

## Core Antigen and Antibody in Woodchucks After Infection with Woodchuck Hepatitis Virus

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**The woodchuck hepatitis virus is a naturally occurring hepatitis B-like virus that infects the eastern woodchuck. Direct immunofluorescence staining for woodchuck hepatitis virus core antigen in liver biopsies demonstrated the presence of this antigen in 14 of 17 chronically infected woodchucks, and in 8 of 10 woodchucks undergoing acute infections. Fluorescent localization of woodchuck hepatitis virus core antigen was typically cytoplasmic, and this was confirmed further by electron microscopy. Experimental infection with woodchuck hepatitis virus was achieved in four of four woodchucks inoculated with serum from chronic carrier woodchucks. All infected animals developed a self-limited disease characterized by seroconversion to antibodies against the major viral antigens (core and surface antigens); naturally acquired acute infection demonstrated a similar course. A chimpanzee seronegative for all markers of hepatitis B virus developed a subclinical infection after inoculation with woodchuck hepatitis virus.**

The eastern woodchuck and the woodchuck hepatitis virus (WHV) are currently under investigation as a naturally occurring model system for hepatitis B virus (HBV) infection in humans. Both WHV and HBV are characterized by similar structural and genomic compositions (9, 11, 12, 28, 29), and infection with either virus can lead to analogous pathology in their respective hosts (22, 26, 27, 29, 30, 31). An important marker for the study of HBV infection in humans has been the detection of viral core antigen in liver biopsies (1, 5, 35). In conjunction with serum antigen and antibody profiles, the status of hepatitis B core antigen in liver tissue is useful in defining the clinical status of disease in humans and chimpanzees (2, 5, 14, 16, 17), and also as a prognostic marker of disease outcome after antiviral therapy (10, 32). Because WHV infection of woodchucks is also associated with disease sequelae (22), the characterization of changes in WHV markers after infection is required to further define the utility of this system as a model for HBV-induced liver disease in humans. In the present study, woodchuck anti-core antibody (anti-WHc) responses and woodchuck hepatitis virus core antigen (WHcAg) expression in the liver were characterized after WHV infection.

### MATERIALS AND METHODS

**Animals.** Woodchucks (*Marmota monax*) were maintained in a colony established in 1978 by the National Institute of Allergy and Infectious Diseases (NIAID) (Meloy Laboratories, Inc., Rockville, Md.). The colony originally consisted of 72 animals trapped in the Delaware, Maryland, and Pennsylvania areas. Twenty-eight more woodchucks from the same areas were added in 1981, together with an additional 38 seronegative animals from New York State (provided by B. Tennent, Cornell University Woodchuck Breeding Colony, New York State College of Veterinary Medicine, Ithaca, N.Y.). The chimpanzee used in this study was from the NIAID primate colony (Meloy).

**WHV transmission.** Adult woodchucks were inoculated with one of two different WHV serum pools. Pool A was prepared by using several DNA polymerase-positive bleedings obtained from a single WHV chronic carrier woodchuck (no. 8). For woodchucks, the inoculum was diluted 1:1,000 and administered intravenously via the femoral vein as a 0.2-ml dose. For the chimpanzee, the inoculum was diluted 1:10 and injected as a 1-ml dose via the cephalic vein. Pool B was a gift of R. L. Synder (Penrose Laboratory, Philadelphia Zoo). The inoculum was diluted twofold and administered intravenously as a 0.5-ml dose.

**Sera and liver biopsies.** Sera were obtained from colony woodchucks on a routine basis or weekly in conjunction with experimental protocols. Sera from an additional 16 chronic carrier woodchucks were supplied by the Cocalico woodchuck farm (Dutchland/Hazelton Laboratories, Denver, Pa.). Sera were stored at  $-70^{\circ}\text{C}$  until assays were performed. Liver biopsies were obtained at selected times from animals under anesthesia, using an 18-gauge Menghini needle. Liver biopsies were obtained for immunofluorescence and electron microscopy (see below).

**Electron microscopy.** For electron microscopy, a portion of each liver sample was fixed with 2.5% glutaraldehyde in cacodylate buffer, postfixed in 1% osmium tetroxide, embedded in Epon, and stained with uranyl acetate and lead citrate.

