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Woodchuck Hepatitis Virus Infections: Very Rapid Recovery after a Prolonged Viremia and Infection of Virtually Every Hepatocyte

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Earlier studies have suggested that transient hepadnavirus infections in mammals are associated with virus replication in a large fraction of hepatocytes. Although the viremia that occurred during transient infections in some individuals would presumably lead to virus replication in all hepatocytes, these studies did not reveal if this was the case. The question of the extent of hepatocyte infection was therefore reinvestigated because of the implications of the results for the mechanisms of virus clearance. Woodchucks were inoculated with woodchuck hepatitis virus, and the course of hepatic infection was determined. These studies indicated that essentially 100% of the hepatocytes became infected in the majority of woodchucks. In 7 of 10 woodchucks, the viral infection was then rapidly cleared from the liver, generally in less than 4 weeks. In another three woodchucks, though productive infection was just as rapidly cleared, viral covalently closed circular DNA remained for weeks to months after other indicators of virus infection had disappeared from the liver. Bromodeoxyuridine labeling and anti-proliferating cell nuclear antigen staining to detect hepatocytes passing through S phase indicated an increase in hepatocyte proliferation during the recovery phase of infection. The rate of cell division appeared to be sufficient to replace no more than 2 to 3% of the hepatocytes per day, at the times at which the biopsies were performed. Histopathologic evaluation of the biopsy samples did not provide evidence for a massive amount of liver regeneration. Models to explain virus clearance, with or without massive immune system-mediated destruction of infected hepatocytes, are reviewed.

Hepatitis B virus infections may be either transient or chronic. In the transient form, liver disease arises and then ceases in a short period without any residual pathological findings (2, 3, 16, 27). In the persistent form, the infection may remain throughout the lifetime of the patient, with chronic disease and ultimately the development of cirrhosis and hepatocellular carcinoma. It is generally believed that in transient infections the virus is cleared from the host by immune mechanisms that result in the destruction of infected hepatocytes (5). However, there is no definitive evidence of how differences in the immune responses to transient and persistent infections serve to differentiate the ultimate outcome for the host.

In a previous study, we found that infection of adult ducks with duck hepatitis B virus is predominately of the transient form and that these infections could be rapidly cleared even if the majority of the hepatocytes were infected (18). Clearance occurred with only mild elevations of liver enzyme levels in the bloodstream, suggesting that cytolysis of liver cells was not the major factor in the disappearance of infected hepatocytes and that most infected hepatocytes were either cured of the infection or were destroyed by a noncytolytic process. Our findings did not serve to distinguish between the relative contributions of cell death and noncytocidal processes for elimination of virus from the hepatocyte population of the liver, though it was clear from analyses of duck liver biopsy samples that, as in transient human hepatitis B virus infection, cell death via eosinophilic degeneration, presumed to reflect apoptosis (6), was enhanced as a result of infection.

In contrast to the situation with the transiently infected ducks, in which viremia was generally of very short duration (<1 week) (18), transient hepadnavirus infections in mammals often involve a viremia with a duration of many weeks or even months (10, 19, 23, 25, 27). Nonetheless, as in the case of the ducks, most hepadnavirus infections induced in adult mammals are transient. This raises several issues concerning these transient hepadnavirus infections in mammals. (i) Can all hepatocytes be infected, or is there a need for infection to be restricted from a significant fraction of the hepatocytes in order for an infection not to become chronic? If so, how large must this fraction be? Earlier studies suggested that >50 to 75% of the hepatocytes might be infected (2, 3, 16, 27). (ii) Is there any significant reduction in the proportion of virus-infected hepatocytes in the liver while a viremia is still present, i.e., can recovery begin through the generation of hepatocytes that are resistant to de novo infection? Alternatively, does virus clearance from the liver only begin after sufficient antibodies are produced to inactivate all virus in the circulatory system? (iii) If all hepatocytes become infected, what is the origin of the uninfected hepatocytes found in the liver following recovery of the host from the infection?

To begin to study these issues, we inoculated adult woodchucks with a dose of woodchuck hepatitis virus (WHV) sufficient, in theory, to infect the majority of liver cells. The subsequent course of infection was monitored through analyses of blood samples taken weekly and through periodic liver biopsies.

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MATERIALS AND METHODS

Woodchucks and WHV. Experiments with woodchucks were reviewed and approved by the Institutional Animal Care and Use Committee of the Fox Chase Cancer Center. Woodchucks, ca. 1 year of age and negative for serologic markers of past or current WHV infection, were purchased from Northeastern Wildlife (South Plymouth, N.Y.). Woodchucks chronically infected with WHV were obtained from the same source. To study transient infection, serologically negative woodchucks were inoculated intravenously with 7.5 to 10 ml of pooled WHV-positive sera from infected woodchucks. Two different serum pools were used in these studies. Woodchucks 22, 23, and 25 were inoculated with serum from pool 1, and woodchucks 33, 34, 35, 36, 38, 40, and 41 were inoculated with serum from pool 2. Both had WHV titers of ca. 5×10^9 virions per ml, as assessed by Southern blot assays for viral DNA in the virions.

Transient infection protocol. Woodchucks were bled and subjected to liver biopsies 2 to 4 weeks before WHV inoculation and at various intervals postinfection. In some cases, the woodchucks were injected with bromodeoxyuridine (BUdR) (50 mg/kg) 8 h prior to biopsy to label hepatocytes in S phase. The procedure for liver biopsy was essentially as described previously (4), except that anesthesia was achieved with a 60-mg/kg intramuscular dose of ketamine-Rompun. Liver biopsy specimens (ca. 0.2 to 0.4 g) were divided into three parts. One aliquot was fixed in formalin overnight; another was fixed for 20 min in acetic acid-ethanol (1:3) and then overnight in ethanol, for immunomicroscopy; and the third was stored at −80°C for subsequent extraction of total and covalently closed circular (CCC) viral DNAs. The procedures for fixation, paraffin embedding, and subsequent processing of tissue sections have been described in detail previously (18). Blood (ca. 1 ml) was drawn from the femoral vein after sedation of the woodchucks with 30 mg of ketamine-Rompun per kg. The serum was collected and stored at −80°C.

Extraction and analyses of viral DNAs in the serum and liver tissue of WHV-infected woodchucks. To detect virus in serum, 50-μl aliquots were layered on 4-ml step gradients of sucrose (10 and 20% [wt/vol]) in 0.15 M NaCl–0.02 M Tris-HCl (pH 7.5), and virus particles were collected by ultracentrifugation for 3 h at 50,000 rpm in an SW-60 rotor (Beckman) at 4°C. The pellets were drained, resuspended in 50 μl of 2 mg of pronase per ml–0.1% (wt/vol) sodium dodecyl sulfate–0.1 M NaCl–0.01 M Tris-HCl (pH 7.5)–0.01 M EDTA, and incubated for 1 h at 37°C. The samples were then subjected to electrophoresis in 1.5% agarose gels and subsequent transfer to nitrocellulose filters, essentially as described previously (32).

