronic WHV carriers. As demonstrated previously by our laboratory, it is possible to induce antibodies against a peptide hapten or protein hapten by immu-

nization with a mixture of FIS and the haptenic peptide (or protein) not covalently linked (8,9). This prompted us to treat chronic WHV infection by attempting to induce anti-WHs antibodies in nine chronically WHV-infected woodchucks. It was found that immunization with WHsAg in conjunction with a TDh peptide (FIS or $HBs_{120-134}$) (Fig. 1 and 2), but not with WHsAg alone or FIS alone (Fig. 2 F and E, respectively), induced detectable levels of anti-WHs antibodies. However, only high anti-WHs antibody titers were induced in 2/7 animals after joint immunization with WHsAg and FIS. The poor detection of anti-WHs antibodies might be related to the formation of immunocomplexes with circulating WHsAg in the serum, although no firm conclusion can be drawn because these immunocomplexes were not measured. Significant levels of IL-2 ($SI \geq 3$) were observed after *in vitro* stimulation of PBMC with FIS but not with WHsAg (Table 1) showing that the T-cell help for anti-WHs antibody induction was provided by FIS and not by a TDh encompassed by WHsAg. We believe that the mechanism by which FIS helps antibody induction (discussed in more detail in refs. 8 and 9) may be as follows: FIS binds directly to empty MHC class II molecules from B lymphocytes having recognized WHsAg via its surface immunoglobulin, and to other antigen-presenting cells. Recognition of the complex TDh-MHC at the surface of WHsAg-specific B lymphocytes by T helper lymphocytes leads to the expansion of WHsAg-specific B lymphocytes and to the production of anti-WHs antibodies.

Among the woodchucks immunized with WHsAg and FIS, the highest anti-WHs antibody titer (woodchuck 1D2I) was associated with a high SI in the IL-2 production assay (Table 1). However, the highest SI observed in this group was found in woodchuck 845, which showed a low titer of anti-WHs antibodies. This apparent discrepancy may be explained assuming a more Th2 type cytokine profile in those animals with the highest anti-WHs antibody responses. In future experiments we shall attempt to measure other cytokines such as IL-4, IL-5 and γ-IFN.

Irrespective of the mechanism by which high anti-WHs antibodies were elicited, it is interesting to note that those animals that were immunized with WHsAg and FIS, and from which liver samples were taken (310, 1D2I, 845 and 848), showed an evident hepatic inflammation (Fig. 3). Greater liver damage was observed in animals with higher anti-WHs antibody titers. Indeed, the two animals that died (1D2I and 310) had anti-WHs antibody titers that were higher than in any of the other immunized chronically infected woodchucks. Also, in these two animals, after a transient peak, the WHV DNA decreased below the basal levels at the time of death (Table 1). It is likely that the fulminant hepatitis that followed immunization of woodchuck 1D2I may be related to the induction of anti-WHs antibodies, which in turn, could be responsible for the induction of a strong, antibody-dependent, cell-mediated cytotoxicity. However, we can not discard the possibility of a direct T cell cytotoxicity induced by WHsAg through the T cell help provided by FIS. The peaks of WHV DNA and WHsAg in the sera that followed immunization might be a consequence of the induced cytotoxicity. However, since this peak was also observed following immunization with FIS alone (woodchuck 079, Fig. 2 E), it could be speculated that it might be due to a transiently enhanced viral replication in infected cells (B and T cells) following a proliferation stimulus provided by FIS. This situation may resemble the *in vitro* production of WHV by *E. coli* lipopolysaccharide-stimulated PBMC (31).

As mentioned earlier, induction of high anti-WHs antibody titers was associated with severe liver damage, suggesting a strong activation of the immune system. A less intense activation may take place when these antibody titers are lower, for instance in woodchuck 845 (Fig. 2 B), for which a flare-up of γ-GTP is clearly observed after joint immunization with FIS and WHsAg. This flare-up of γ-GTP may resemble the episode of ALT observed by Wen et al. (5) and Pol et al. (6) in vaccinated chronically infected patients showing a decrease or clearance of HBV DNA in their sera.

Our results indicate that antibodies against WHsAg can be induced in chronic WHV infection, by joint immunization with surface antigen and an adequate TDh peptide like FIS. As mentioned in the introduction, therapeutic vaccination of chronic HBV carriers has been attempted before (4–6). An alternative to this approach was tested in the woodchuck using complete recombinant WHV core protein (13). However, only one out of six woodchucks eliminated WHV after vaccination with recombinant WHV core protein. These strategies differ from the one reported here, mainly in that the T cell help must necessarily be induced via TDh peptide sequences that are an integral part of the viral antigens, while in our study T cell help is induced by co-immunization with a conveniently chosen TDh peptide not covalently linked to the surface antigen.

We believe that our strategy may have the advantage over other methods of immunization (4–6) of providing help via a TDh for which tolerance has not been induced, as might be the case for some or all the TDh from viral antigens. Also, our immunization protocol uses a great excess of TDh, which may favor direct

binding to empty MHC class II molecules from surface antigen-specific B lymphocytes. This protocol may potentiate the induction of anti-surface antibodies more efficiently than immunization with vaccine alone, where no antibodies were detected (4–6). Unfortunately, as seen in woodchuck 1D2I, this procedure of inducing anti-WHs antibodies may lead to significant hepatic injury, including fulminant hepatitis. Thus, our approach needs to be modulated in such a way that the rate of lysis of infected hepatocytes is lower than hepatocyte regeneration.

To modulate the immune response, co-immunization with an adequate TDh peptide (as in the present study), could be envisaged. Another alternative might be to administer cytokines or antibodies against cytokines that, as in the case of murine leishmaniasis, might alter the pattern of the immune response (32,33). Along a similar line of reasoning, Milich et al. (34) have suggested that a goal of immunomodulatory therapy in patients with chronic HBV infection might be to shift the balance of T cells from Th2 to Th1 predominance. Our joint immunization procedure might be conducted in such a way as to choose a TDh peptide to induce the desired pattern of Th1/Th2 cell response. This could be done by selecting peptides that elicit mainly Th1 or Th2 responses (35). This may bypass nonresponsiveness to TDh peptides from viral antigens, and offer the possibility of a tailor-made immunization according to the HLA-DR of the chronic patient. A more efficient approach would be to select a promiscuous TDh peptide covering a greater number of HLA-DR alleles.

Another strategy to treat chronically infected patients might be to induce a cytotoxic response using peptide constructs containing a helper and a cytotoxic cell determinant covalently linked (10,11,36,37). Similar peptide constructs of the core protein have been tested by Vitiello et al. (38) to try to induce cytotoxic responses in human volunteers.

Vaccination of chronically HBV-infected patients with vaccines containing the surface antigen have been reported to be safe and without side-effects (4–6). In contrast, although ablation of persistent hepatitis B, due to adoptive transfer of specific immunity against HBV, has been reported after bone marrow transplantation (39), this procedure may also lead to fatal hepatitis B reactivation (40). This result, in conjunction with our experiments in the woodchuck reported here, suggests that an enhancement of the immune response in chronic carriers could have deleterious consequences for liver function. Caution should be used in human immunization trials to avoid the possibility of similar problems.

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