Transmission of the hepatitis B virus-associated δ agent to the eastern woodchuck

(chronic viral hepatitis/animal model/hepadnavirus)

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ABSTRACT   δ agent of human origin was inoculated into four woodchucks chronically infected with woodchuck hepatitis virus (WHV). The animals developed δ infections with serologic patterns similar to those previously observed in human and chimpanzee infections. δ antigen was detected transiently in serum and liver and was followed by seroconversion to anti-δ antibody. Analogous to the chimpanzee model of δ infection, serum and hepatocyte markers of WHV were suppressed in the woodchuck during acute δ infection. The suppression of WHV DNA in serum was evident only during the time of δ-antigen positivity, while the inhibition of other WHV markers was more protracted. The δ antigen in woodchuck sera circulated as an internal component of a particle similar in size to the human δ particle (36-nm diameter) and was encapsidated by the woodchuck hepatitis virus surface antigen; δ antigen from infected woodchuck and chimpanzee livers had similar biophysical properties. Histologic analysis showed that experimental δ infection is associated with a transient acute hepatitis in woodchucks and loss of hepatocytes carrying WHV antigens. The lesions differed from the conspicuous hepatitis associated with reappearance of WHV replication. Hepatitis B-like viruses, therefore, appear to provide the requisite helper functions for δ replication and the woodchuck represents a useful model for study of the virology and pathology of the δ agent.

The δ agent is a virus-like human pathogen that superinfects individuals with type B viral hepatitis (1-3). Infectivity studies in chimpanzees have established the helper role of hepatitis B virus (HBV) in δ infection (3). The agent consists of the δ antigen (δAg) and an associated low molecular weight RNA (1.7 kilobases) encapsidated by hepatitis B virus surface antigen (HBsAg) (4). Epidemiologic data indicate that δ is distributed worldwide (5) and that, in HBV infected individuals, the agent causes acute and chronic liver disease (6, 7).

The design of therapy and prevention of δ infections has been hampered by the lack of information concerning δ replication and pathogenicity. Because of the unavailability of cell culture systems for HBV and the requirement for HBV helper functions, the δ agent can be propagated and studied only in an animal model. Extensive use of the chimpanzee model is restricted by cost, biohazard control, and the paucity of HBV carrier animals. A virus similar to HBV has been found in nature in the eastern woodchuck (*Marmota monax*), the woodchuck hepatitis virus (WHV) (8). Like HBV, WHV can cause acute and chronic infections associated with hepatitis and other long-term sequelae, such as hepatocellular carcinoma (HCC) (8-10). Therefore, we investigated the possibility of using the WHV-infected woodchuck as a surrogate host for propagation of δ agent of human origin.

MATERIALS AND METHODS

Animals. Woodchucks (*M. monax*) were maintained in a colony established in 1978 by the National Institute of Allergy and Infectious Diseases (Meloy Laboratories, Rockville, MD). Details of the WHV status of colony animals and histologic findings have been reported elsewhere (10). The chimpanzees referred to were from the National Institute of Allergy and Infectious Diseases primate colony (Meloy Laboratories).

δ Infection Transmission. Woodchucks were injected with sera shown to contain high levels of δ Ag by RIA. The standard infectious pool of δ agent was derived from plasma from chimpanzee 57; it was obtained from the peak of δ antigenemia during serial transmission studies (11) of human δ in chimpanzees (second passage). The undiluted inoculum contained at least 10^{11} chimpanzee infectious doses of δ (5). One milliliter of δ inoculum was administered i.v. to each of three woodchucks chronically infected with WHV: one adult male (no. 80), one adult female (no. 69), and one young female (no. 35). For the second passage of δ in woodchucks, 1 ml of δ Ag-positive serum from woodchuck 69 was inoculated i.v. into an adult female woodchuck (no. 86) with chronic WHV infection.