**Direct immunofluorescence.** Serial bleedings from a WHV chronic carrier woodchuck having high titers of anti-WHc were pooled and centrifuged (5 h, 40,000 rpm,  $4^{\circ}\text{C}$ , 50 Ti Beckman rotor). The immunoglobulin fraction of the supernatant was prepared by ammonium sulfate salt precipitation (40%, 30 min,  $4^{\circ}\text{C}$ , pH 7.0) and dialysis against a carbonate-bicarbonate buffer (pH 9.5). The protein concentration was adjusted to approximately 10 mg/ml, and the preparation was conjugated with fluorescein isothiocyanate (FITC, isomer I, Beckton Dickinson and Co., Cockeysville, Md.) (18). The FITC conjugate was adsorbed overnight with guinea pig and rabbit liver powder (Cappel Laboratories, Cochranville, Pa.). The preparation was centrifuged, and the supernatant was filtered through a 0.45- $\mu\text{m}$  Millipore filter and stored at  $-70^{\circ}\text{C}$ . For use, the FITC conjugate was diluted between

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1:40 and 1:80 with phosphate-buffered saline. Liver tissue samples obtained from biopsies and autopsies were fixed as 5- $\mu$ m cryostat sections, using anhydrous ether (5 min, 25°C). They were then stained with FITC anti-core (25  $\mu$ l, 30 min, 25°C), washed with phosphate-buffered saline (30 min, 25°C), and examined under an epifluorescence photomicroscope III (Carl Zeiss, New York). The stained preparations were observed with a BP 485 interference filter, an FT 510 chromatic splitter, and an LP 520 barrier film. Photographs were taken with ASA/ISO 160/25 tungsten Ektachrome film (Eastman Kodak Co., Rochester, N.Y.).

**Serological assays.** Hepatitis B surface antigen, hepatitis B surface antibody, and hepatitis B core antibody were assayed by Ausria II, Ausab, and Corab, respectively (Abbott Laboratories, North Chicago, Ill.). Serological assays were conducted as described for DNA polymerase (20) and for woodchuck hepatitis virus surface antigen (WHsAg) (7, 8, 34) and antibody (anti-WHs) (34). Serum WHV DNA was detected with a blot-hybridization procedure similar to that developed for HBV DNA (4). Anti-WHc was determined by competitive inhibition radioimmunoassay (A. Ponzetto, P. Cote, R. Engle, J. Cicmanee, M. Shapiro, R. Purcell, and J. Gerin, submitted for publication). Briefly, microtiter plate wells were coated with a 1:1,000 dilution of anti-WHc serum (centrifuged as described above), incubated with a standard amount of WHcAg-positive liver homogenate, and washed free of unbound antigen. For the anti-WHc assay, 10  $\mu$ l of test sera was added to the wells with 90  $\mu$ l of  $^{125}$ I-labeled anti-WHc immunoglobulin ( $2 \times 10^5$  cpm per well, 18 h, 25°C). Samples demonstrating 50% or greater inhibition of binding of  $^{125}$ I-labeled anti-WHc were considered positive.

The anti-WHc titer was the final serum dilution producing 50% inhibition of binding of the iodinated probe.

## RESULTS

**Specificity and use of the immunofluorescence test for core antigen.** The specificity of the immunofluorescence (IF) test was shown by complete inhibition of fluorescence staining of a standard liver substrate with high-titer anti-WHc serum and by adsorption of FITC-conjugated anti-WHc activity with a core antigen-rich preparation from infected woodchuck liver. The activity was not adsorbed by preparations made from uninfected liver. Furthermore, kidney, pancreas, spleen, and gonads from a WHV chronic carrier woodchuck were not stained with the FITC anti-WHc (data not shown). In addition, the same anti-WHc preparations were used in the RIAs for WHcAg and anti-WHc, and no cross-reactivity with WHsAg and anti-WHs was detected (Ponzetto et al., submitted for publication).

Two hundred liver specimens collected from 57 woodchucks were examined for core antigen by IF. Livers of uninfected and convalescent woodchucks were WHcAg-negative, whereas the antigen was demonstrated in more than 80% of the animals undergoing acute and chronic infections (8 of 10 and 14 of 17, respectively). Hepatitis B core antigen-positive chimpanzee livers were not stained by the FITC anti-WHc; however, WHcAg was stained weakly in some woodchuck livers with an FITC hepatitis B core antibody conjugate (13).