DNA extracted from liver biopsy specimens was prepared for electrophoresis and filter blot hybridization as described previously (18). Briefly, 20 to 100 mg of liver tissue was disrupted in 1.5 ml of 0.01 M Tris-HCl (pH 7.5)–0.01 M EDTA by using a Dounce homogenizer with a loose-fitting pestle. A 10-μl aliquot was stained with ethidium bromide, and nuclear counts were determined in a hemocytometer under fluorescent illumination (580 nm). The remainder was divided into two parts and used for the preparation of total DNA- and CCC DNA-enriched fractions (18), respectively. The concentration of DNA in the total DNA fraction was determined by the procedure of Labarca and Paigen (22). Following hybridization of blots with a ^{32}P-labeled DNA probe, the bound radioactivity was determined by using either an AMBIS radioanalytic imaging system or a Fuji Image Analyzer.

Immunoperoxidase staining and in situ hybridization (ISH) of tissue sections. Paraffin sections containing tissue fixed with

TABLE 1. Time course of viremia in woodchucks inoculated with WHV

No. of wks postinoculation	Virus titer, 10^9 virions/ml (neutralization score) for woodchuck[a]:									
	22	23	25	33	34	35	36	38	40	41
0	0 (−)	0 (−)	0 (−)	0 (−)	0 (−)	0 (−)	0	0 (−)	0 (−)	0 (−)
1	0.2	0.02	0.04	0.007	0.054	0.14	0	0	0	0
2	1.3	0.4	0.3	0.002	0.01	2.4	0.2	0.3	0.005 (+/−)	0.07
3	2.8	0.6	0.7	0.017	0.004	3.8	2.7	5.2	0	0.6
4	3.7	0.6	1.2 (+)	0.004	0 (−)	1.6	1.4	ND[b]	0 (−)	2.3
5	2.9	0.4 (−)	0.2 (+/−)	0	0	2.9	1.2	4.5	0	2.5
6	3.3	0.06 (+)	0 (+)	0	0	1.0	1.3	4.0 (−)	0 (−)	0.7 (−)
7	0.9	0 (+++)	0 (+++)	0	0 (−)	0.3	0.6	4.8	0	0 (−)
8	0.9	0 (+++)	0 (+++)	0	0	0.06	0.2	5.9	0	0 (−)
9	0.2 (+/−)	0	ND	0 (+/−)	0	0.01 (+++)	0	5.6	0	0
10	ND (+/−)	ND	ND	0	0 (−)	0 (+++)	0	3.9	0	0 (−)
11	0	0	ND	ND (+/−)	ND (++)	0	0	4.2		
12	ND	ND	0		ND (++)			ND		
13	ND	ND	ND	ND (+/−)				1.5 (−)		
14	0 (+/−)	0	0					ND		
15								0.7		
16	ND (+/−)							0.03 (−)		
17								0		
18								0 (+)		
19								ND		
20								0 (++)		
21								0		

[a] Virus titers were determined by quantitating, by Southern blot hybridization, the amount of viral DNA contained in virus particles collected from serum samples by ultracentrifugation. Virus neutralization (i.e., the ability of serum samples to block infection of woodchuck hepatocyte cultures by a standard virus preparation) was determined by mixing 25 μl of serum with 10 μl of virus (titer, ca. 10^9 virions per ml), incubating the mixture for 1 h at 37°C, and then adding it to primary woodchuck hepatocytes. Virus infectivity was determined by quantitating viral DNA present in cells after 2 to 3 weeks of culture in medium containing suramin, added beginning at 1 day postinoculation, to prevent secondary rounds of infection (26). −, <25% inhibition of infection; +/−, 25 to 50% inhibition of infection; +, 50 to 75% inhibition of infection; ++, 75 to 90% inhibition of infection; +++, >90% inhibition of infection.

[b] ND, not determined.

acetic acid-ethanol were processed for anticore staining by the peroxidase antiperoxidase assay, as previously described (18). Rabbit serum reactive to WHV core protein synthesized in *Escherichia coli* was used as the primary antibody to detect WHV-infected cells in the liver sections and was the generous gift of Christoph Seeger (Fox Chase Cancer Center). BUdR-labeled DNA was detected in acetic acid-ethanol-fixed liver sections by using a monoclonal antibody reactive to the BUdR ligand (Boehringer-Mannheim), as described previously (24). Hepatocyte nuclei containing proliferating cell nuclear antigen (PCNA) were detected in sections of formalin-fixed liver tissue by incubation with mouse monoclonal anti-PCNA immunoglobulin (DAKO) in 0.1% bovine serum albumin in phosphate-buffered saline (PBS) at 37°C for 1 h and overnight at 4°C, followed by detection of the first antibody with peroxidase-labeled rabbit anti-mouse immunoglobulin G and visualization with 0.5 mg of diaminobenzidine per ml in 0.03% H_2O_2 in PBS at room temperature for 5 min. Sections were counterstained with hematoxylin, dehydrated in ethanol, and mounted in Depex. In situ hybridization was performed essentially as described before (18), except that the hybridization probe contained 2.5 ng of digoxigenin-dUTP (DIG-dUTP; Boehringer-Mannheim)-labeled DNA representing the complete WHV genome per μl. Probe was prepared by the random priming method. Plasmid pUC19 DNA similarly labeled with DIG-dUTP was used as a control for hybridization specificity. Visualization of DIG-dUTP was performed according to the manufacturer's instructions by immunoreaction with alkaline phosphatase-labeled anti-DIG-dUTP immunoglobulins, followed by detection with 5-bromo-4-chloro-3-indolyl-phosphatase and nitroblue tetrazolium salt. Following development, sections were counterstained with hematoxylin, rapidly dehydrated in ethanol, washed in xylene, and mounted in 66% Depex in xylene.

Histopathology. Liver sections stained with hematoxylin and eosin were graded by a subjective scale. Liver injury was assessed on the basis of the degrees of hepatic inflammation, hepatocyte death, vacuolization, biliary hyperplasia, Kupffer cell activation, and variation in hepatocyte nuclear size. On the basis of these criteria, a score was assigned as follows: −, no evidence of inflammation; +/−, scant numbers of inflammatory cells in portal tracts with minimal inflammation in the liver parenchyma; +1, mild accumulations of lymphocytes in portal areas and focal accumulations in the parenchyma, with individual hepatocyte necrosis, Kupffer cell aggregates, and variation in hepatocyte nuclear size also present; +2, moderate inflammation of portal tracts with sites of extension into the terminal distributing vasculature; and +3, moderate to extensive inflammatory infiltrate extending from the portal tract into adjacent parenchyma or portal inflammation accompanied by moderate to extensive parenchymal inflammation.

Serology. Serum samples were analyzed for sorbitol dehydrogenase (SDH) levels with a multichannel analyzer (Monarch 2000; Instrumentation Laboratory, Lexington, Mass.). Results in Fig. 7 are expressed in international units per liter.

The ability of woodchuck sera to block infection of woodchuck hepatocyte cultures by WHV (e.g., because of the presence of virus-neutralizing antibodies) was assayed essentially as described previously (18). Primary cultures of woodchuck hepatocytes were prepared, infected, and maintained as described previously (1), with the exception that the hepatocytes were purified by low-speed centrifugation through 90% Percoll (Sigma, St. Louis, Mo.) after filtration of the dissociated liver tissue through cheesecloth. Ten microliters of a WHV-positive serum containing ca. 10^9 virions per ml was mixed with 25 μl of the serum to be tested, and the mixture was incubated for 1 h at 37°C. The mixture was then used to infect the cultures. Suramin was added at 1 day postinfection to prevent the spread of virus within the cultures (26). The extent of infection was quantitated by assaying for replicative forms of viral DNA within the infected hepatocytes.