Serologic Studies. δ Ag was detected by a "sandwich" solid-phase RIA after treatment of the sample with 0.3% Nonidet P-40; sample/negative control cpm ratios (S/N) ≥ 2.1 were considered positive for δ Ag (12). Antibody to the δ Ag (anti-δ) was measured by competitive-binding RIA as described (12). Results are reported as percentage of inhibition of counts bound by the test sample compared with those for control sera negative for anti-δ. The titer of anti-δ was defined as the highest dilution that exhibited ≥50% inhibition. Woodchuck hepatitis virus surface antigen (WHsAg) and antibody (anti-WHs) were monitored by specific RIAs (13–15). HBsAg and anti-HBs were tested by commercial Ausria II and Ausab (Abbott) procedures. HBsAg was also detected by RIA with a monoclonal antibody specific for HBsAg (16). Endogenous DNA polymerase activity was determined as described (17). Sera were also assayed for WHV DNA by

Abbreviations: WHV, woodchuck hepatitis virus; δ Ag, delta antigen; HCC, hepatocellular carcinoma; WHsAg, woodchuck hepatitis virus surface antigen; WHcAg, woodchuck hepatitis virus core antigen; HBV, hepatitis B virus; HBsAg, hepatitis B virus surface antigen; S/N ratio, sample/negative control ratio (in cpm); IF, immunofluorescence.
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molecular hybridization techniques (18, 19) similar to those developed for HBV DNA detection (20) using a nick-translation product of cloned WHV DNA as the ^{32}P-labeled probe. The amount of WHV DNA present in woodchuck sera was estimated by constructing a standard relating cpm per gel lane to known quantities of cloned WHV DNA (see Fig. 2).

Liver Immunofluorescence (IF). Five micrometer cryostat sections of frozen liver tissue were fixed with anhydrous ether (5 min, 25°C), stained with either fluorescein isothiocyanate-conjugated anti-δ IgG (human) or fluorescein isothiocyanate-conjugated anti-woodchuck hepatitis core IgG (woodchuck). Stained sections were examined with an epifluorescence photomicroscope (Zeiss).

Histologic Studies. Part of each liver sample was fixed with formalin, paraffin embedded, and stained with hematoxylin/eosin. Victoria blue staining was also carried out (21); this substance stains hepadnavirus surface antigens similar to the Shikata stain. Analysis was carried out under code without prior knowledge of serological findings and was repeated after disclosure of the code. Lesions were scored as + (moderate), ++ (medium), or +++ (severe).

Biophysical Characterization of δ Particles and δ Antigen. δ-associated particles from plasma and δ Ag from liver were characterized by ultracentrifugation methods as described in the figure legends; plasma samples and liver tissues were obtained from one woodchuck and one chimpanzee. Gradient fractions were assayed for δ Ag, HBsAg, WHsAg, and CsCl concentration and examined by electron microscopy.

RESULTS

Transmission of the δ Agent in Woodchucks. The course of events after inoculation of woodchuck 35 is detailed in Fig. 1. The δ Ag was found in a small proportion of hepatocytes by IF as early as 1 wk after inoculation, with maximum levels occurring over the next 2 wk (Fig. 1B). The distribution of δ Ag by IF was predominantly nuclear with faint cytoplasmic staining. Only a small percentage of hepatocytes were δ Ag positive at any time; a maximum of ≈10% of liver cells were stained at the peak of δ Ag expression (see Fig. 3a).

δ antigen was detected in Nonidet P-40-treated sera short-

ly after its appearance in the liver (e.g., 2 wk after inoculation; Fig. 1A). Levels of circulating δ Ag were low during the entire course of antigenemia (maximum S/N = 10). Antibody to δ Ag (anti-δ) appeared 1 wk after the clearance of the δ Ag from serum and liver and eventually reached a maximum titer of 1:3000 (data not shown). δ infection was accompanied by a decrease in the levels of serum WHsAg and DNA polymerase activity; liver WHV core antigen (WHcAg) also disappeared (Fig. 1 A and B). WHcAg did not reappear for 7 wk, despite the lack of detectable δ Ag in serum and liver (Fig. 1B). Serum DNA polymerase became positive when WHcAg was again detected in the liver (Fig. 1A). Unlike chimpanzee plasma (4), woodchuck plasma did not contain δ-associated RNA detectable by ethidium bromide staining techniques. This might be due to the lower concentrations of circulating δ particles in woodchucks or to particle instability associated with adaptation of δ to the woodchuck.