**Distribution of core antigen in hepatocytes.** Core antigen was present almost exclusively in the cytoplasm of hepatocytes from chronic carrier woodchucks and from wood-

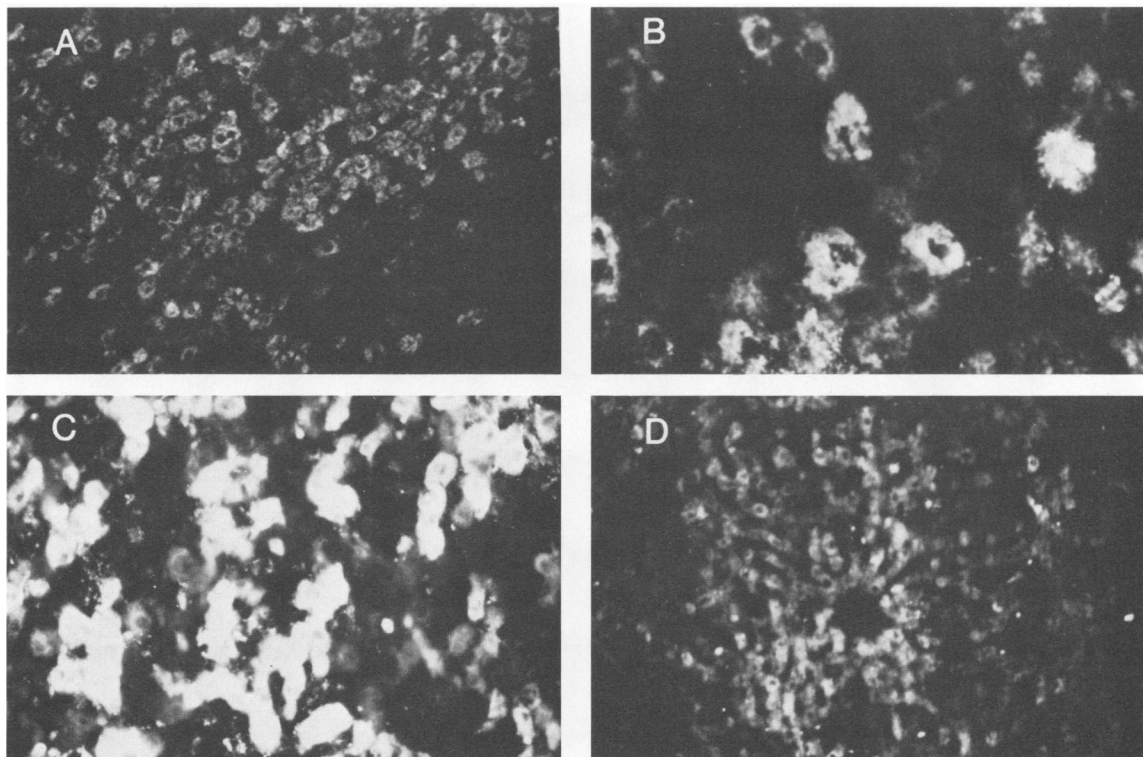


FIG. 1. Localization of WHcAg by IF in woodchuck hepatocytes. Shown are liver specimens from WHV chronic carrier woodchucks no. 7 (A;  $\times 160$ ) (B;  $\times 400$ ) and no. 69 (C;  $\times 250$ ), and from woodchuck no. 15 during the acute phase of WHV infection (D;  $\times 160$ ).

chucks undergoing acute infection. The fluorescence staining was of a fine granular type throughout the entire cytoplasm, sometimes appearing confluent over the entire cell (Fig. 1A to D). Perinuclear fluorescence without staining of the remaining cytoplasm was observed in early acute cases. The perinuclear ring disappeared with progression of the disease, which was characterized by a more diffuse staining pattern. As a rule, nuclei were stained only faintly in hepatocytes of animals undergoing acute infections and were negative in chronic carriers. These observations were confirmed by electron microscopy; the core structures were found throughout the cytoplasm in association with the endoplasmic reticulum (Fig. 2). On the average, ca. 50% of hepatocytes were stained in infected animals; however, a greater proportion of cells were stained in animals undergoing acute infection compared with chronic carriers.

**Distribution of core antigen in livers.** The distribution of WHcAg in livers of infected animals varied considerably. During acute infections, antigen expression was diffuse and homogeneously distributed throughout the organ; this was demonstrated by staining several areas of autopsied woodchuck livers obtained during the acute phase of infection. In contrast, core antigen expression was found in localized areas of the liver in chronic carriers; other areas of the liver were core antigen negative. Hepatoma tissue obtained from 10 animals was negative for WHcAg, even though core antigen expression was found in localized areas of nontumor tissue. In two of the three core antigen-negative chronic

carrier animals, a large proportion of the liver tissue was replaced by hepatoma.