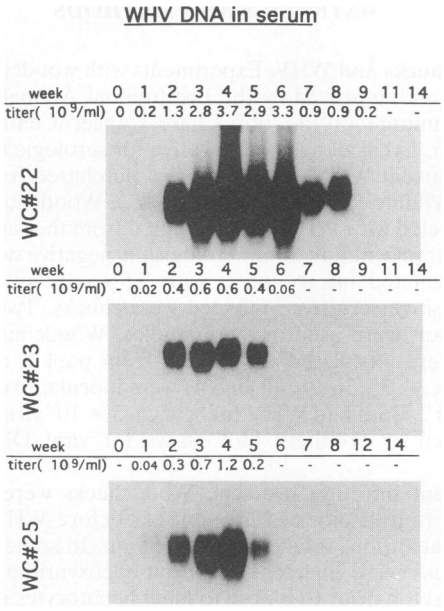

FIG. 1. Time course of viremia in experimentally WHV-infected woodchucks. Woodchucks were inoculated with ca. 7×10^{11} virions of WHV. Blood samples were taken at the indicated times postinoculation, serum was collected, and virus in 50-μl aliquots of sera was pelleted by ultracentrifugation. Viral DNA was detected by electrophoresis and filter blot hybridization, and the amount of viral DNA (equivalents of 3 kbp of viral DNA) was determined by comparison with a cloned hybridization standard. The approximate viral titers predicted by this assay are indicated. WC, woodchuck.

RESULTS

Inoculation of woodchucks with a large dose of WHV produced a rapidly appearing, high-titer viremia. In order to achieve rapid infection of the liver, we used a procedure previously found useful in infecting adult ducks with duck hepatitis B virus (18). Woodchucks were infected with ca. 5×10^{10} virions, which, if completely infectious and efficiently taken up by the hepatocytes, would be sufficient to infect a large fraction of the hepatocytes, assuming that there are ca. 10^{10} hepatocytes in the adult woodchuck liver. In the majority of woodchucks, a viremia was detected in the first week postinoculation, with titers ranging from 2×10^7 to 2×10^8 virions per ml (Fig. 1; Table 1). Whether this very early viremia was derived from the inoculum or from de novo production of virus was not determined. In most woodchucks, virus levels in the serum increased dramatically over the next few weeks, reaching titers in excess of 10^9 virions per ml. Virus titers then began to drop dramatically in most woodchucks beginning at 4 to 6 weeks postinoculation, and they fell to undetectable levels within a few weeks. Attempts were made to correlate this decline with the appearance of antibodies that could block virus spread, by using the infection of woodchuck hepatocyte cultures (1) to detect this blocking activity essentially as described previously (18). As summarized in Table 1, we were able to detect blocking activity in some but not all of the

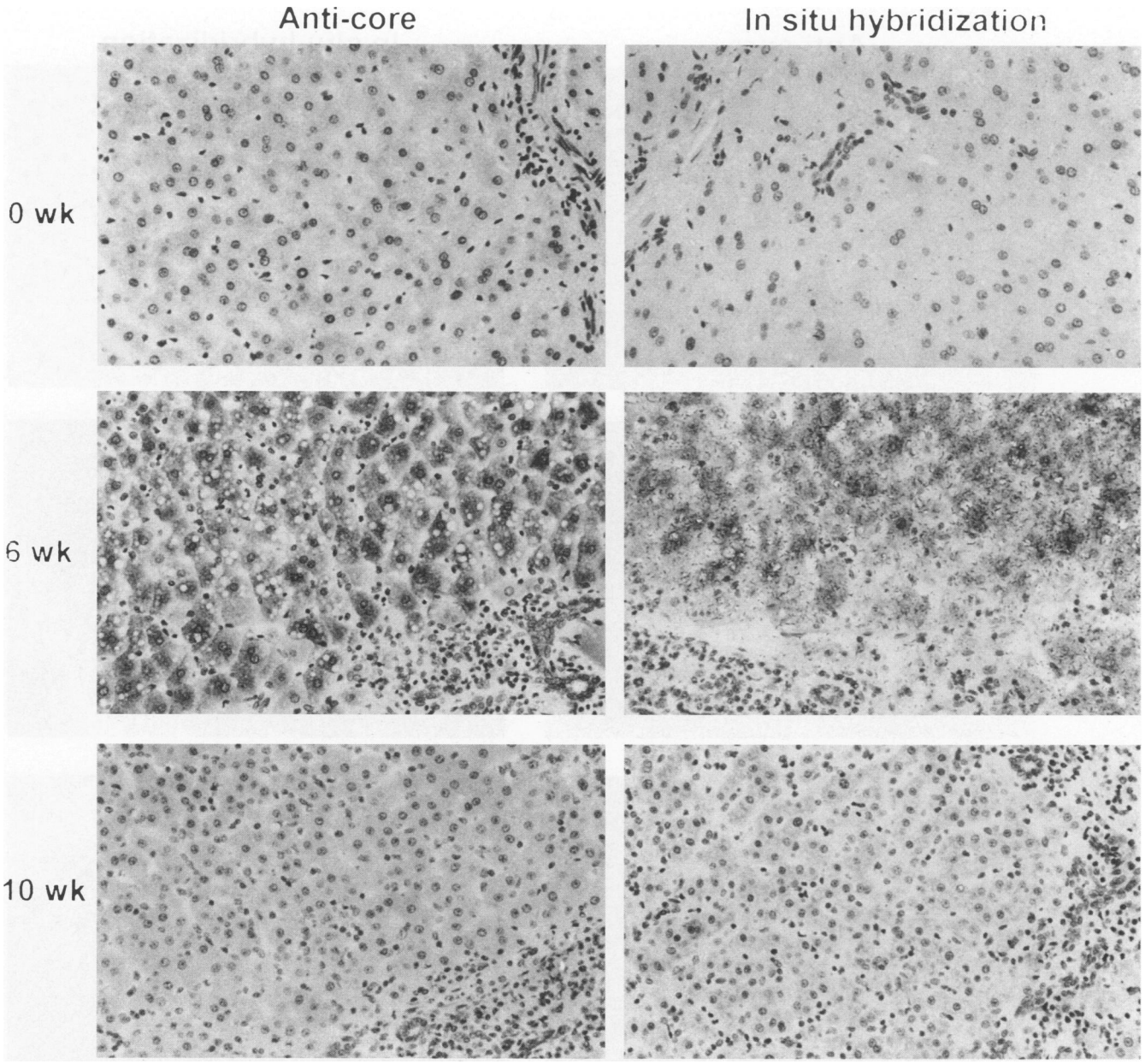

FIG. 2. Detection of WHV core antigen and nucleic acids in the liver of woodchuck 22. Sections of ethanol-acetic acid-fixed liver tissue from woodchuck 22, sampled by liver biopsies performed preinoculation and at 6 and 10 weeks postinoculation, were reacted for the detection of cytoplasmic WHV core antigen by immunoperoxidase staining with rabbit anticore antibodies. For detection of cytoplasmic WHV nucleic acids, sections were subjected to ISH with DIG-dUTP-labeled WHV DNA. Nuclei were counterstained with hematoxylin. Magnification, ×160.

recovery-phase sera that were assayed. Our ability to detect this activity, which is presumably caused by virus-neutralizing antibodies, in some but not all woodchucks is consistent with the observations of Ponzetto et al. (27), who were unable to detect an anti-woodchuck hepatitis virus surface antigen (anti-WHsAg) response in some woodchucks during the recovery phase of an infection by using a radioimmune assay. These observations suggested that hepatocytes may sometimes lose the capacity to support WHV replication during the recovery phase of an infection and/or that the liver may be repopulated by proliferation of a pool of virus-resistant hepatocytes.