The first passage of the δ agent in a second woodchuck (no. 69) was similar to that described above. The δ Ag appeared in serum for 2 wk (Fig. 2A) and was also detected by

FIG. 2. First and second passages of the δ agent in WHV-carrier woodchucks. (A) Infection of woodchuck 69 with δ agent from chimpanzee 57. The woodchuck was killed 4 wk after inoculation. (B) Infection of woodchuck 86 with δ agent from woodchuck 69. An estimate of serum WHV DNA content was obtained by constructing a standard curve of the ratios of cpm per gel lane to known quantities of cloned WHV DNA on an electrophoresed, hybridized, and autoradiographed Southern (22) blot that also contained WHV DNA from woodchuck sera. By excising equal-sized pieces of nitrocellulose corresponding to the radioactive regions of the standard and experimental samples, we determined cpm (by scintillation spectrometry) of [^{32}P]DNA on each piece; replicate pieces of the same size were analyzed to determine the background, which was subtracted. In the example given here, 0.2 ng of DNA yielded 10^3 cpm. Woodchuck serum samples (200 μl) yielded from 4×10^4 to 1.4×10^3 cpm. IF symbols, as in Fig. 1B.
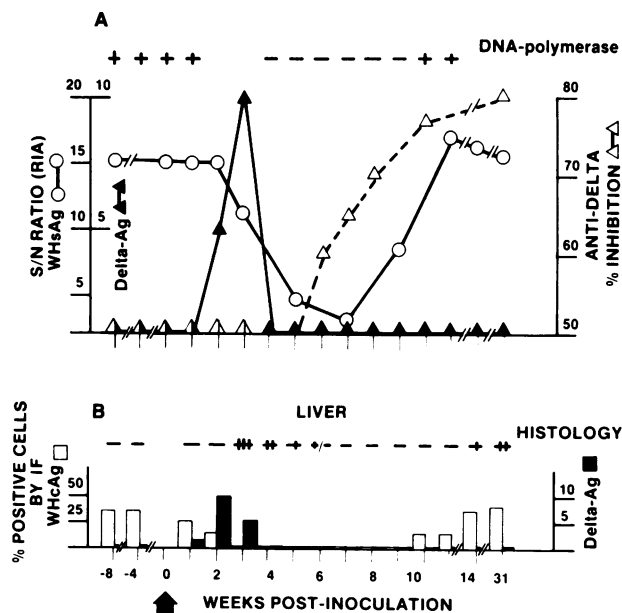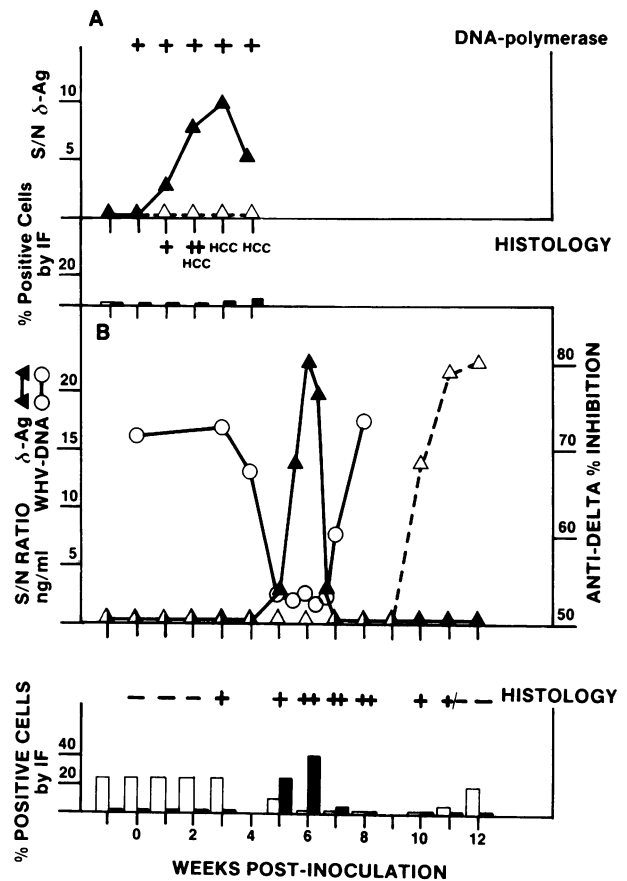

FIG. 1. First passage of the δ agent in a woodchuck chronically infected with WHV. Woodchuck 35 was inoculated with chimpanzee (no. 57) plasma that contained δ agent. (A) Serological events. (B) Liver histopathology and immunofluorescence.