**Natural and experimental infection of woodchucks with WHV.** The serological events following inoculation of a woodchuck with WHV pool A are shown in Fig. 3; the serum WHsAg and anti-WHs profiles of this animal were described previously (34). The animal developed serum WHsAg by week 6, followed by seroconversion to anti-WHs. WHs antigenemia was associated with the appearance of intrahepatic WHcAg, although liver WHcAg was detected over a somewhat longer period of time compared with that of serum WHsAg. The latter may be due to a greater protraction of liver WHcAg expression relative to WHs antigenemia or to a greater sensitivity of the former assay. Both liver WHcAg and serum anti-WHc were detected during the period of WHs antigenemia. Liver WHcAg disappeared coincident with seroconversion to anti-WHs. In this animal, anti-WHc preceded the appearance of anti-WHs, analogous to the hepatitis B core and surface antibody patterns in human HBV infection (15). In a second animal inoculated with the same WHV pool, surface antigenemia was not detected, but the woodchuck developed brisk antibody responses to the core and surface antigens starting at weeks 5 and 12, respectively (data not shown); liver core antigen was detected by IF at both 6 and 7 weeks postinoculation. Thus, serum anti-WHc was a more sensitive indicator of WHV infection than was serum WHsAg.

Two additional woodchucks were inoculated with the