Transient infection with WHV was associated with virus replication in virtually all hepatocytes. To determine if a substantial fraction of hepatocytes might be resistant to WHV, the fraction of infected hepatocytes was measured. Liver biopsies were performed before virus inoculation and at selected intervals postinoculation. From these, we quantitated the proportion of productively infected hepatocytes, as reflected by the cellular accumulation of core antigen and viral nucleic acids. In addition, the total accumulation of replicative intermediates in viral DNA synthesis and of CCC DNA, the template for viral RNA transcription, was measured. The results of assays for core antigen and viral nucleic acid accumulation in individual hepatocytes are illustrated in Fig. 2

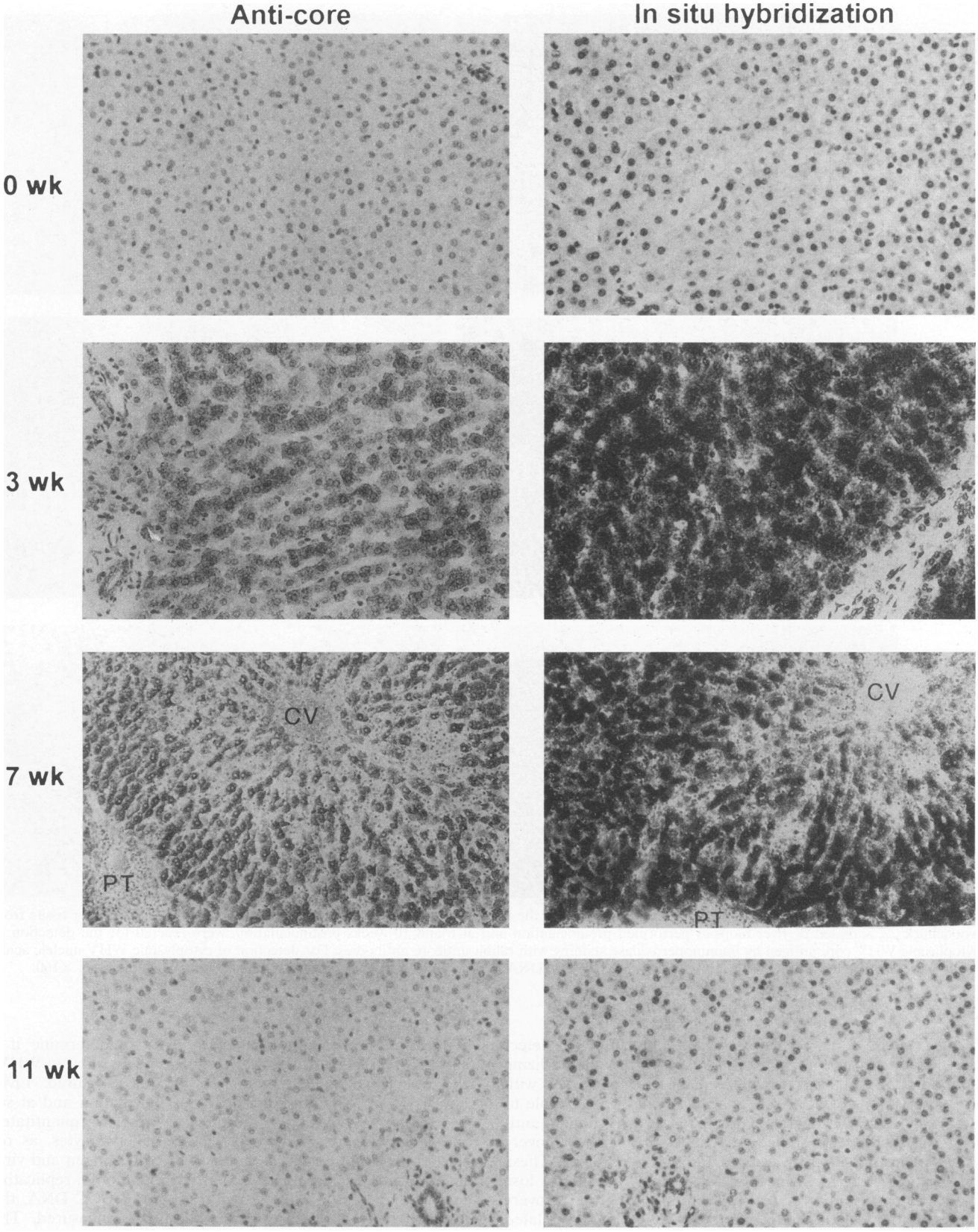
Anti-core
In situ hybridization
0 wk
3 wk
7 wk
11 wk
CV
PT
CV
PT

TABLE 2. Detection of WHV antigens and nucleic acids in liver sections of WHV-inoculated woodchucks

Woodchuck	No. of wks postinoculation	% WHV-positive hepatocytes		Liver injury score	% Positive hepatocytes by anti-PCNA staining	Average CCC DNA copy no.[a]	WHV replicative-intermediate DNA copy no.[a]
		Anticore staining	ISH				
22	Preinoculation	0	0	+/−	0.11	0	0
	2	34	55	+/−	<0.01	15.3	380
	6	83	100	+2	0.07	26.5	460
	10	0	0	+2	0.03	0	0
	19	0	0	+/−	ND[b]	0	0
23	Preinoculation	0	0	+/−	<0.01	0	0
	2	52	44	+/−	<0.01	13.9	240
	6	92	100	+3	0.14	15.7	400
	10	0	0	+1	<0.01	0	0
	19	0	0	+/−	ND	0	0
25	Preinoculation	0	0	+/−	ND	0	0
	2	72	62	+/−	ND	10.0	260
	6	56	44	+3	ND	6.4	200
	12	0	0	+/−	ND	0	0
	19	0	0	+/−	ND	0	0

[a] Copy numbers are corrected to the higher of the two estimates of the number of infected hepatocytes. Hepatocytes comprise ca. 83% of the liver cell population, assuming that there is one nucleus per hepatocyte. Because a significant number of cells are binucleate, the actual fraction of hepatocytes will be somewhat lower than that used for our calculations. A zero indicates that viral DNA was no longer detectable by Southern blot analysis.

[b] ND, not done.

through 4, while Southern blot analyses for viral replicative intermediate DNA and CCC DNA are illustrated in Fig. 5 and 6. The results of these assays for all of the woodchucks are summarized in Tables 2 and 3.

The data implied that in at least 7 of 10 woodchucks all of the hepatocytes were infected during the viremic stage of the infection. Infected hepatocytes then rapidly disappeared from the liver. Eight of ten of the woodchucks began to clear infected hepatocytes from the liver at about the time when virus disappeared from the blood stream. The exceptions were woodchucks 33 and 34, in which large fractions of infected hepatocytes were apparent several weeks after viremia was no longer detectable (cf. Tables 1 and 3). Infection was even cleared in woodchuck 38, in which apparently complete infection of the hepatocyte population extended over a period of at least 8 weeks (Fig. 4 and 6; Table 3). In summary, transient infections did not appear to depend upon the existence of a fraction of virus-resistant hepatocytes and, moreover, essentially complete infection of the hepatocyte population did not appear to prevent woodchucks from rapidly clearing the WHV infection.