IF in liver tissue in a few hepatocytes. This animal was near death from HCC at the time of inoculation and most of its hepatic parenchyma was replaced by tumor. This hampered the demonstration of viral antigens in the liver and overall histological assessment for hepatitis. The animal was killed prior to the appearance of anti-δ to obtain blood and liver tissue containing δ agent. The third animal (woodchuck 80) inoculated with δ agent from chimpanzee 57 exhibited δ Ag in the liver at wk 4–6, but δ Ag and anti-δ were not detected in the circulation (data not shown). Thus, three of three inoculated woodchucks were infected with δ during primary passage from the chimpanzee.

A second passage of δ in a WHV carrier woodchuck (no. 86) was achieved with acute-phase serum from woodchuck 69. Second passage was associated with increased expression of δ Ag. Liver δ Ag was observed in 40% of hepatocyte nuclei (Figs. 2B and 3b) and the level of δ Ag in the circulation was higher than that attained in the first passage (Fig. 2B). WHcAg in hepatocytes, detected by IF, declined with the first appearance of liver δ Ag and then rapidly disappeared. WHcAg reappeared 4 wk after the last δ Ag-positive liver biopsy (Fig. 2B). Circulating WHV DNA was markedly depressed during the period of δ antigenemia (e.g., from an estimated 17 ng of WHV DNA per ml before appearance of δ Ag to about 2 ng/ml during the peak of δ Ag expression; Fig. 2B).

Histologic Observations. Serial biopsies from woodchuck 35 (first passage; Fig. 1B) and woodchuck 86 (second passage; Fig. 2B) revealed two bouts of hepatitis and the histologic appearances were, in principle, similar in both animals. The preinoculation specimens had minor portal inflammation and essentially normal-appearing hepatocytes in routine stains (Fig. 4a), although the cytoplasm of many hepatocytes reacted with Victoria blue, indicating WHV-specific antigens in analogy to "healthy" human HBV carriers. In woodchuck 35, the initial hepatic alterations set in faster, were more severe, and regressed more quickly. The lesions consisted mainly of cytoplasmic alterations reflected in the accumulation of small fat droplets and in irregular eosinophilic clumping, in places progressing to acidophilic bodies (Fig. 4b). Necrosis bridging central and portal canals was

FIG. 4. (a) Healthy WHV carrier woodchuck (no. 86) before inoculation with woodchuck-passaged δ agent (see Fig. 3b). The appearance of the liver is essentially normal. (Hematoxylin/eosin; × 60.) (b) Same woodchuck 7 wk after inoculation. (Hematoxylin/eosin; ×140.) Note the irregular eosinophilic clumping of hepatocytic cytoplasm, which often contains many small fat droplets (curved arrow), the acidophilic bodies (straight arrow), and the increase of mainly macrophagic sinusoidal cells.
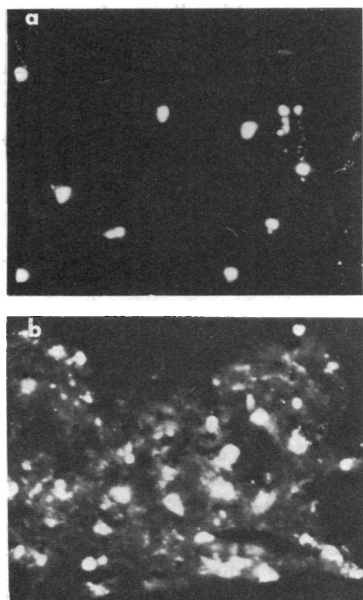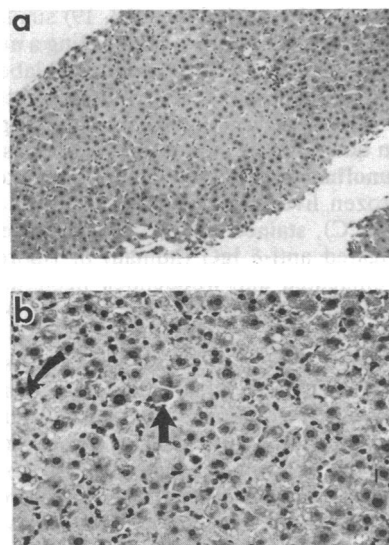

