Correlation of virus and host response markers with circulating immune complexes during acute and chronic woodchuck hepatitis virus infection

Running title: Circulating immune complexes in WHV infection

Dieter Glebe, Heike Lorenz, Wolfram H. Gerlich, Scott D. Butler, Ilia A. Tochkov, Bud C. Tennant, Paul Cote, and Stephan Menne

Institute of Medical Virology, Justus-Liebig-University Giessen, Giessen, Germany
Gastrointestinal Unit, Department of Clinical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, New York, and Department of Microbiology and Immunology, Georgetown University Medical Center, Washington, District of Columbia.

* Corresponding author. Mailing address: Institute of Medical Virology, Justus-Liebig University Giessen, Frankfurter Str. 107, D-35392 Giessen, Germany. Phone: +49 (641) 9941209. Fax: +49 (641) 9941209. E-mail: Dieter.Glebe@viro.med.uni-giessen.de.

§ Both authors contributed equally to this work.
ABSTRACT

Woodchuck hepatitis virus (WHV) is an established model for human hepatitis B virus. The kinetics of virus and host responses were studied in serum and liver during acute, self-limited WHV infection in adult woodchucks. Serum WHV DNA and surface antigen (WHsAg) were detected as early as one to three weeks following experimental infection and peaked between one and five weeks post-infection. Thereafter, serum WHsAg declined rapidly and became undetectable while WHV DNA became undetectable much later between four and twenty weeks post-infection. Decreasing viremia correlated with transient liver injury marked by an increase in serum sorbitol dehydrogenase (SDH). Clearance of WHV DNA from serum was associated with normalization of serum SDH. Circulating immune complexes (CICs) of WHsAg and antibodies against WHsAg (anti-WHs) were detected that correlated temporarily with the peaks in serum viremia and WHs antigenemia. CICs were no longer detected in serum once free anti-WHs became detectable. The detection of CICs around the peak in serum viremia and WHs antigenemia in resolving woodchucks suggests a critical role for the humoral immune response against WHsAg in the early elimination of viral and subviral particles from the peripheral blood. Individual kinetic variation during WHV infections in resolving woodchucks, infected with the same WHV inoculum and dose, is likely due to the outbred nature of the animals indicating that the onset and magnitude of the individual immune response determine the intensity of virus inhibition and the timing of virus elimination from serum. (240 words)
INTRODUCTION

Infection of adult humans with the hepatitis B virus (HBV) often results in acute hepatitis followed by recovery based on serological and clinical parameters (2). Progression to chronic HBV infection and associated diseases later in life, including liver cirrhosis and hepatocellular carcinoma (HCC), occurs infrequently in infected immunocompetent adults, but HBV infection often persists in unvaccinated infants born to HBV-carrier mothers or infected horizontally (2). Self-limited HBV infection usually involves a robust primary immune response, acute hepatitis with limited liver injury, and a substantial clearance of virus and viral antigens from the peripheral blood and liver (3, 49). Several studies indicate that clearance of acute HBV infection relies on the development of an adequate immune response against HBV, including protective, virus-neutralizing antibodies against HBV surface antigen (HBsAg), virus antigen-specific responses of T helper (Th) cells and cytolytic T lymphocytes (CTL), and the expression of antiviral cytokines in liver, such as interferon-gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) (1, 9, 13, 20, 24, 26, 40). In contrast, patients with established chronic HBV infection involving persistent viral replication frequently exhibit weak and inefficient humoral and cellular immune responses, resulting in a continual virus replication and HBs antigenemia (1, 9, 13, 20, 24, 26, 40).

The kinetic development of appropriate immune responses (or the failure thereof) during the earliest stages of adult and neonatal HBV infection, and their differential influence on the onset and outcome of infection, is characterized to some extent in animal models (see below), but not in humans. This is mainly because patients usually present
with clinical symptoms several weeks after the HBV transmission event (except for a few rare cases involving known exposure times; e.g., (49)), and chronicity is a less frequent outcome in these cases. Moreover, neonates born to chronic carrier mothers have not been subjected to detailed kinetic studies, so the contribution of the earliest immune responses promoting viral elimination versus persistence in humans is not fully understood.

The woodchuck hepatitis virus (WHV) is like HBV an orthohepadnavirus of the Eastern woodchuck (Marmota monax) with essentially identical biological properties, genomic organization, and replicative strategy (10). Experimental infection of woodchucks with WHV is a well-accepted animal model for many aspects of the pathogenesis of human HBV infection including viral and host factors involved in the outcome of acute infection, disease progression, immune response, and antiviral therapy (22, 29, 33, 34, 43, 44). Infection of neonatal or adult woodchucks with a standardized inoculum of WHV produces predictable proportions of acute, self-limited (i.e., resolved) infections versus chronic infections (6). This mimics the effects of age on outcome of HBV infection in humans (2). WHV infections of adult woodchucks resolve in more than 95% of the cases, whereas 60-75% of neonatal woodchucks develop chronic infections (6). In neonatal and adult woodchucks, resolution of WHV infection is associated with seroconversion to protective, virus-neutralizing antibodies to WHV surface antigen (anti-WHs) (5, 6, 8, 30-32). Clearance of WHV during the acute phase of infection is associated further with strong and frequently detectable Th cell responses to WHV antigens in the peripheral blood, and also with strong Th1 cytokine expression in the liver (5, 8, 30-32, 38, 47, 48). In contrast, the persistence of WHV replication, and the onset
and subsequent progression to chronicity as an outcome of experimental neonatal WHV infection is associated with deficiencies in virus-specific B and Th cell responses in the periphery, deficiencies in Th1 cytokine expression in the liver, and reduced immune-mediated liver injury (5, 8, 30, 32, 38, 47, 48). These results indicate a crucial role of humoral and cellular immune responses in the outcome of WHV infection. Detailed kinetic studies of WHV viremia and WHs antigenemia in correlation to acute liver injury and resolution are not available. Likewise, the presence and possible role of circulating immune complexes (CICs) involving the viral surface antigen (WHsAg) and its antibodies (anti-WHs) are not characterized sufficiently in woodchucks (or in humans) in order to better understand the process of recovery or its failure leading to chronicity.

To investigate these issues in greater detail, the kinetics of WHV viremia, WHs antigenemia, CICs, and ‘free’ antibody response against WHsAg were determined in experimentally-infected adult woodchucks. For WHs antigenemia, the ratios of the different surface proteins and their glycosylation patterns within subviral particles were compared. In addition to typical anti-WHs responses (detected as free antibody), CICs were measured in resolving woodchucks and correlated subsequently with other virus and host response markers, including the severity of acute liver injury. For additional comparisons, attempts were made to detect CICs in woodchucks with established chronic WHV infection, which lack detectable free anti-WHs.