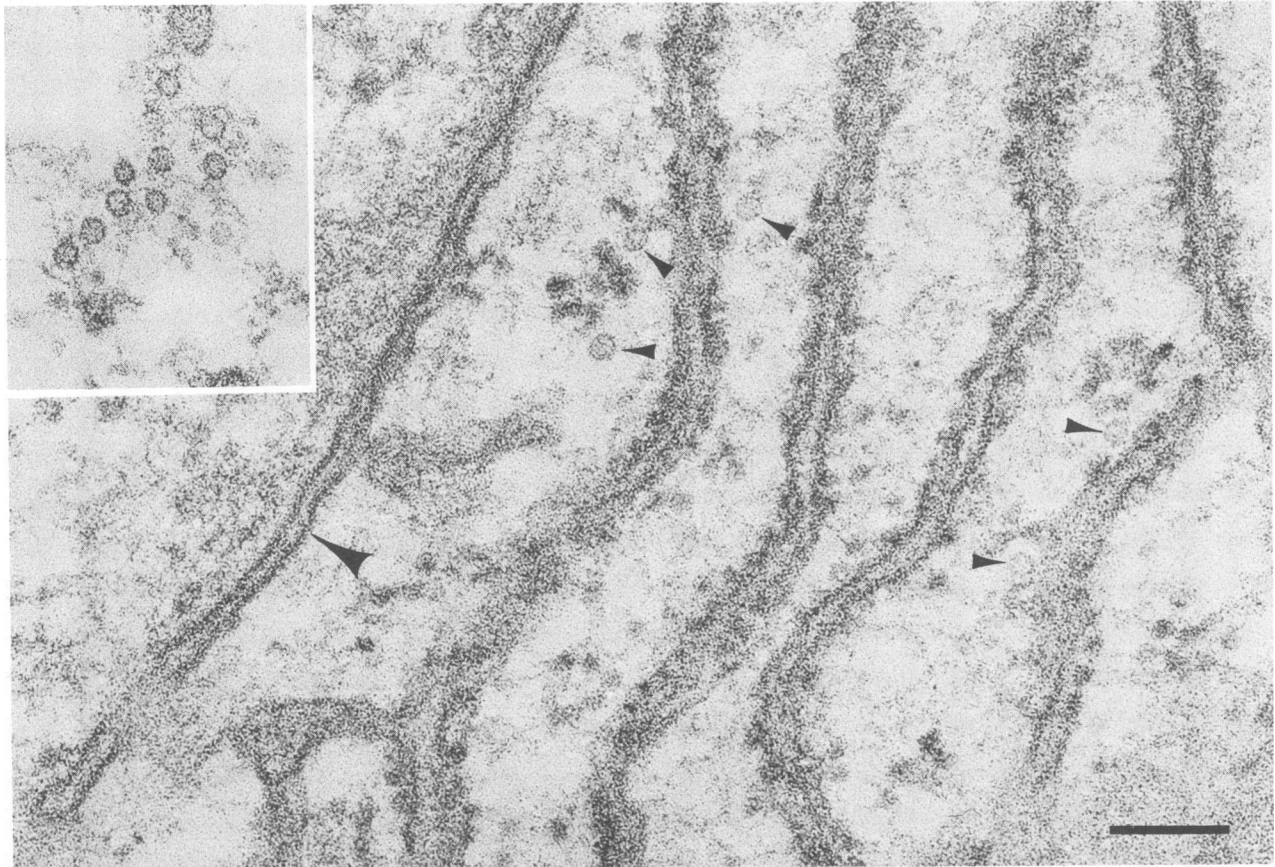


FIG. 2. Electron micrograph of a WHV-infected hepatocyte. The liver specimen was obtained from woodchuck no. 15 at autopsy. WHV core particles are shown in the cytoplasm of the cell ( $\times 150,000$ ); the inset shows a cluster of core particles within the endoplasmic reticulum ( $\times 150,000$ ). The large arrow identifies the nuclear membrane, and the small arrows indicate core particles. Bar = 100 nm.

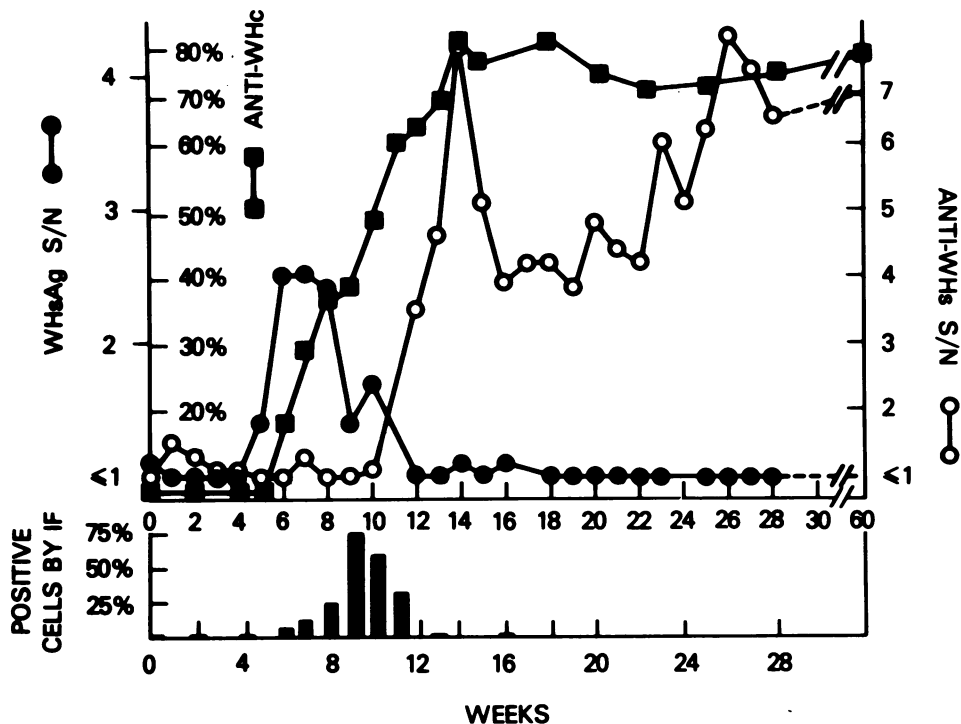


FIG. 3. Liver core antigen expression and serum anti-WHc activity during acute experimental WHV infection. Woodchuck no. 12 was inoculated with WHV serum pool A; values shown in the figure for WHsAg and anti-WHs were derived in a previous study (34).

WHV pool B (Fig. 4). Again, only one of the two woodchucks developed WHs antigenemia (Fig. 4A). The animal developing WHs antigenemia seroconverted to low-level anti-WHs that was only detected by double antibody ra-

dioimmune precipitation (data not shown). The second woodchuck that was surface antigen negative developed an anti-WHs response by week 8 (Fig. 4B).