For three woodchucks (Table 3, woodchucks 33, 40, and 41) viral CCC DNA persisted at significant levels after replicative-intermediate DNAs and core antigen- and viral nucleic acid (ISH)-positive hepatocytes were no longer detectable. In woodchuck 33, the absolute amount of CCC DNA in the liver had dropped about 10-fold between 7 and 29 weeks postinoculation; in woodchuck 40, there was a 4-fold drop between 3 and 17 weeks postinoculation. These observations may reflect retention of nontranscribed CCC DNA in hepatocytes or in lymphoid cells in the liver, as suggested by the earlier work of Korba and colleagues (19–21). Persistence of CCC DNA was not observed in an earlier study of transient duck hepatitis B virus infections (18).

Evidence for an enhanced rate of hepatocyte turnover during transient WHV infection. Since infection did not appear to be restricted from a significant fraction of hepatocytes, it was possible that recovery involved massive destruction of infected hepatocytes together with rapid polyclonal proliferation of rare uninfected hepatocytes and/or progenitor cells. The importance of a period of cell death and replacement in clearance of an infection was supported by serologic assays for SDH, an abundant hepatocellular enzyme (17). Hepatocyte injury, on the basis of elevated serum SDH levels, persisted throughout the recovery period of an infection, as illustrated in Fig. 7 (cf. Tables 2 and 3).

In order to determine if there was also an enhanced, perhaps focal, proliferation of hepatocytes during infection, as an independent indication of cell killing by the virus or by the immune response to viral antigens, liver biopsy specimens were immunostained to detect nuclear localization of PCNA, an indicator that cells have recently progressed into S phase (11). Woodchucks described in Table 3 were also injected with BUdR 8 h before the first (preinoculation) biopsy and again before the biopsy at 7 weeks postinoculation (before the biopsies at 7 and 17 weeks for woodchuck 38), and BUdR-labeled nuclear DNA, as an indicator of hepatocytes progressing through S phase, was detected, again, by immunostaining. The results of PCNA and BUdR immunostaining assays are illustrated in Fig. 8 and summarized in Tables 2 and 3. A definite increase in the rate of cell turnover was predicted by these two assays, although the increase was modest (ca. 3- to

FIG. 3. Detection of WHV core antigen and nucleic acids in the liver of woodchuck 36. Sections of ethanol-acetic acid-fixed liver tissue from woodchuck 36, sampled by liver biopsies performed preinoculation and at 3, 7, and 11 weeks postinoculation, were reacted for the detection of cytoplasmic WHV core antigen by immunoperoxidase staining with rabbit anticore antibodies. For detection of cytoplasmic WHV nucleic acids, sections were subjected to ISH with DIG-dUTP-labeled WHV DNA. At 7 weeks postinoculation, core antigen and nucleic acid signals in centrilobular hepatocytes (zone 3) adjacent to central veins (CV) were reduced compared with signals in hepatocytes located in zone 1 adjacent to portal tracts (PT). Nuclei were counterstained with hematoxylin. Magnification for samples taken at 0, 3, and 11 weeks (wk), ×160; for samples taken at 7 weeks, ×80.

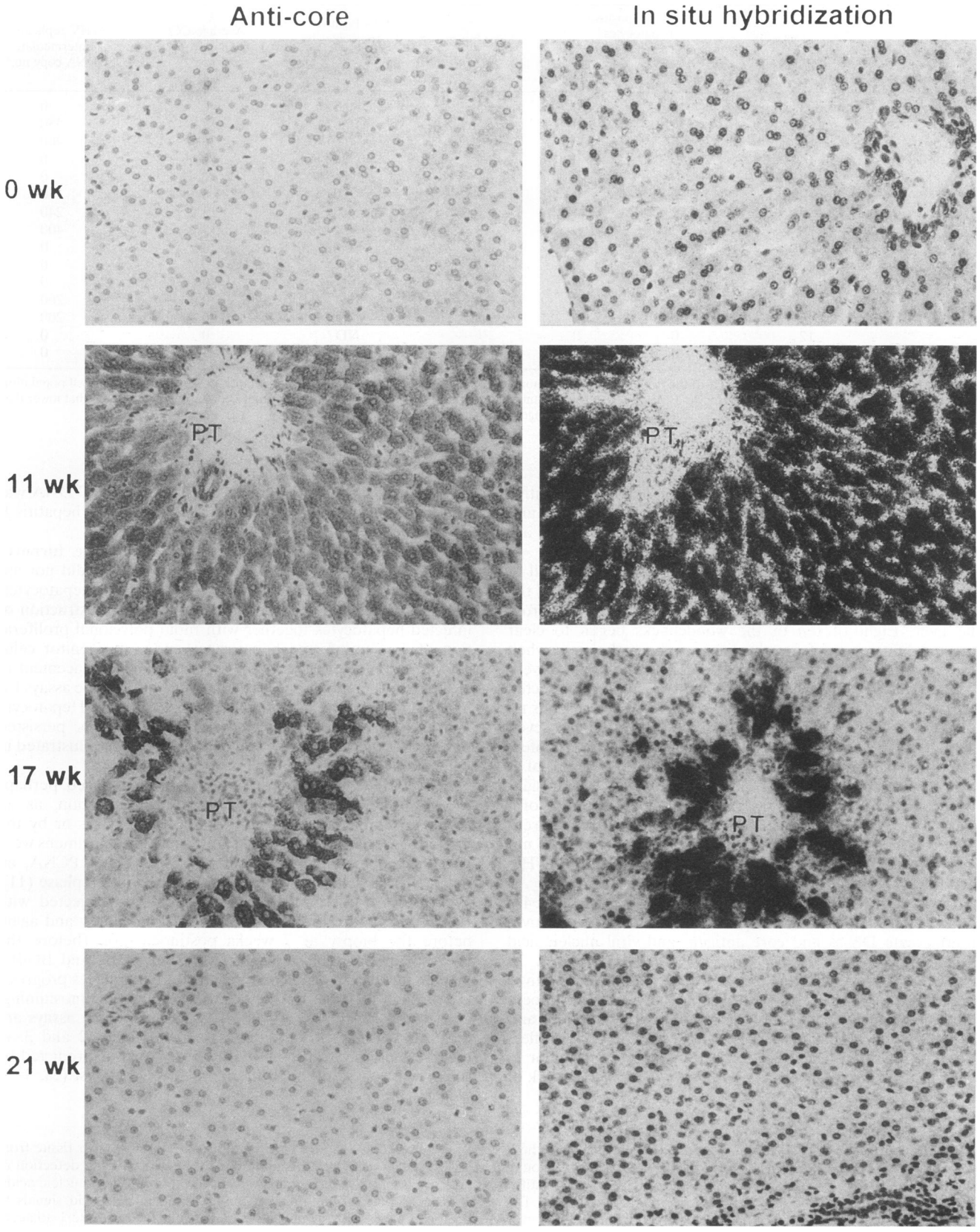
Anti-core
In situ hybridization
0 wk
11 wk
17 wk
21 wk
PT
PT
PT
PT