MATERIALS AND METHODS

Experimental animals and serum samples. Woodchucks were born to WHV-negative females and reared in environmentally controlled laboratory animal facilities at
Cornell University. All experiments involving woodchucks were performed under protocols approved by the Cornell University Institutional Animal Care and Use Committee. Six adult woodchucks of both sexes, one to six years of age, all seronegative for markers of WHV infection, were experimentally infected with WHV to study virus-host kinetics (see below). Stored serial serum samples were also available from three additional experimentally-infected adult woodchucks, three to five years of age, with self-limited WHV infection. These woodchucks had been infected experimentally as adults by intravenous inoculation with $1 \times 10^4$ woodchuck infectious doses ($\text{WID}_{50\%}$) of WHV of a standardized WHV inoculum (cWHV7P2) (6). Diagnosis of resolution was based on the loss of detectable WHV DNA and WHsAg in serum, and on the detection of serum antibodies to WHV core antigen (anti-WHc) and WHsAg (anti-WHs) following infection. Stored serum samples were also available from nine adult chronic WHV carrier woodchucks, approximately one to two years of age. These woodchucks had been infected experimentally as neonates at three days of age by subcutaneous inoculation with $5 \times 10^6$ $\text{WID}_{50\%}$ of WHV of a standardized WHV inoculum (WHV7P1) (6). Diagnosis of persistent WHV infection was based on the consecutive detection of WHV DNA and WHsAg in serum from three months of age. At the time of sample collection, woodchucks had minimal chronic hepatitis based on serum liver enzyme profiles. All animals were free of HCC at the time of sampling, as determined by hepatic ultrasound examination and normal serum activity of $\gamma$-glutamyl-transferase (GGT).

**Experimental WHV infection kinetics.** Six adult woodchucks were inoculated intravenously with $1 \times 10^7$ $\text{WID}_{50\%}$ of WHV7P1 (6). Blood samples were obtained under general anesthesia (ketamine 50 mg/kg and xylazine 5 mg/kg intramuscularly) two weeks
before experimental WHV infection, prior to inoculation at T₀ (“week 0”), and thereafter at weekly or biweekly intervals until the end of the study at weeks 16 or 28 post-infection. For determining markers of WHV infection within the liver, hepatic specimens were obtained by percutaneous needle biopsy starting two weeks prior to infection. Following the infection, one additional biopsy was obtained from some of the woodchucks at week eight or ten post-infection. For other woodchucks, liver biopsies were obtained more frequently at weeks 4, 8, 12, 16, 20, 24, and 28 post-infection. Biopsies were performed while animals were under general anesthesia (ketamine 50 mg/kg and xylazine 5 mg/kg intramuscularly) with 16-gauge Bard Biopty-Cut (C.R. Bard Inc., Covington, GA) disposable biopsy needles directed by ultrasound imaging (39, 42).

**Serum WHV nucleic acids.** Serum WHV DNA was determined by real time PCR (assay sensitivity, ≥ 1×10² WHV ge/ml), with primers located within the S-region of the envelope protein, that recognized all orthohepadnaviral genomes, as described (41). Briefly, WHV DNA from 200 µl serum was extracted in 50 µl buffer using the High Pure Viral Nucleic Acid Kit (Roche, Mannheim, Germany). PCR reactions were normalized as described (41) by using a WHV reference serum and WHV plasmid pW8 which contains a full-length WHV genome (6).

**Serum WHsAg.** WHsAg in serum was measured quantitatively by the electroimmunodiffusion technique (Laurell electrophoresis) using a polyclonal antiserum against this antigen (46), as described (11). Briefly, 0.5 % agarose containing a 1:25 dilution of rabbit anti-WHs antiserum was layered on a glass-slide. Thereafter, 10 µl of serum or appropriate dilutions of serum were transferred to the wells on the agarose-layered glass-slide and separated by electrophoresis. The migrating antigen within the
immuno-agar forms immune precipitates until the antigen is used up, resulting in precipitation ‘arches’. The length of the ‘arches’ in this assay is proportional to the amount of the applied antigen. Reactions were normalized by using serum from a chronic WHV carrier woodchuck (F5413) with known WHsAg concentration. This serum was calibrated in µg WHsAg protein using purified WHsAg (46). The detection limit for WHsAg is approximately 2 µg/ml (11).

Circulating immune complexes. WHsAg-containing CICs in serum were detected by polyethylene glycol (PEG)-precipitation followed by Western-blotting of WHs proteins as described (25). Briefly, immune complexes of 100 µl cleared sera were precipitated by adding 50 µl of 7.5% PEG 6000 (w/w). After incubation at 4°C for 12 hours, the immune complexes were pelleted at 6800 × g in a v-vial for 5 minutes at 4°C. The precipitate was washed two times with 200 µl of cold 2.5% PEG 6000 (w/w) and the resulting pellet was resuspended in 30 µl phosphate buffered saline (PBS). WHsAg within the precipitated immune complexes was detected after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by Western-blotting (see below).

Isolation of subviral particles from serum. Subviral particles in serum of WHV-infected woodchucks were isolated as described (46). Briefly, three to six ml of serum from individual woodchucks were pelleted through two layers of 10% and 15% sucrose at 25,000 rpm for 16 hours at 10°C using the SW41 swing-out rotor from Beckmann (Munich, Germany). The pellet was dissolved in PBS and adjusted with solid cesium chloride (CsCl) to a buoyant density of 1.30 g/ml and layered within a CsCl gradient ranging from 1.16 to 1.35 g/ml. After centrifugation at 25,000 rpm for 36 hours at 10°C with the SW41 rotor (Beckmann), the gradient was fractionized and tested for the
presence of WHsAg by SDS-PAGE and silver-staining as described (46). WHsAg-containing fractions were pooled and concentrated using an ultrafiltration-device (Vivascience, Sartorius, Germany). The concentration of WHsAg was estimated from the optical density at 280 nm (OD280), with an OD280 value of 5.1 equaling 1.0 mg WHsAg per ml (46).

**SDS-PAGE and immunoblotting of WHsAg.** Purified subviral particles or resuspended CICs were treated with Laemmli buffer containing 8% dithiothreithol for 15 minutes at 70°C and analyzed on 12% precast polyacrylamide gels (Invitrogen, Karlsruhe, Germany). Following SDS-PAGE, the gel was blotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Eschborn, Germany). The membrane then was blocked in 3% low-fat milk powder in PBS and incubated with the same polyclonal rabbit anti-WHs antiserum as above diluted 1:1000 in PBS with 1% low-fat milk powder for one hour at 37°C as described (46). This was followed by incubation with a peroxidase-conjugated donkey anti-rabbit antibody (Dianova, Hamburg, Germany) at a dilution of 1:10000 in PBS. WHsAg bands were visualized with the enhanced chemiluminescent-light detection kit (Roche, Mannheim, Germany). In immunoblots with reduced and SDS-denatured WHsAg, this antiserum reacts strongly with the glycans of M protein, and weakly with deglycosylated M protein, L protein and glycosylated S protein. Unglycosylated denatured S protein does not react (46).

**WHV antibodies.** Anti-WHc and anti-WHs were measured by qualitative enzyme-linked immunosorbent assay (ELISA) using a 1:10 dilution of serum as described (7). The cutoff of these assays was defined as ≥ 0.05 optical density units (ODU).
**Serum biochemistry.** Serum biochemical measurements included serum GGT, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and sorbitol dehydrogenase (SDH) (39, 42). Serum activities of these liver enzymes were quantified at the New York State Diagnostic Laboratory at Cornell University using a Hitachi autoanalyzer. Serum activities of AST, ALT, and SDH are markers of hepatocellular injury in woodchucks. Serum GGT is a marker of HCC.