FIG. 3. IF of δ antigen in woodchuck hepatocytes after δ infection. (a) First passage of δ agent in woodchuck 35 (2 wk) after inoculation. (b) Second passage of δ agent in woodchuck 86 (6 wk) after inoculation.

present in woodchuck 35. In addition, inflammatory cells, many of them macrophages containing p-aminosalicylic acid-positive diastase-resistant granules, and other inflammatory cells were present focally in the parenchyma and expanded the portal tracts. The number of hepatocytes with Victoria blue-stained cytoplasm decreased but excess Victoria blue-positive material was present in macrophages. p-Aminosalicylic acid-positive macrophages and portal inflammation persisted after decline of the hepatic changes attributable to the δ-infection. Neoplastic nodules and HCC eventually developed, preceded by a second attack of hepatitis associated with the increase in serum WHV markers and seemingly characterized by periportal inflammation, hepatocellular nuclear changes, and accumulation of lymphocytes rather than p-aminosalicylic acid-positive macrophages. On first passage of δ to woodchuck 69, HCC was already present (Fig. 2A) and, in woodchuck 80 (first passage; data not shown), in which hepatic δ Ag was the only marker of δ-infection, a transient mild hepatitis developed.

Characterization of δ Ag Particles from Woodchuck Plasma. Plasma from the peak of δ Ag activity from one woodchuck and one chimpanzee was isopycnically banded in CsCl density gradients. The δ Ag activity in chimpanzee serum banded as a HBsAg subpopulation at the expected density of 1.25 g/cm³ (Fig. 5B; ref. 4) while the peak of δ Ag from woodchuck plasma was recovered at a lower density in a parallel gradient (1.23 g/cm³; Fig. 5A). The detection of δ Ag activity in both gradients required pretreatment of fractions with Nonidet P-40. Electron microscopic examination of the samples containing the peak of δ Ag activity from each gradient (fractions 20 and 18 in Fig. 5 A and B, respectively) showed morphologically similar forms (36 nm in diameter) (Fig. 6); fewer particles were observed in the woodchuck sample, consistent with the lower S/N values for δ Ag. Electron micrographs showed the presence of 22-nm forms of WHsAg and HBsAg, respectively. The use of WHsAg- and HBsAg-specific RIAs (Fig. 5A) indicated that δ Ag in woodchuck plasma was associated with a subpopulation of WHsAg, as no HBsAg activity was detected in the gradient
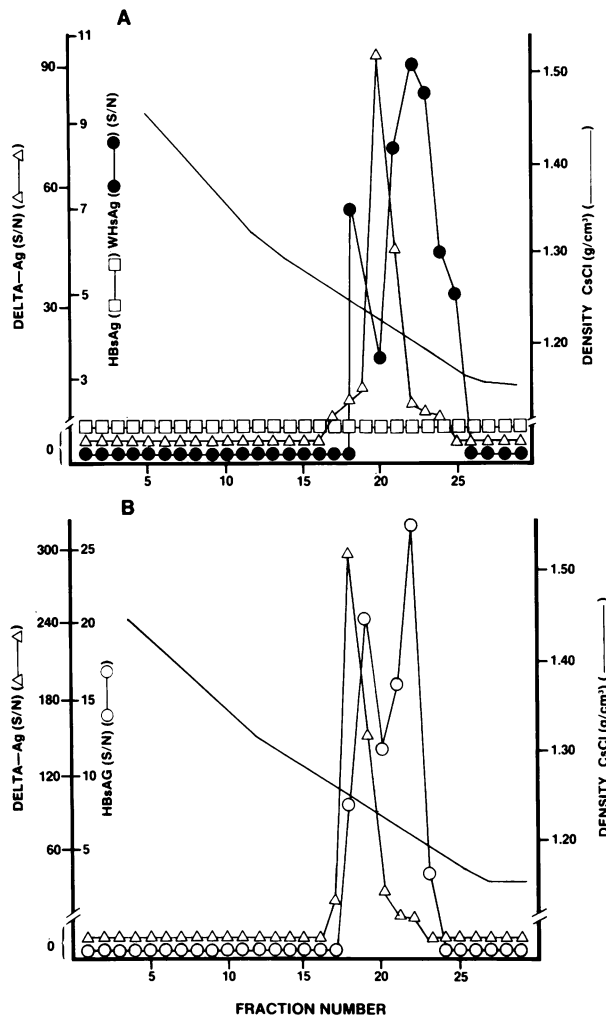
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FIG. 5. Isopycnic banding of woodchuck and chimpanzee δ-associated particles in CsCl. A 1.5-ml sample of δ Ag-positive plasma from woodchuck 86 (A) and from chimpanzee A-20 (B) was centrifuged over a 20% sucrose cushion (35,000 rpm, 4°C, 5 hr, Beckman SW41 rotor). The pellets were suspended in 0.5 ml of 0.85% NaCl/0.01 M phosphate buffer, pH 7.4, and centrifuged on a discontinuous CsCl gradient (1.15–1.40 g/cm³, 24 hr, 35,000 rpm, 4°C, Beckman SW41 rotor). Gradient fractions were collected by bottom puncture and assayed for δ Ag (△) after Nonidet P-40 treatment and CsCl density by refractometry. (A) WHsAg (●) and HBsAg (□) in gradient fractions (1:2 dilution) were determined using site-specific monoclonal immunoadsorbents that were WHsAg and HBsAg specific, respectively; bound antigen was detected using an ¹²⁵I-labeled monoclonal antibody that crossreacts with WHsAg and HBsAg (13, 14). (B) HBsAg (○) was determined by a commercial radioimmunoassay, Ausria II.