TABLE 3. Detection of WHV antigens and nucleic acids in liver sections of WHV-inoculated woodchucks

Woodchuck	No. of wks postinoculation	% WHV-positive hepatocytes		Liver injury score	% Positive hepatocytes		Average CCC DNA copy no.[a]	WHV replicative-intermediate DNA copy no.[a]
		Anticore staining	ISH		Anti-BUdR[b] method	Anti-PCNA staining		
33	0	0	0	−	<0.01	0.11	0	0
	3	31	47	−	(0.04)	0.01	2.1	100
	7	26	44	−	0.08	0.11	2.2	82
	11	11	14	+1	(0.04)	0.8	3.4	100
	21	0	0	−	(0.04)	0.13	0.2[c]	0
	29	ND[d]	ND	−	ND	ND	0.1[c]	0
34	Preinoculation	0	0	+/−	0.07	<0.01	0	0
	3	68	100	+1	(0.04)	0.14	4.7	170
	7	100	89	+1	0.93 [58]	1.1	4.0	62
	11	0	0	+/−	(2.3 [82])	0.34	0	0
	19	ND	ND	+/−	ND	ND	ND	ND
35	Preinoculation	0	0	−	<0.01	<0.01	0	0
	3	70	100	+1	(<0.01)	0.09	14.5	210
	7	83	100	+2	0.09	1.1	53	230
	11	0	0	+2	(0.03)	1.1	0	0
	22	0	0	+2	(0.01)	0.8	ND	ND
	29	ND	ND	+1	ND	ND	ND	ND
36	Preinoculation	0	0	−	<0.01	0.03	0	0
	3	100	100	+/−	(0.05)	0.1	10.0	320
	7	74[e]	95[e]	+1	0.26	1.6	32	290
	11	0	0	+/−	(0.05)	0.02	0	0
38	Preinoculation	0	0	−	<0.01	0.05	0	0
	3	100	100	−	(<0.01)	0.01	15.7	570
	7	100	100	+1	0.05	0.25	28	770
	11	100[f]	95[f]	+1	(0.03)	0.3	36	610
	17	16[g]	19[g]	+1	0.97 [28]	1.8	63	190
	21	0	0	+/−	(0.72 [42])	0.25	0	0
40	Preinoculation	0	0	+/−	<0.01	0.05	0	0
	3	8	6.4	+/−	(<0.01)	0.01	19.6	62
	7	0	0	−	0.05	0.16	0.8[c]	0
	11	0	0	+/−	(0.02)	<0.01	0.6[c]	0
	17	ND	ND	+/−	ND	ND	0.4[c]	0
41	Preinoculation	0	0	+1	<0.01	0.05	0	0
	3	67	100	+1	(<0.01)	0.30	11.7	120
	7	0	0	+2	1.3 [70]	0.52	0.5[c]	0
	11	0	0	+1	(1.4 [65])	0.06	0	0
	17	ND	ND	+1	ND	ND	0	0

[a] Copy numbers were corrected to the higher of the two estimates of the number of infected hepatocytes. A zero indicates that viral DNA was no longer detectable by Southern blot analysis.

[b] The results after BUdR pulses are not in parentheses. The results of BUDR pulse-chases are in parentheses. In some cases, the percentages of BUdR-positive cells appearing in pairs are indicated (in brackets).

[c] Not corrected to the fraction of viral core antigen- or nucleic acid-positive hepatocytes.

[d] ND, not done.

[e] Woodchuck 36, at 7 weeks postinoculation, had very strong ISH and anticore signals in zone 1. Centrilobular areas (zone 3) had a reduced-intensity signal with increased numbers of apoptotic cells and inflammatory cells and decreased numbers of ISH- and anticore antigen-positive cells.

[f] Woodchuck 38, at 11 weeks postinoculation, showed a reduced signal intensity (anticore and ISH) in centrilobular areas (zone 3).

[g] Woodchuck 38, at 17 weeks postinoculation, contained ISH- and anticore antigen-positive cells in zone 1, whereas zones 2 and 3 were WHV negative.

10-fold) in most woodchucks. The labeled hepatocytes were scattered throughout lobular zones 1 and 2, rather than being focal in localization, suggesting that proliferation to replace dying cells occurred in the general population of mature hepatocytes and not in a rare hepatocyte type. The nuclear labeling index determined by either assay represented less than 2% of the hepatocyte population (Table 3). Moreover, neither these assays nor histopathologic evaluations (presented below) revealed a marked proliferation of oval cells, a population of facultative stem cells that is often activated in response to major hepatocellular injury (7–9, 30, 31).

To further assess hepatocyte turnover during recovery from an infection, we also carried out BUdR pulse-chase analyses (Table 3). In three woodchucks (34, 35, and 36), the pulse-

FIG. 4. Detection of WHV core antigen and nucleic acids in the liver of woodchuck 38. Sections of ethanol-acetic acid-fixed liver tissue from woodchuck 38, sampled by liver biopsies performed preinoculation (0 weeks [wk]) and at 11, 17, and 21 weeks postinoculation, were reacted for the detection of cytoplasmic WHV core antigen by immunoperoxidase staining with rabbit anticore antibodies. For detection of cytoplasmic WHV nucleic acids, sections were subjected to ISH with DIG-dUTP-labeled WHV DNA. At 17 weeks postinoculation, WHV core antigen and nucleic acid signals were detected only in zone 1 hepatocytes adjacent to portal tracts (PT), whereas hepatocytes located in zones 2 and 3 were WHV negative. Nuclei were counterstained with hematoxylin. Magnification, ×160.

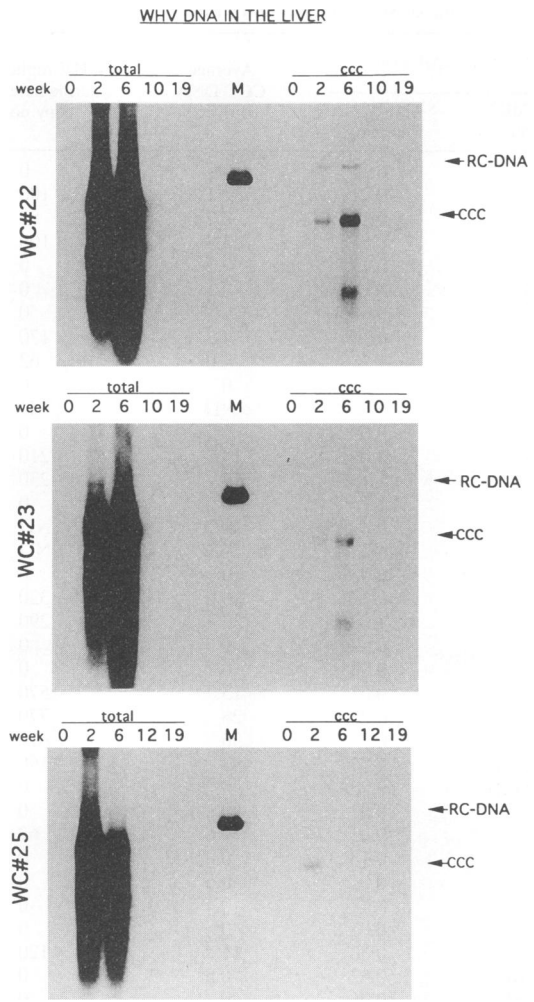

FIG. 5. Detection of viral DNA replication intermediates and CCC DNA in liver biopsy samples of WHV-inoculated woodchucks. Assessment of total WHV DNA, represented predominately by replicative intermediates, in 8 μg of liver DNA is shown at the left. Viral CCC DNA extracted from a liver specimen containing 4×10^5 nuclei is shown at the right. The marker (M) was 50 pg of cloned, 3.3-kbp (unit length) WHV DNA. RC-DNA, 3.3 kbp of relaxed circular WHV DNA; CCC, 3.3 kbp of closed circular WHV DNA. In some cases, the CCC DNA signal is not apparent in the film exposure used for this reproduction (cf. Table 2).