**Histopathology and immunohistochemistry.** Aliquots of liver biopsy specimens were fixed in phosphate-buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histopathological analysis (i.e., portal hepatitis, lobular hepatitis, bile duct proliferation, and liver cell dysplasia), as described (23, 39, 42). According to their severity and distribution, individual histological changes associated with WHV infection were scored on a scale from 0 (not present) to 4 (most pronounced). Sections of these tissues were also stained for intrahepatic expression of WHV core antigen (WHcAg) and WHsAg using immunohistochemical methods and polyclonal rabbit antibodies for the respective antigens (23, 39, 42). Other sections of these tissues were stained for the proliferating cell nuclear antigen (PCNA) using a cross-species reactive monoclonal mouse anti-human PCNA antibody (Roche Diagnostics Corp., Indianapolis, IN) (23, 39, 42). Apoptotic activity in liver tissues was determined by the detection of DNA strand cleavage in hepatocytes using terminal deoxynucleotidyl transferase-mediated uridine deoxynucleotide nick end labeling (TUNEL) technique (Roche Diagnostics Corp.) (23, 39, 42). CD3-positive lymphocytes in liver were stained with a cross-reacting polyclonal rabbit anti-human CD3 antibody (DAKO Inc.,
Carpinteria, CA) (39, 42). Macrophages in liver were stained with a cross-reacting monoclonal mouse antibody (DAKO Inc.) (6, 38).

RESULTS

Kinetic study of self-limited experimental WHV infection in adult woodchucks. Markers of WHV infection and host immune responses to WHV antigens were characterized in two repeat experiments, each containing three adult animals. All six woodchucks were inoculated intravenously with $1 \times 10^7 \text{ WID}_{50\%}$ of WHV7P1. Following inoculation, woodchucks of group 1 (F3524, F3538, M3540) were followed weekly until the end of the study in week 16. Woodchucks of group 2 (F3391, F3539, M3553) were followed for a longer period and monitoring was performed weekly during the initial twelve weeks and then every other week until the end of the study in week 28. Within the differential detection limits of assays used, these close monitoring intervals enabled a detailed characterization of both the uniformity and variation in the responses of viral markers, CICs, and antibody responses in the peripheral blood from the early to late acute stages of resolving WHV infection, as described below.

(i) Serum WHV DNA. All six woodchucks resolved their infection based on the inability to detect WHV DNA in serum at the end of the study, as determined by real time PCR. Individual variation in the kinetics of WHV DNA was noted during the course of infection. WHV viremia was high and long-lasting in F3538 and F3539, intermediate in
F3524, and low and of short duration in F3391, M3540, and M3553 (Figure 1). All woodchucks became positive by PCR for WHV DNA in serum by one to two weeks following inoculation. The initially detectable WHV DNA concentrations ranged between $1 \times 10^4$ WHVge/ml in M3540 and $4 \times 10^6$ WHVge/ml in F3539, with all concentrations clearly detectable above the respective assay cutoff values.

In F3524, F3538, and F3539, WHV DNA concentrations increased over time and peaked between four and five weeks post-infection. In M3540, M3553, and F3391 WHV DNA also was detected initially at one or two weeks post-infection, but those levels corresponded to (or were very near to) the maximal observed WHV DNA concentrations. In these woodchucks, maximal WHV DNA concentrations were considered low viremic, ranging between $1 \times 10^4$ and $4 \times 10^5$ WHVge/ml. In contrast, in F3524 maximum WHV DNA concentration was considered intermediate viremic at $3 \times 10^7$ WHVge/ml (week 5), and in F3538 and F3539, maximal WHV DNA concentrations were considered high viremic at $2 \times 10^9$ and $1 \times 10^{10}$ WHVge/ml (weeks 5 and 4, respectively).

The time of WHV DNA elimination from serum (i.e., duration until WHV DNA concentrations were below the assay cutoff) varied among the woodchucks, but in general correlated with the level of viral replication: In F3391, M3540, and M3553 (low viremic), WHV DNA became undetectable between five and six weeks post-infection. In F3524 (intermediate viremic) WHV DNA became undetectable by week 12, while in F3538 and F3539 (high viremic) WHV DNA became undetectable by weeks 14 and 20, respectively. Overall, the half-life of serum WHV DNA after the peak in viremia was estimated to be two to three days for woodchucks with high, intermediate, or low viremia, with the exception of F3391 that had a shorter half-life.
The influence of animal age on the magnitude and course of viremia was negligible because rapid or prolonged clearance of WHV from serum was equally observed among younger and older woodchucks; i.e., the high viremic woodchucks F3538 and F3539 were two and three years of age, the intermediate viremic woodchuck F3524 was three years of age, and the low viremic woodchucks F3391, M3540, and M3553 were six, one, and two years of age at the time point of WHV inoculation.

(ii) Serum WHsAg. Using electroimmunodiffusion, free serum WHsAg was detected in the high viremic woodchucks F3538 and F3539 (shown for F3538 in Figure 2). Free WHsAg was detected for a shorter duration in serum of both woodchucks compared to the respective durations for serum WHV DNA (i.e., detected for three to four weeks, starting at three weeks post-infection; Figures 1 and 2). In F3538, serum WHsAg increased within two weeks, from an initial concentration of 3 µg/ml at three weeks post-infection to a maximum concentration of 367 µg/ml at week five post-infection; i.e., the doubling time was approximately two days. Somewhat higher levels were detected initially in F3539 at week three (74 µg/ml) suggesting an even shorter doubling time (from below 2 µg/ml to 74 µg/ml within 7 days), and the maximal concentration of 414 µg/ml at week four was quite remarkable as well. In both woodchucks, the timing of the observed peak in WHs antigenemia correlated with the peak in serum WHV viremia. Serum WHsAg concentrations decreased much more rapidly than WHV viremia in both woodchucks; it was undetectable in F3538 one week after the peak (i.e., six weeks post-infection; Figure 2) and by one to two weeks after the peak in F3539 (i.e., five to six weeks post-infection). This corresponds to a half-life of less than one day.
(iii) **Circulating immune complexes (CICs).** Using PEG-precipitation followed by Western-blotting, CICs consisting of WHsAg ‘complexed’ with anti-WHs were detected in four of the six woodchucks (Figure 3). CICs in F3391, F3539, and F3524 were detected initially between four and five weeks post-infection, and their appearance correlated with the peaks in WHV viremia and WHs antigenemia (Figure 1). F3538 tested positive for the first time for serum CICs one week following maximum viral replication, at six weeks post-infection. With the exception of F3391, in which serum CICs were detected for only one week and at a relative low level, CICs in the other three woodchucks were observed for five to six weeks. In all woodchucks, the levels of CICs appeared to be highest during the initial two to four weeks of detection, and then declined thereafter during the remaining one to two weeks. Disappearance of CICs from serum in F3539 correlated with the first detection of free anti-WHs (Figure 1). In the other three woodchucks, serum anti-WHs became detectable between two and four weeks following the disappearance of CICs.