Liver WHcAg was not assessed in these studies. Preinoc-

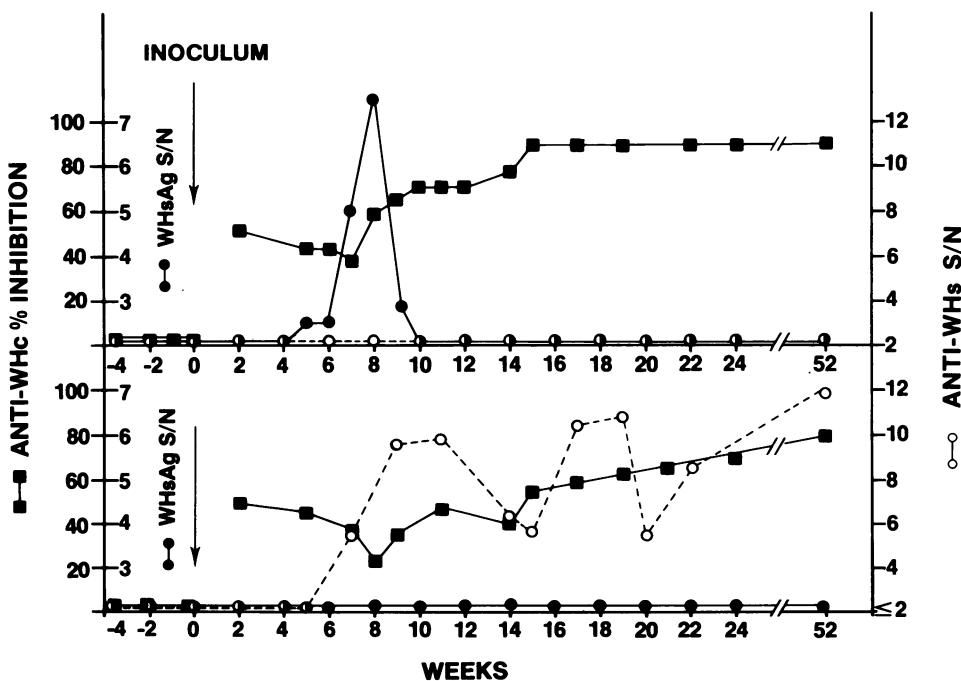


FIG. 4. Variation in the course of acute experimental WHV infection. Data were derived after experimental infection of woodchucks no. 3 (upper) and no. 2 (lower) with WHV serum pool B. Anti-WHc in the first sample postinoculation was passively acquired from pool B serum.

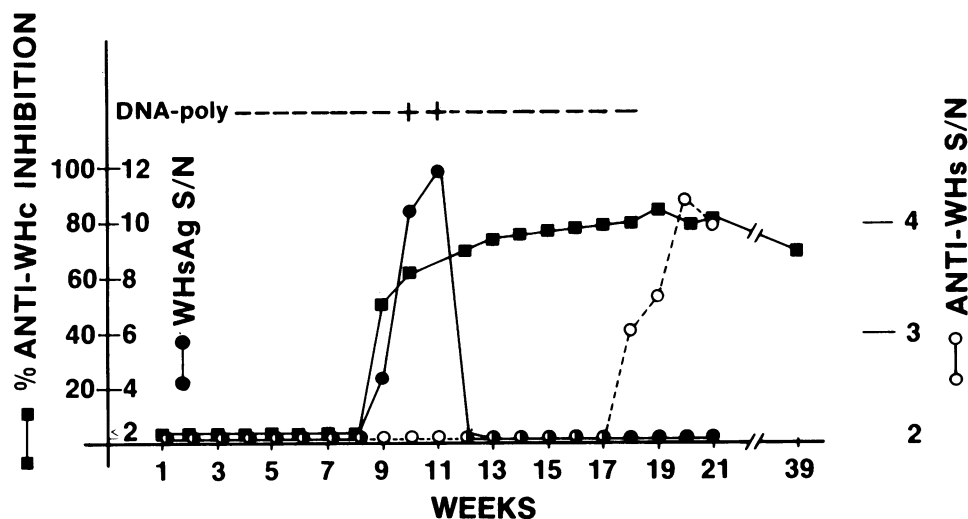


FIG. 5. Course of a naturally occurring acute WHV infection. Data were derived from woodchuck no. 16 after contact infection.

ulation sera were negative for anti-WHc. Low levels of anti-WHc activity (1:10 titer) were detected in both animals at 2 weeks postinoculation; these levels decreased passively and probably represented anti-WHc acquired from the relatively large volume of the pool B inoculum. The subsequent rise in anti-WHc titers in both woodchucks indicated successful transmission of WHV; the titers of anti-WHc at weeks 20 (Fig. 4A) and 24 (Fig. 4B) were 1:10,000 and 1:320, respectively (data not shown). In view of the variable sensitivities of different assays for anti-WHs, it appeared that serum anti-WHc was the more reliable marker of prior WHV infection.