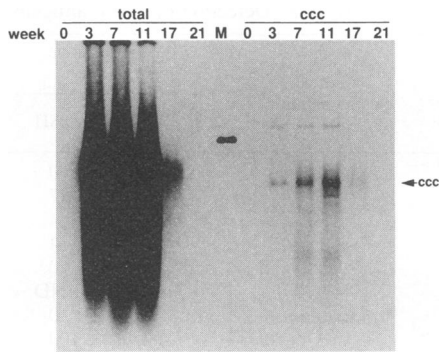

FIG. 6. Detection of viral DNA replication intermediates and CCC DNA in liver biopsy samples of WHV-inoculated woodchuck 38. See legend to Fig. 5.

chase (from 7 to 11 weeks postinoculation) spanned the period from essentially complete infection of hepatocytes to total virus clearance. Surprisingly, the fraction of labeled hepatocytes remained large in two of these woodchucks at the end of the chase (34 and 35). This observation raised the possibility that virus is sometimes removed from the liver without the destruction of all infected hepatocytes and/or their immediate progeny. To examine this possibility further, double staining with anti-BUdR and anti-WHV-core immunoglobulins was carried out with liver sections cut from biopsy specimens taken at 7 and 11 weeks postinoculation from woodchucks 34 and 35. Though the acid and protease treatments of the tissue sections, required for the anti-BUdR staining, reduced the sensitivity of the assay for the detection of core antigen, we were still able to determine in the case of woodchuck 34 that the BUdR-labeled hepatocytes detected at 7 weeks postinoculation were also infected with WHV (core antigen positive) (data not shown). By 11 weeks, core antigen was no longer detected in the hepatocytes, though the same proportion of these cells were anti-BUdR positive. In the case of the other woodchuck, the acid and protease treatments inhibited core antigen detection too much for reliable measurements. Though preliminary, the observations with woodchuck 34 again support the possibility that recovery from an infection sometimes involves the loss of virus from infected hepatocytes and/or the progeny of infected hepatocytes, i.e., the curing of hepatocytes or their progeny.

Histopathology. Prior to infection, the livers of most animals were histologically similar. Livers were characterized by mild mononuclear, mainly lymphocytic infiltrates of the portal areas, occasional mononuclear cell aggregates in the parenchyma, uniform size of hepatocyte nuclei, and small amounts of pale brown pigment in less than 10% of the hepatocytes and Kupffer cells. Two weeks following virus inoculation, the histology of the livers was not changed. However, at 6 to 7 weeks postinoculation, the livers of woodchucks 22, 23, 25, 35, and 41 were moderately to severely inflamed (Tables 2 and 3; subjective scores of +2 to +3), whereas in the case of the remaining woodchucks analysis of biopsy specimens did not produce evidence of a significant liver injury (i.e., their subjective scores never exceeded +1). Inflammation in the five woodchucks with moderate to severe liver injury at the time of biopsy was characterized by lymphocytic and plasmacytic portal infiltrates; these infiltrates expanded portal areas and extended beyond the limiting plates in three of the woodchucks. Parenchymal inflammation was characterized by diffusely distributed lymphocytes within sinusoids and in small aggregates around individual hepatocytes. Small aggregates of hypertrophic Kupffer cells were scattered throughout the parenchyma. Lobular architecture was essentially intact. However, in the more inflamed liver specimens centrilobular hepatocytes were determined to be atrophic and periportal hepatocytes were determined to be regenerative on the basis of the presence of two-cell-thick hepatic plates, deeply eosinophilic cytoplasm, and enlarged nuclei. Apoptotic hepatocytes, identified on the basis of rounded deeply eosinophilic cytoplasm and deeply basophilic nuclear remnants, were most numerous in the 6- to 7-week biopsy samples but constituted less than 1% of the hepatocytes. There were fewer apoptotic hepatocytes in later biopsy samples and in those woodchucks with less hepatic inflammation. By 10 to 12 weeks postinoculation, the hepatic inflammation had subsided in four of the woodchucks, but inflammation persisted in woodchuck 22. Liver injury was determined to be mild in subsequent biopsies.

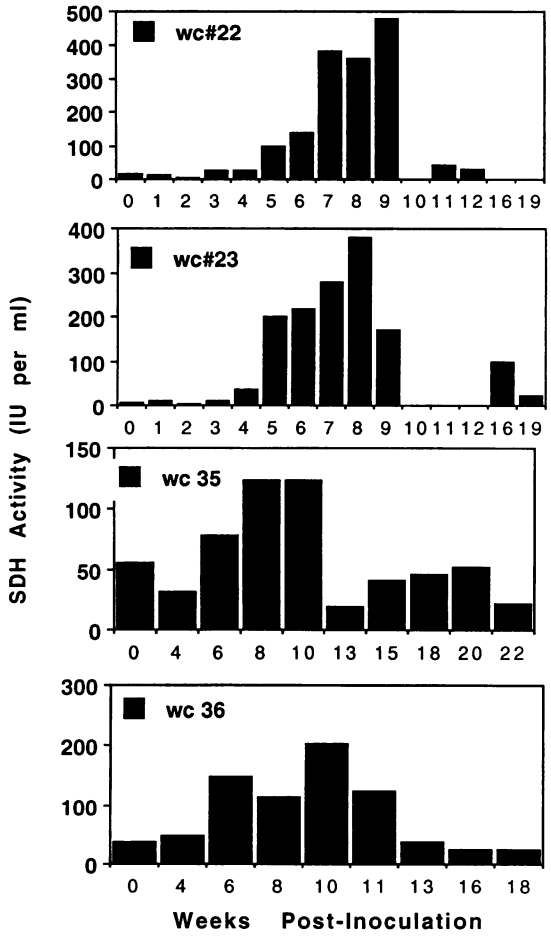

FIG. 7. Increased SDH activity in sera of WHV-infected woodchucks. Serum samples were collected 2 to 3 weeks before WHV inoculation (indicated by 0) and at the indicated number of weeks postinoculation. SDH activity was measured as described in Materials and Methods.