(iv) **Antibody responses to WHcAg and WHsAg.** All WHV-infected woodchucks developed serum anti-WHc between one and two weeks after the peak in WHV viremia and WHs antigenemia (i.e., between five and seven weeks post-infection). Anti-WHc in M3553 appeared at the time of maximum viral replication at two weeks post-infection. Another notable exception was M3540 in which serum anti-WHc was detected starting four weeks after the peak in WHV DNA and WHsAg (i.e., at seven weeks post-infection). In all woodchucks, anti-WHc was present in serum until the end of the study.

The appearance of conventional ‘free’ serum anti-WHs (i.e., not ‘complexed’ with WHsAg) varied between individual woodchucks, but, in most woodchucks, it
became detectable between two and five weeks following the first appearance of anti-WHc, between three and six weeks following the disappearance of WHsAg, and immediately or between two and four weeks following the disappearance of CICs. No anti-WHs was detected in serum of M3540; this woodchuck also had no detectable free serum WHsAg, and no CICs involving WHsAg and anti-WHs. M3553 had a long-lasting anti-WHs response, with no detectable serum WHsAg or CICs. Free anti-WHs was present in serum from most of the woodchucks until the end of the study, with the exception of F3391, where anti-WHs was detected once at eight weeks post-infection.

(v) Liver injury response. Serum SDH activity was determined in all woodchucks as a marker of hepatocellular injury (Figure 1), and then correlated with elevations of other liver enzymes in serum, and also with the expression of WHV antigens and histopathological changes in hepatic biopsy specimens (Tables 1-3). Serum SDH increased remarkably starting at eight weeks post-infection in the two high viremic woodchucks (F3538 and F3539) to a peak activity at twelve weeks post-infection of 725 and 632 international units (IU)/L. Serum SDH activities were less pronounced in the other four woodchucks, with maximum activities between five and ten weeks post-infection ranging between 90 IU/L (M3540) and 222 IU/L (F3524). It is important that peaks in serum SDH occurred after serum WHV DNA and WHsAg already started to decline, and after anti-WHc became detectable. Also, at peak SDH activity, most woodchucks already had detectable anti-WHs. SDH declined relatively rapidly following the peak in all woodchucks and normalized within one to four weeks, including the high viremic woodchucks F3538 and F3539.
As with SDH, the serum activities of ALT and AST became transiently increased in most woodchucks (Table 1). Peak ALT and AST activities were observed at the same time as peak SDH activity. High or intermediate viremic woodchucks (F3524, F3538, F3539) had greater increases in serum ALT and AST activity than low viremic woodchucks (F3391, M3553, and M3540). These markers are generally less sensitive than SDH as markers for the severity of liver injury in woodchucks. At the time of maximum liver injury (indicated by SDH, ALT, and AST) the activities of other enzyme markers like ALP and GGT remained unchanged from pre-infection levels in most woodchucks, except for F3539 and M3553, where marked increases in these two enzymes were noted.

During peak liver injury, high and intermediate viremic woodchucks (F3524, F3538, F3539) had increased hepatic expression of WHcAg and cytoplasmic WHsAg, which were not evident in the liver of low viremic woodchucks (F3391, M3553, and M3540) (Table 2). Most woodchucks had histological changes including portal and lobular hepatitis that were scored as mild to more severe for this species (Table 2). No liver changes were observed in M3540, where the liver biopsy was obtained four weeks after the peak in SDH activity (i.e., at eight weeks post-infection), at a time when hepatic injury had already abated. No liver changes were observed in the low viremic woodchuck M3553. Most woodchucks were clear of histological changes in bile duct proliferation and liver cell dysplasia at the time of biopsy, except for F3524 and F3538, which each had a mild change in one or both markers, respectively (Table 2).

Liver cell proliferation and apoptosis in hepatocytes became increased in woodchucks at the time of maximum acute liver injury, except in the low viremic
woodchuck F3991 where proliferation but no apoptosis were noted (Table 3). High and intermediate viremic woodchucks (F3524, F3538, F3539) demonstrated greater increases in liver regeneration and/or elimination of hepatocytes than low viremic woodchucks (F3391, M3553, and M3540). Consistent with the patterns of liver injury indicated above, the numbers of CD3+ lymphocytes and macrophages within the liver were increased in most woodchucks (Table 3). Hepatic inflammation by these cell types was more remarkable in the high and intermediate viremic woodchucks than in low viremic woodchucks. Interestingly, however, in F3991 and M3553, the respective numbers for macrophages versus CD3+ lymphocytes were actually less post-infection compared to pre-infection, even though the overall degree of inflammation and liver injury were increased post-infection. A lower number of these cell types was also observed in the biopsy from M3540 in which hepatic injury had already abated.

Detection of CICs in serum of woodchucks with self-limited versus chronic WHV infection. For further correlation of the appearance and duration of free WHsAg, CICs, and free anti-WHs in relation to outcome of infection, serum samples were assayed from woodchucks previously infected with WHV (three adult woodchucks with self-limited WHV infection; nine neonatally-infected woodchucks with chronic WHV infection). Woodchucks F6143, F6239, and M5015 had been experimentally infected with $10^4$ WID$_{50\%}$ of cloned WHV7P2, which has the same biological outcome properties as the parental uncloned WHV7P1 inoculum used in the above kinetic study (6). These woodchucks were followed for a total of 32 weeks through their recovery from acute WHV infection and hepatitis. CICs were detected in serum of all three woodchucks for three to six weeks duration, but with variable timing in the responses (Figure 4).
F6143, CICs were detected at eleven weeks post-infection, and their appearance correlated with the first increase in free serum WHsAg (i.e., with the beginning of WHs antigenemia). CICs in F6239 and M5015 were detected at six and ten weeks post-infection, respectively, and their appearance correlated with the peak of WHs antigenemia. This was associated further with the initial detection of free anti-WHs. In F6143, free anti-WHs became detectable only after CICs had disappeared from serum. Relative to the signal for the antigen standard (0.5 µg WHsAg), CIC levels in F6143 and F6239 were higher than in M5015. The variation in CIC levels was unexpected because F6239 and M5015 had no remarkable differences in their levels of free WHsAg and free anti-WHs. Comparison of appearance and duration of WHsAg, CICs, and anti-WHs in these three woodchucks with those in the six woodchucks from the above kinetic study indicated that the inoculum dose used for experimental WHV infection had no effect on the kinetic of standard viral and host markers.