The serological course of a contact infection in one woodchuck is shown in Fig. 5. Liver core antigen was detected at weeks 9, 14, 15, and 16, but not at week 18. This pattern of infection demonstrates again that liver core antigen expression can occur well beyond the disappearance of WHsAg from the serum. Hepatic expression of WHcAg in this animal was detected up to the time of seroconversion to anti-WHs. Viremia, as determined by serum DNA polymerase activity, was apparently limited to the period of WHs antigenemia. In HBV infection, however, the detection of infectious virus in serum is often beyond the limits of sensitivity of both DNA polymerase and surface antigen assays (15). Thus, coincident liver core antigen and serum

anti-WHc are good indicators of ongoing WHV infection in the woodchuck animal model.

A survey of colony woodchucks revealed that endpoint titers for anti-WHc were higher in animals with chronic and acute phase infections than in those convalescent or recovered from WHV infection. Twenty-eight chronic WHV carrier woodchucks had anti-WHc titers between  $10^{-4}$  and  $10^{-6}$ . Twenty-eight WHsAg-negative woodchucks with anti-WHs (recovered animals) had anti-WHc titers between  $10^{-1}$  and  $10^{-3}$ , with only one such animal having a titer approaching  $10^{-4}$ . Three woodchucks in the late phase of an acute experimental WHV infection (convalescent animals) had anti-WHc titers of  $10^{-4}$  (Fig. 6).

**Apparent experimental transmission of WHV to a chimpanzee.** The standard WHV inoculum shown to be infectious in woodchucks at a  $10^{-3}$  dilution (pool A) was diluted 1:10 and administered in 10-fold-greater volume to an HBV-seronegative chimpanzee. Surface antigenemia was not evident, but the animal developed anti-WHc and then anti-WHs, suggesting productive but subclinical WHV infection (Fig. 7). There were no changes in serum enzymes in this animal. The sera were not reactive in the Corab and Ausab assays.

## DISCUSSION

IF analysis and electron microscopy demonstrated that WHcAg was located predominantly in the cytoplasm of WHV-infected hepatocytes. This feature contrasts in part with that found in HBV infection of primates, in which core particles are found primarily in the hepatocyte nuclei (1, 14, 17). The greater cytoplasmic expression of WHcAg could indicate more rapid coupling between core assembly and the exit of assembled core particles from the nucleus in the woodchuck model. Hepatitis B core antigen has been observed occasionally in the cytoplasm of infected human hepatocytes by either IF or electron microscopy (19, 21, 23, 35, 36); also, in situ hybridization studies have shown that HBV DNA is found in the hepatocyte cytoplasm (6). This probably reflects assembly of HBV virion in the cisternae of the endoplasmic reticulum (19, 36). The woodchuck core particles may be more rapidly assembled into virion by virtue of their greater preponderance in the cytoplasm; this could account for the generally higher titers of virion-

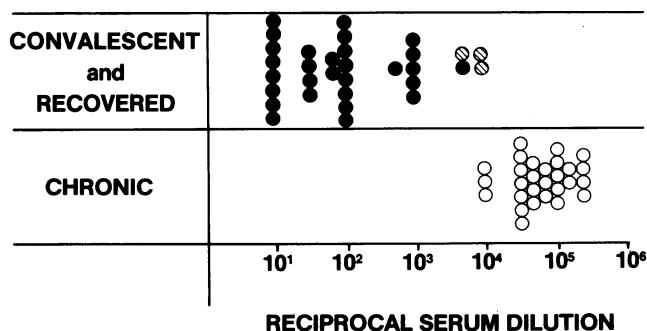


FIG. 6. Anti-WHc titers in WHV-infected woodchucks. Solid circles represent woodchucks recovered from WHV infection; hatched circles represent woodchucks in the late phase of an acute infection; open circles represent woodchucks chronically infected with WHV.