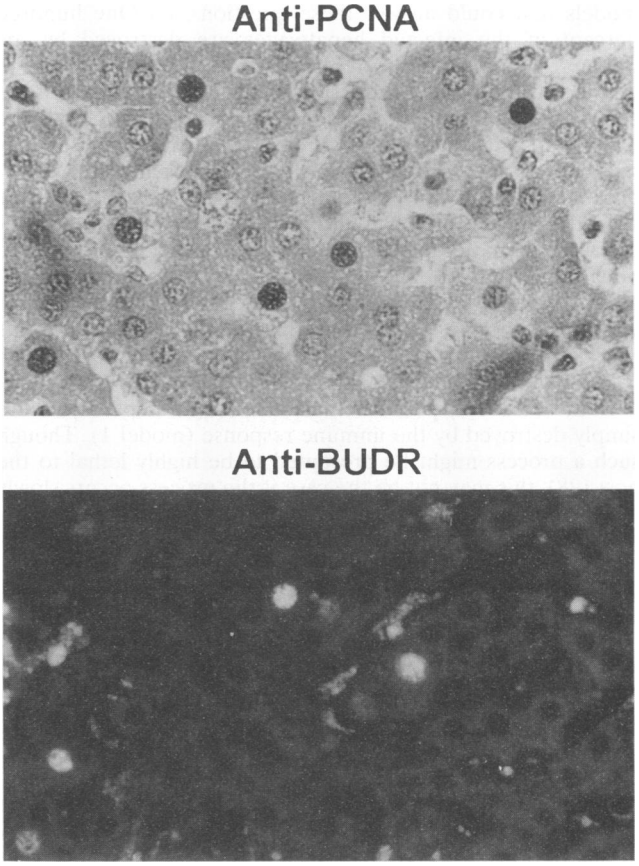

FIG. 8. Detection of proliferating cells in liver tissue. Liver biopsy tissue from woodchuck 38, taken at 17 weeks postinoculation, was stained for the presence of PCNA (1.8% of hepatocyte nuclei) in formalin-fixed tissue and the presence of BUdR (0.97% of hepatocyte nuclei) in ethanol-acetic acid-fixed tissue. Magnification, ×294.

In summary, histologic evidence indicated that hepatic inflammation peaked while WHV DNA levels were declining in infected liver tissue and serum and supported the view that elimination of infected hepatocytes was promoted by the inflammatory response. Because of the limited amount of histologically evident cell death, it again seemed unlikely but cannot be ruled out that elimination of WHV infection could be explained by the death of all infected cells. However, a problem in making this determination on histopathologic grounds is the remarkable ability of the liver to recover from injury and the lack of clear criteria for assessing cumulative cell death and replacement in this organ.

DISCUSSION

Perhaps the major peculiarity about transient hepadnavirus infections in mammals is that such infections are often associated with a viremia having a duration of many weeks, suggesting that there would at least be the opportunity for infection of all hepatocytes. In fact, early studies with woodchucks and chimpanzees (2, 3, 16, 27) suggested that this might be the case. The present study has confirmed the general conclusions of these earlier studies, that infection involved >50 to 75% of all hepatocytes, and also yielded a more precise quantitation of the extent of hepatocyte infection. By either ISH to detect WHV nucleic acids or immunoperoxidase staining to detect the viral core antigen, it was determined that 100% of the hepatocytes were apparently infected at the peak of infection in the majority of woodchucks evaluated in the present study. It was found that virus was then cleared from the liver rapidly and, within the limits of sensitivity of our assays, completely, in 7 of 10 woodchucks. CCC DNA persisted in three woodchucks, though it disappeared in one and declined in amount in the other two during the course of the study. This residual CCC DNA did not appear to support virus replication and neither ISH nor anticore staining of tissue sections revealed evidence of the cells that contained this DNA. Our observation that clearance of infection from the liver was not always complete and that viral CCC DNA sometimes persisted for many weeks or months after other markers of infection had disappeared agrees with the earlier observations of Korba and colleagues (19, 20).

The evidence of virtually complete infection of the hepatocyte population raises a number of questions about how infections are cleared. First, what is the ultimate fate of the infected hepatocytes, and second, considering that virtually all hepatocytes are infected at the peak of the infection, what is the source of the uninfected hepatocytes that populate the liver when the infection has been eliminated? There are at least four

models that could answer these questions. (i) One hundred percent of the infected hepatocytes are destroyed by the immune system and are replaced by division of a pool of uninfected progenitor cells. (ii) Infected hepatocytes are destroyed by the immune response and are replaced by the division of adjacent, mature hepatocytes, which may or may not be infected. Infection is lost as a consequence of cell division. (iii) Hepatocytes that are destroyed by the immune response are replaced by division of adjacent mature hepatocytes, which may or may not be infected. Viral DNA in infected hepatocytes is distributed to daughter cells, but virus replication is inhibited once hepatocytes enter the cell cycle, so that virus is eventually lost by dilution as cells undergo multiple rounds of division. (iv) Virus is spontaneously cleared from individual, nondividing hepatocytes.

It is, of course, possible that all infected hepatocytes are simply destroyed by the immune response (model 1). Though such a process might be presumed to be highly lethal to the host (28), this may not be the case if the process occurs slowly enough. The preliminary evidence for the survival of initially infected hepatocytes or their progeny through the recovery phase, with loss of the virus, may rule out model 1. It should also be noted that the histopathological analyses did not reveal signs of massive liver regeneration.

Considering that infection was cleared very rapidly and that there was at least some evidence for an increased rate of hepatocyte turnover as a result of cell death during clearance, perhaps the simplest explanation is model 2 or 3, though there is no evidence that a hepadnavirus infection would fail to pass to daughter cells. There is, however, evidence that replication of a mammalian hepadnavirus may be inhibited in hepatocytes that enter the cell cycle. Hepatitis B virus replicates very poorly in stably transfected HepG2 cells until the culture becomes stationary (29), which may reflect a loss of some essential host proteins (e.g., transcription factors) in dividing cells. Moreover, as observed previously by Ponzetto et al. (27), and also noted in our experiments, there is some evidence that viremia may cease and infections may clear without the production of sufficient neutralizing antibodies to protect the hepatocytes from reinfection. One consequence of model 3 would be that the amounts of virus core antigen and nucleic acids observed in individual hepatocytes during the period in which it could be inferred that replicating virus was being cleared from the liver (e.g., in woodchuck 25, at 6 weeks; in woodchuck 33, at 7 and 11 weeks; and in woodchuck 38, at 17 weeks [Fig. 5]) should be less, per cell, than those observed at the peak of the infection. This was not generally observed, albeit the microscopic methods used may lack sensitivity to small changes. The exception was with woodchuck 36 (at 7 weeks postinoculation; Fig. 4), for which a reduction of signal intensity in zone 3 (centrilobular) was noted, though all cells remained virus positive.

It is also not inconceivable that hepatocytes could be spontaneously cured of an infection (model 4). For instance, if viral CCC DNA has a short half-life in hepatocytes, then inhibiting viral replication within stationary cells would cause the virus to eventually disappear. It is not, in fact, known if there is any mechanism for inhibition of virus replication during the recovery phase of a transient infection. Chisari and colleagues (12–15) have, however, shown, by using a hepatitis B virus-transgenic mouse model, that injection of cytotoxic T lymphocytes reactive to hepatitis B virus envelope protein causes a rapid reduction in viral RNA levels in surviving hepatocytes. This loss of viral RNA was shown to be mediated by tumor necrosis factor alpha, which was hypothesized to be produced by macrophages in the liver in response to interleukin-2 that was released, e.g., by cytotoxic T lymphocytes reactive to viral antigen. Therefore, it is possible that virus replication is directly inhibited by some aspect of the immune response and that destruction of infected hepatocytes may not be essential to eliminate replicating virus. It remains to be determined, however, if viral CCC DNA does actually have a finite lifetime in infected hepatocytes, either normally or in response to cytokines released as a result of the immune response to infection.

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