Individual serum samples were analyzed for CICs also from nine woodchucks during the chronic phase of experimental neonatal infection with uncloned WHV7P1 inoculum. The age of the woodchucks at the time of sampling was either 15 months (M6510, M6539) or 22 months (F5883, F5890, F5904, F5927, F5929, M5951, F5954). In five of the nine woodchucks, CICs were detected in serum at variable levels (Figure 5). Compared to the antigen standard (0.5 µg WHsAg), M6539 had the most remarkable levels of CICs. CIC levels in F5954 and M6510 were intermediate, whereas the levels were low in woodchucks F5904 and F5927. CICs were not detected in the sample from each of the remaining chronic WHV carrier woodchucks. Comparison of CIC levels with serum WHV DNA, free WHsAg, and anti-WHc (Table 4) indicated that the presence of
CICs in chronic WHV carrier woodchucks does not correlate with the magnitude of WHs antigenemia, WHV viremia, and antibody response to WHcAg. However, a possible direct correlation between CICs and free WHsAg in serum (similar to woodchucks with self-limited WHV infection) was observed, with M6539 having the highest levels of CICs and WHsAg, followed by that for F5954, F5927, and F5904. In contrast, M6510 had intermediate CIC levels with relatively low levels of free WHsAg, and F5883 and M5951 had relatively high levels of free WHsAg with no CICs. The apparent lack of correlation in these animals, however, may relate more to the immune status of the carrier (e.g., immune tolerant versus partial immune response).

Comparison of S, M, and L surface proteins of WHsAg in acute and chronic WHV infections. Subviral WHs particles representing primarily excess surface antigens of WHV were isolated from serum of woodchucks with acute, self-limited or established chronic WHV infection. The purified particles were separated by SDS-PAGE, and the L, M, and S proteins of the WHsAg analyzed by silver-staining and/or Western-blotting (Figure 6).

In both the acute, self-limited and chronic WHV infections, silver-staining indicated two strong protein bands in all woodchucks with molecular weights below 31 kDa (Figure 6A). These represent the non-glycosylated (smaller) and the single N-glycosylated (larger) forms of the S protein, with molecular weights of 24 and 27 kDa, respectively. Two weaker bands above the S protein with molecular weights of 41 and 45 kDa were also observed for all woodchucks, which represent multiple N- and O-glycosylated forms of the M protein. Comparison of S and M proteins between woodchucks with acute, self-limited or established chronic WHV infections demonstrated
no differences in the ratio of individual proteins within subviral particles, with the S protein as the predominant protein. Because of the weak signals for L protein, a comparison of this protein between woodchucks with different outcome of experimental WHV infections was not possible.

In further comparisons of S and M proteins by Western blot using a polyclonal rabbit anti-WHs antiserum that can react with denatured WHsAg, all woodchucks demonstrated the multiple N- and O-glycosylated forms of the M protein, and the non-glycosylated and the single N-glycosylated forms of the S protein (Figure 6B). The polyclonal antibody reacted much better with the sequential epitopes in the preS domains of the M and L proteins than with the S protein which contains mainly conformational, disulfide-dependent epitopes. Again, no differences in the protein ratio and glycosylation pattern within subviral particles were observed between woodchucks in the different outcome settings or times post-infection, but some microheterogeneity of the M and L proteins between the different samples was present.

**DISCUSSION**

Adult, WHV-susceptible woodchucks were infected experimentally with WHV to investigate the dynamically changing relationships between WHV viremia and antigenemia, humoral immune responses also involving CICs, and acute liver injury. Although all six woodchucks received the same relatively high inoculum and dose, the course of acute, self-limited WHV infection varied remarkably between animals. Individual differences based on the markers were minimal immediately after inoculation. WHV DNA was already detectable at one or two weeks post-infection. Thereafter, the
kinetics of serum WHV viremia differed remarkably in individual woodchucks. The peak
of viral replication had already occurred after two weeks in two woodchucks at a low
level, whereas WHV viremia in the other woodchucks continued to increase and
maximum viral replication of up to $1 \times 10^{10}$ WHV ge/ml was observed between four and
five weeks post-infection. Peak concentration and duration of WHV DNA in serum
suggested to classify viremia as high in two woodchucks, intermediate in one and low in
three. With the PCR assay used it was not possible to differentiate whether WHV DNA
detected in serum of low viremic woodchucks following inoculation was part of the
inoculum or progeny WHV DNA. The fact that low viremic woodchucks had a
productive WHV infection was demonstrated by the detection of anti-WHc in all animals,
in addition to CICs, anti-WHS, and acute liver injury in some. Furthermore, comparison
of the half-life for clearance of WHV DNA following the peak in WHV viremia indicated
a similar viral clearance rate in low, intermediate, and high viremic woodchucks during
the recovery phase, thus strongly suggesting that new WHV was still being produced in
face of the developing host response.

Free WHsAg was detectable by electroimmunodiffusion only in serum of the high
viremic woodchucks, and the peak in WHs antigenemia correlated with the peak in serum
WHV viremia (Figure 2). Due to the low sensitivity of the assay, WHsAg was probably
not detected in the remaining woodchucks or in the high viremic woodchucks at later
time points. Free WHsAg in serum of both high viremic woodchucks was only present
for two to three weeks. WHsAg was cleared more rapidly from serum compared to serum
WHV DNA in both woodchucks. This difference in kinetics may be explained by the fact
that WHsAg was detected only as free antigen whereas WHV DNA could be extracted
both from free and anti-WHs-complexed WHV. Furthermore, the antigenicity of WHV particles may be different from that of spherical WHsAg subviral particles. The envelope of HBV and HBsAg filaments contain a higher proportion of L protein than HBsAg spherical particles. The preS domains partially mask the S domain which becomes more accessible to monoclonal antibodies after removal of preS by trypsin (16). The WHsAg filaments contain also a higher proportion of WHs L protein than spherical WHs particles (46). Thus, WHV may escape easier WHsAg S antibody recognition than the subviral particles.

Following the peak in WHV viremia, serum WHV DNA concentrations declined continuously over nine (F3538) or 14 weeks (F3539), respectively, before becoming undetectable (Figure 1). Presence of WHV DNA in serum of the remaining woodchucks was shorter, and WHV DNA declined to undetectable levels over two (M3540) to seven weeks (F3524) following the peak in WHV viremia. Obviously, viral clearance rates (i.e., half-lives of serum WHV DNA) were similar between woodchucks, irrespective of peak viremia.

The magnitude of maximum WHV DNA concentrations and duration of viral clearance in individual woodchucks is dependent on several factors. Viral factors that influence the course of acute, self-limited WHV infection include the inoculum dose, viral strain within the inoculum, and route of inoculation (6). The age and status of the immune system of an individual woodchuck are host factors that determine the outcome and course of WHV infection (6, 8, 32, 38, 47, 48). Studies in humans and woodchucks suggest that the fine balance between viral load and quality of the individual, antiviral immune response is important for an efficient suppression of viral replication resulting in
eradication of the virus from the host (3, 8, 32, 38, 47, 48). The quality of the antiviral
immune response appears the main determining factor for the observed differences in the
course of WHV infection in the present study, because all group 1 and group 2 adult
woodchucks had been infected with the same inoculum and dose.

The protein composition of subviral surface antigen particles was determined in
resolving and chronic carrier woodchucks in order to explore whether differential host
immune responses or replication-dependent processes might skew the ratios of viral and
subviral particles or their component proteins. Comparison of the subviral protein
composition by silver-staining revealed no significant differences between woodchucks
with different outcome of WHV infection (Figure 6) and confirmed previous results
obtained with chronic WHV carriers (46). Furthermore, minor differences in the
migration pattern of M protein were detected by Western-blotting in subviral particles
from resolving woodchucks and chronic WHV carrier woodchucks or from F3538 during
the acute phase of WHV infection (Figure 6) These are probably caused by slight
differences in the glycosylation (42).