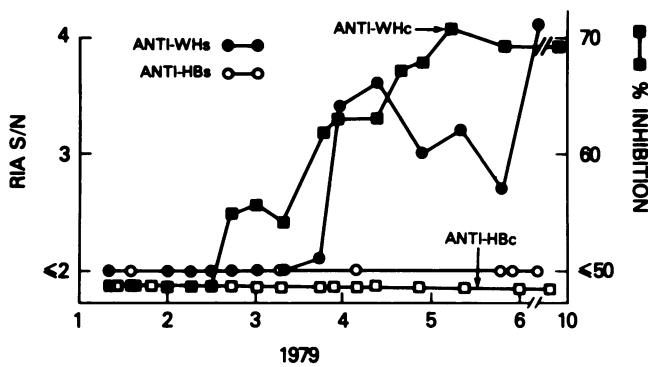


FIG. 7. Apparent transmission of WHV to a chimpanzee. Values shown in the figure for WHsAg and anti-WHs were derived in a previous study (34).

associated DNA polymerase found in sera of chronic WHV carrier woodchucks compared with chronic HBV carrier chimpanzees and humans (24).

The percentage of core antigen-positive liver biopsies from chronically infected woodchucks (82%) was somewhat higher than that found overall among chronic HBV carriers (14, 23). In humans, chronic HBV infection may be inactive or active, the latter form being characterized by persistent virion-associated DNA polymerase in the circulation. A high percentage of patients with chronic active HBV infections express liver core antigen (1, 14). We and others have found that DNA polymerase persists in the circulation of chronic carrier woodchucks (22, 24) and that a high percentage of these animals demonstrate liver core antigen. Therefore, the chronic hepatitis found in the woodchuck more closely resembles the aggressive form of chronic type B hepatitis of humans (type IV according to Bianchi and Gudat [5]).

The availability of recently developed serological assays allowed us to correlate liver WHcAg expression with serum markers of WHV infection. Present observations indicate that the expression of liver core antigen and the appearance of serum anti-WHc are more closely associated with each other than either is to serum WHsAg. Because WHs antigenemia may not be detected during some infections, it appears that serum anti-WHc and liver core antigen expression are the better markers to indicate acute WHV infection. Summers et al. (30) detected anti-WHs before anti-WHc in experimentally infected woodchucks; however, our results show an early anti-WHc response followed by anti-WHs; the latter is more consistent with the hepatitis B core and surface antibody patterns in humans after acute HBV infection. Although the anti-WHs response in one woodchuck was not detected by the solid-phase RIA used previously (34) (Fig. 4A), the response was detected by a recently developed radioimmuno precipitation assay employing  $^{125}\text{I}$ -labeled WHsAg and rabbit anti-woodchuck immunoglobulin G (25); the first anti-WHs was noted 2 weeks after WHs antigenemia and the initial rise in serum anti-core titer. Moreover, the core-anti-core window demonstrated in one naturally infected woodchuck (Fig. 5) resulted from a delay in the anti-WHs response after WHs antigenemia. The core-anti-core window has been observed in the chimpanzee model of HBV infection (2, 3) and is also typical of acute HBV infections in humans.

It appears that serum anti-WHc can serve as a useful marker to indicate both ongoing and prior WHV infection when liver core antigen is not assessed or detected. For

example, not all WHV-infected woodchucks having anti-WHc had detectable WHcAg in the liver; the latter may be related to differences in detection sensitivities, sampling of core-negative regions of the liver, or virus dose. Also, we show that higher endpoint titers for anti-WHc are evident during chronic phase infection as compared with the recovery period (Fig. 6). Thus, the detection of anti-WHc (or a rise in anti-WHc titer) represents a useful indicator of successful WHV transmission, which obviates the need for invasive biopsy techniques. The overall observations correlate well with those found in chimpanzees infected with HBV (2, 3, 16), and, therefore, further support the usefulness of the WHV system in modeling HBV infection in humans.

The seroconversion to anti-WHc by a chimpanzee inoculated with the WHV was presumed to indicate successful transmission. Liver biopsies were not available for observation, and the lack of detectable surface antigenemia and of increased serum enzymes suggested that the animal experienced a subclinical infection. Although cross-reactivities between the major antigen-antibody systems of WHV and HBV have been reported (7, 8, 11, 13, 33), we did not detect cross-reactive antibodies in this chimpanzee when we used the commercial HBV RIA systems. The antibody responses of the chimpanzee, detected by the present woodchuck-specific RIAs (25, 34; Ponzetto et al., submitted for publication), indicated that the responses were specific for WHV infection. The apparent susceptibility of this single chimpanzee to WHV would be the first demonstration of interspecies cross-infectivity among hosts of the hepadnavirus family. In all likelihood, the potential susceptibility of primates to WHV infection has little epidemiological significance for humans; however, precautions should be taken in the handling of WHV infectious material until further studies on WHV host range are completed.

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