Host responses correlated temporally with viral markers as expected. Antibody
responses against WHV antigens were elicited in individual woodchucks and, as with Th
cell responses that develop first against WHsAg and then to WHcAg (5, 30, 31), the
earliest antibody responses (detected as CICs) were directed against WHsAg. CICs
containing WHsAg and anti-WHs were detected around the time of peak WHV viremia
and WHs antigenemia (Figures 3 and 4). Due to the preferential reactivity of the CIC
detecting antiserum with WHsAg M protein in immunoblots, we cannot exclude the
possibility that a subfraction of WHs-containing CICs with very low or absent proportion
of M protein was missed, but as shown in Figure 6 B, there is no indication for such a heterogeneity. Although not tested in this study, it is possible that anti-WHs within CICs represents the IgM class, whereas the later appearing free anti-WHs represents the IgG class. However, in contrast to the core antigen, which readily elicits measureable IgM antibodies in a T cell-independent manner (35), the surface antigen of hepadnaviruses is a strictly T cell-dependent antigen, and poorly immunogenic directly for B cells \textit{in vitro}.

The rapid decline of free WHsAg and the presence of CICs demonstrate directly that antibodies against WHsAg are involved in the clearance of viral and subviral particles from peripheral blood in resolving woodchucks. This finding is comparable to acute, self-limited HBV infection in humans in which the neutralization of virus is clearly associated with the presence of antibodies against HBsAg (2, 3). Anti-HBs, however, appears not to play as important a role in the early clearance of subviral particles during resolving HBV infection (see below).

CICs in patients with chronic HBV infection have been described (25). In contrast to chronic WHV carrier woodchucks, amounts of CICs were high in chronic HBV carriers and low or absent in acute HBV infection (27, 28). In chronic WHV carrier woodchucks with comparable levels of free WHsAg, smaller amounts of CICs were present than during acute infection (Figure 5; Table 4). The reason for this difference between WHV and HBV is unknown but may relate to the immune system of woodchucks and/or the structural differences between HBV and WHV. Furthermore, the sensitivity of the used assay and the immune status of woodchucks at the time of serum sample collection (e.g., normal versus elevated liver enzyme activities indicating exacerbations of liver injury) may have been different.
One to two weeks following the initial appearance of CICs, antibodies against WHcAg became detectable (Figure 1) and their presence lasted until the end of the study (i.e., up to 7 months in some woodchucks). The constitutive presence of anti-WHc in all resolving woodchucks is in contrast to the much shorter detection of anti-WHs in woodchucks with WHs seroconversion (Figures 1 and 4). The differences in the humoral response against both WHV antigens may be explained by the higher immunogenicity of WHcAg over WHsAg. Free anti-WHs, as a marker of the more favorable outcome of WHV infection, appeared only during the later stages of infection, and were associated with almost undetectable serum WHV DNA and normalized serum activity of the liver enzyme SDH. Besides elimination of viral particles from blood, anti-WHs are also important for the protection of hepatocytes from WHV reinfection within the regenerating liver (15). In this respect, the role of the antibody responses is similar in woodchucks and humans.

All woodchucks in the present experimental infections resolved their WHV infection based on undetectable serum WHV DNA at the end of the study. Comparable to adult chimpanzees with acute, self-limited HBV infection (37), serum WHV DNA in woodchucks started to decline substantially before the peak in acute liver injury was observed as determined by increases in serum SDH activity (Figure 1). The reduction in serum WHV DNA before liver injury suggests that non-cytotoxic immune mechanisms are mainly responsible for the initial decrease of WHV replication. As demonstrated in HBV-transgenic mice and HBV-infected chimpanzees, massive production of the cytokines IFN-γ by HBV-specific CD8+ T cells followed by TNF-α from macrophages and hepatic Kupffer cells is responsible for the initial reduction of HBV DNA, and was
also described for the self-limited outcome of acute WHV infection in woodchucks (12, 14, 15, 19, 21, 37, 38, 48). Inhibition of HBV replication by these cytokines is a result of destabilized HBV mRNA and HBV covalently-closed circular DNA (cccDNA) in infected hepatocytes (17, 37), and by analogy in WHV-infected hepatocytes of woodchucks.

One of the later steps in terminating acute WHV infection in woodchucks is the induction of necrosis and apoptosis of WHV-infected hepatocytes by CTLs resulting in eradication of virus-infected cells from the liver. All six woodchucks had increases in the serum activity of SDH (Figure 1) and/or other liver enzymes (Table 1) as markers of hepatocellular injury, although peak SDH concentrations varied between animals and across the time interval. Correlating with the magnitude of serum SDH activity, differences between individual woodchucks were also observed for the hepatic expression of WHcAg and WHsAg (Table 2), for histological changes in liver (Table 2), for apoptosis of hepatocytes and hepatic cell proliferation (Table 3), and hepatic inflammation by CD3+ cells and macrophages (Table 3). Termination of viral infection was associated with killing of (infected) hepatocytes and subsequent regeneration of the liver via proliferation of (uninfected) hepatocytes as described in HBV-infected chimpanzees and in WHV-infected woodchucks (8, 15, 18, 21, 37, 45, 47, 51).

Comparison of the above parameters demonstrated that acute liver injury, as determined by the peak in serum SDH activity, was more pronounced in woodchucks with higher peak viremia than in woodchucks with lower peak viremia. Serum SDH concentrations declined rapidly following the peak in enzyme activity and became normal thereafter, suggesting that the duration of acute liver injury is relatively short.
This suggests further that woodchuck liver has a high potential to regenerate, and that rapid regeneration may compensate for the severity of liver damage in resolving woodchucks. This assumption is supported by the results for proliferation and apoptosis of hepatocytes (Table 3), with a much higher percentage of hepatocytes undergoing cell division than apoptosis at the peak of serum SDH activity. The above results indicate furthermore that suppression of WHV replication in the liver is mainly caused by non-cytotoxic rather than cytotoxic mechanisms as also described in other studies of resolving WHV infection in woodchucks (15, 21, 38, 47, 48). One possible explanation for the different course of acute, self-limited WHV infection in individual woodchucks may relate to the individual immune response genes leading to different antigen recognition on virus-infected hepatocytes and different regulation, level, and action of cytokine production. Inbred woodchucks are not available and no studies have been undertaken so far that measure cytokine levels in blood during the peak of WHV viremia and acute liver injury.

The course of acute, self-limited WHV infection in adult woodchucks is generally comparable to HBV infection in adult humans and chimpanzees and remarkably variable in individuals from the three species (4, 37, 49). In patients with resolving HBV infection, viremia at the onset of clinical symptoms ranged between $10^3$ and $10^8$ ge/ml (4), which is similar to the WHV viremia observed at the onset of strong SDH elevations (Figure 1). Furthermore, the apparent half-life of HBV DNA during the elimination phase is 1.6 to 4 days (4, 50), and, thus, similar to the apparent WHV DNA half-life of 2 to 3 days. The apparent half life of HBV or WHV measured in our and previous studies (4, 46) is the sum of eliminated and newly exported virus and does not reflect the true half
life due to elimination (33). The true half-life of HBV DNA was found to be much shorter (4 hours) in chimpanzees with acute HBV infection (36). We do not know whether this applies also to acute WHV infection, because we have not quantitatively determined the intrahepatic WHV DNA concentration. Major differences in the course of WHV and HBV infection concern the appearance, concentration, and elimination of subviral particles from the peripheral blood. Ninety percent of all patients with acute resolving hepatitis B had 10 to 100 µg/ml free HBsAg in the first available serum sample at the onset of disease symptoms (i.e., during a rather late stage of infection (4)), whereas only about half of the resolving woodchucks were WHsAg positive (> 2 µg/ml) in serum samples obtained during the entire observation period (Figures 1 and 4). However, the maximum WHsAg concentration in the serum of two woodchucks reached up to 400 µg/ml and was 4- to 40-fold higher than those in patients with 10 to 100 µg HBsAg per ml of serum (4, 11).

Free HBsAg appears more resistant to elimination from peripheral blood than free WHsAg, because subviral particles in serum of patients that eventually resolved were detectable over several weeks following inoculation, and before acute hepatitis was observed (49). Using the same type of assay (i.e., electroimmunodiffusion (11)), the half-life time of HBsAg during acute hepatitis was determined to be 6 to 8 days (4), whereas the half-life time of free WHsAg was less than one day despite much higher antigen concentrations. Results of this study suggest that the rapid development of an antibody response against WHsAg is mainly responsible for the fast elimination of subviral particles from serum, as demonstrated by the appearance of large amounts of CICs around the peak in WHs antigenemia, followed by the appearance of free anti-WHs
around the time of undetectable serum WHV DNA. Generation of CICs during or even before the ‘anti-core window’ (i.e., the time between loss of free WHsAg and appearance of anti-WHs during which anti-WHc is the only detectable serological host response marker) suggests a more rapid elicitation of a humoral response during WHV infection in woodchucks than during HBV infection in patients in which anti-HBs become detectable mainly during the end stages of resolving infection, and sometimes much later after complete reconvalescence (2, 3).

In summary, this study in woodchucks shows that resolution from acute WHV infection involves a process with variable but appropriate virus-specific immune responses in the peripheral blood and liver. Rapid development of a humoral response against free WHsAg in form of CICs contributes to the early elimination of subviral and viral particles from the periphery. Marked reductions in WHV from peak viremia do begin before the peak in acute liver injury is detected by serum biochemical criteria. Eventual elimination of WHV DNA from the periphery is seen with the appearance of free anti-WHs and following removal of WHV-infected hepatocytes from liver and replenishment with uninfected hepatocytes. Thus, the onset and magnitude of host control is an important determinant for eradication of WHV infection. Understanding the kinetics of virological and immunological responses that are involved in resolution of WHV infection will reveal the mechanisms for the persistence of WHV in chronic infection and thus facilitate the development of strategies for effective antiviral therapy against established chronic HBV infection and its sequelae.

ACKNOWLEDGEMENTS
This work was supported by grant SFB535/A2 from the German Research Foundation (DFG) to D.G. and W.H.G., and by contract N01-AI-05399 from the National Institute of Allergy and Infectious Diseases (NIAID) to the College of Veterinary Medicine at Cornell University.

We gratefully acknowledge the expert assistance of Betty Baldwin, Lou Ann Graham, Erin Graham, David Dietterich, and Dr. Chris Bellezza of Cornell University. We thank Sigrun Broehl and Ulrike Wend from the Institute of Medical Virology, Justus-Liebig University for excellent technical assistance.
REFERENCES


and gene expression in chronically infected woodchucks (Marmota monax).


FIGURES

Figure 1. Course of acute, self-limited WHV infection in adult woodchucks following experimental WHV infection. A. Woodchuck F3538. B. Woodchuck F3539. C. Woodchuck F3524. D. Woodchuck M3540. E. Woodchuck F3391. F. Woodchuck M3553. All woodchucks were infected with $1 \times 10^7$ WID$_{50\%}$ of the WHV7P1 inoculum at week zero. The kinetics of WHV DNA, free WHsAg, and SDH in serum are presented in the graphs. Bars below the graphs indicate the appearance and duration of CICs, anti-WHc, and free anti-WHs in serum. Serum WHV DNA was quantitated by a real time PCR-based assay with a cutoff value of $1 \times 10^3$ WHVge/ml for F3524, F3538, M3540, and of $1 \times 10^2$ WHVge/ml for F3391, F3539, and M3553 as indicated by the blotted line. Free WHsAg was quantitated by electroimmunodiffusion with a detection limit of 2 µg/ml. SDH was quantitated using a Hitachi autoanalyzer. The baseline value for SDH observed in healthy, adult, WHV-negative woodchucks is 40 IU/L. CICs were detected qualitatively by PEG-precipitation followed by Western-blotting. Anti-WHc and anti-WHs were measured qualitatively by ELISA with an assay cutoff value of $\geq 0.05$ optical density units (ODU). -○-, serum WHV DNA (WHVge/ml); ▲, serum WHsAg (µg/ml); ●-, serum SDH activity (IU/L).

Figure 2. Detection of free serum WHsAg during the course of acute, self-limited WHV infection in adult woodchucks. A. Standard dilutions of purified WHsAg and reference serum from chronic WHV carrier woodchuck F5413. Free WHsAg, circulating in serum, was quantitated by Laurell electrophoresis (electroimmunodiffusion). Using
endpoint titration it was estimated that the reference serum contained 729 µg WHsAg per ml. B. 1:5 dilutions of serum from woodchuck F3538 obtained at different time points during the course of WHV infection. Values were compared against a 1:10 dilution of the reference serum from F5413. The cutoff of the assay was defined as 2 µg/ml.

Figure 3. Detection of circulating immune complexes during the course of acute, self-limited WHV infection in adult woodchucks. A. Woodchuck F3538. B. Woodchuck F3539. C. Woodchuck 3524. D. Woodchuck 3391. CICs in serum were detected by PEG-precipitation followed by Western-blotting with rabbit anti-WHs antiserum. Purified serum WHsAg (0.5 µg) was used as a standard. IgG, light (25 kDa) and heavy chains (50 kDa) of immune globulin G; MWHs, WHV middle (pre-S2) surface protein; SWHs, WHV small/major (S) surface protein. Arrows in graph D indicate CICs detected in serum of F3391 at four weeks post-infection.

Figure 4. Correlation of free WHsAg, CICs, and anti-WHs during the course of acute, self-limited WHV infection in adult woodchucks. A. Woodchuck F6239. B. Woodchuck F6143. C. Woodchuck M5015. Woodchucks were infected with $1 \times 10^4$ WID$_{50\%}$ of the cWHV7P2 inoculum at week zero. The kinetics of WHsAg and anti-WHs in serum are presented in the graphs. Bars below the graphs indicate the appearance and duration of CICs in serum. WHsAg and anti-WHs were measured qualitatively by ELISA with an assay cutoff value of $\geq 0.05$ optical density units (ODU). CICs in serum were detected by PEG-precipitation followed by Western-blotting with rabbit anti-WHs antiserum. Purified serum WHsAg (0.5 µg) was used as a standard. IgG, light (25 kDa)
and heavy chains (50 kDa) of immune globulin G; MWHs, WHV middle (preS2) surface
protein; SWHs, WHV small/major (S) surface protein. ●, serum WHsAg (ODU); ○, serum anti-WHs (ODU).

**Figure 5. Detection of circulating immune complexes in adult woodchucks with established chronic WHV infection.** CICs in serum were detected by PEG-precipitation followed by Western-blotting with rabbit anti-WHs antiserum. Purified serum WHsAg (0.5 µg) was used as a standard. IgG, light (25 kDa) and heavy chains (50 kDa) of immune globulin G; MWHs, WHV middle (preS2) surface protein; SWHs, WHV small/major (S) surface protein.

**Figure 6. Comparison of S, M, and L surface proteins of the WHsAg within subviral particles isolated from serum of adult woodchucks with acute, self-limited WHV infection and woodchucks with established chronic WHV infection.** Subviral particles isolated from serum were separated by SDS-PAGE, and the S, M, and L proteins of the WHsAg detected by silver-staining using 0.5 µg of subviral particles (A) or by Western-blotting with a polyclonal rabbit anti-WHs antisera using 1.0 µg of subviral particles (B). LWHs, WHV large (preS1) surface protein; MWHs, WHV middle (preS2) surface protein; SWHs, WHV small/major (S) surface protein.
Table 1. Comparison of serum liver enzyme activities prior to experimental WHV infection and at the time of maximum acute liver injury following infection.

<table>
<thead>
<tr>
<th>Woodchuck No.</th>
<th>Viremia</th>
<th>Serum liver enzyme activities (IU/L)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ALP</td>
<td>ALT</td>
<td>AST</td>
<td>GGT</td>
</tr>
<tr>
<td>F3538</td>
<td>high</td>
<td>4</td>
<td>7</td>
<td>39</td>
<td>46</td>
</tr>
<tr>
<td>F3539</td>
<td>high</td>
<td>11</td>
<td>4</td>
<td>5</td>
<td>62</td>
</tr>
<tr>
<td>F3524</td>
<td>intermediate</td>
<td>3</td>
<td>6</td>
<td>4</td>
<td>62</td>
</tr>
<tr>
<td>M3540</td>
<td>low</td>
<td>6</td>
<td>5</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>F3991</td>
<td>low</td>
<td>6</td>
<td>5</td>
<td>4</td>
<td>13</td>
</tr>
<tr>
<td>M3553</td>
<td>low</td>
<td>17</td>
<td>30</td>
<td>4</td>
<td>9</td>
</tr>
</tbody>
</table>

1 The first value in each column was determined prior to experimental WHV infection at week zero. The second value was determined at the time of maximum serum SDH activity as a marker of acute liver injury. Peak serum SDH activity varied between woodchucks and was detected at four weeks post-infection in M3540, at five weeks post-infection in F3991 and M3553, at ten weeks post-infection in F3524, and at twelve weeks post-infection in F3538 and F3539 (see also Figure 1). IU, international unit.
Table 2. Comparison of WHV antigen expression and histological changes in liver prior to experimental WHV infection and at the time of maximum acute liver injury following infection.

<table>
<thead>
<tr>
<th>Woodchuck</th>
<th>WHV antigen expression (%) and histological changes (scores)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>WHcAg</td>
</tr>
<tr>
<td>------------</td>
<td>-------</td>
</tr>
<tr>
<td>F3538</td>
<td>high</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>F3539</td>
<td>high</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>F3524</td>
<td>intermediate</td>
</tr>
<tr>
<td>M3540</td>
<td>low</td>
</tr>
<tr>
<td>F3991</td>
<td>low</td>
</tr>
<tr>
<td>M3553</td>
<td>low</td>
</tr>
</tbody>
</table>

1 The first value in each column was determined in hepatic specimens obtained two weeks prior to experimental WHV infection. The second value was determined in hepatic specimens obtained at or around the time of maximum serum SDH activity (see Table 1 for details). Liver biopsies were obtained at four weeks post-infection from F3991 and M3553, at eight weeks post-infection from M3540, at ten weeks post-infection from F3538 and F3524, and at twelve weeks post-infection from F3539.

43
Maximum SDH activity for M3540 was at week four post-infection whereas the presented data were derived from liver tissue obtained four weeks after the SDH peak (i.e., at eight weeks post-infection).
Table 3. Comparison of hepatocyte proliferation, apoptosis in hepatocytes, and liver inflammation prior to experimental WHV infection and at the time of maximum acute liver injury following infection.

<table>
<thead>
<tr>
<th>Woodchuck</th>
<th>Liver proliferation, apoptosis, and inflammation (%)</th>
<th>Woodchuck</th>
<th>Liver proliferation, apoptosis, and inflammation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>Viremia</td>
<td>PCNA</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>F3538</td>
<td>high</td>
<td>0.9</td>
<td>11.0</td>
</tr>
<tr>
<td>F3539</td>
<td>high</td>
<td>0.9</td>
<td>2.6</td>
</tr>
<tr>
<td>F3524</td>
<td>intermediate</td>
<td>0.5</td>
<td>8.2</td>
</tr>
<tr>
<td>M3540</td>
<td>low</td>
<td>0.8</td>
<td>1.7</td>
</tr>
<tr>
<td>F3991</td>
<td>low</td>
<td>0</td>
<td>0.2</td>
</tr>
<tr>
<td>M3553</td>
<td>low</td>
<td>0.5</td>
<td>0.8</td>
</tr>
</tbody>
</table>

1 The first value in each column was determined in hepatic specimens obtained two weeks prior to experimental WHV infection. The second value was determined in hepatic specimens obtained at or around the time of maximum serum SDH activity (see Tables 1 and 2 for details).

2 Maximum SDH activity for M3540 was at four weeks post-infection whereas the presented data were derived from liver tissue obtained four weeks later (i.e., at eight weeks post-infection).
Table 4. Comparison of circulating immune complexes with WHs antigenemia, WHV viremia, and anti-WHc response in woodchucks with established chronic WHV infection.

<table>
<thead>
<tr>
<th>Woodchuck</th>
<th>Serum markers of WHV infection and humoral response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CICs (signal strength)</td>
</tr>
<tr>
<td>M6539</td>
<td>+++</td>
</tr>
<tr>
<td>F5954</td>
<td>++</td>
</tr>
<tr>
<td>M6510</td>
<td>++</td>
</tr>
<tr>
<td>F5904</td>
<td>+</td>
</tr>
<tr>
<td>F5927</td>
<td>+</td>
</tr>
<tr>
<td>M5951</td>
<td>-</td>
</tr>
<tr>
<td>F5883</td>
<td>-</td>
</tr>
<tr>
<td>F5890</td>
<td>-</td>
</tr>
<tr>
<td>F5929</td>
<td>-</td>
</tr>
</tbody>
</table>
Woodchucks were infected experimentally as neonates at three days of age with $5 \times 10^6$ WID$_{50\%}$ of the WHV7P1 inoculum. ODU, optical density unit.
Figure 2
Figure 4
Figure 6