fractions. Further, significant precipitation of δ Ag from a gradient fraction (fraction 21, Fig. 5A) by WHsAg- and not HBsAg-specific antibodies established (data not shown) that the δ Ag in woodchuck plasma was at least partially encapsidated by WHsAg.

Characterization of the δ Ag from Livers of Woodchuck and Chimpanzee. Earlier studies have indicated that δ Ag from human liver has physical properties of a protein (12). The buoyant densities of δ Ag extracted from livers of a woodchuck (no. 69) and a chimpanzee (no. 56) in CsCl gradients (Fig. 7) did not differ, indicating similar physical properties.

DISCUSSION

δ agent is a unique human pathogen that requires helper functions from HBV for its replication and expression. The
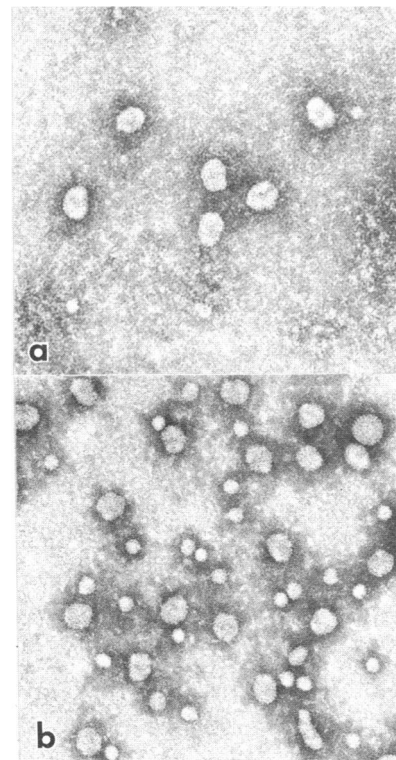

FIG. 6. Electron microscopy of δ-associated particles from the woodchuck (*a*) and chimpanzee (*b*). Samples are fractions corresponding to the peak of δ Ag activity in Fig. 5 *A* and *B* and were negatively stained with 1% phosphotungstic acid. (Bar = 100 nm.)

course of δ infection and disease has been established in HBV-infected chimpanzees (3, 4). Experimental transmission of δ agent in HBV-carrier chimpanzees caused degenerative lesions that coincided with the transient appearance of δ Ag in serum and liver, marked suppression of markers of active HBV replication, and destruction of hepatocytes loaded with HBV-specific antigens. Serum δ Ag is an internal component of a 36-nm particle encapsidated by HBsAg derived from the host HBV infection.

We have studied whether chronic infection of woodchucks with a hepatitis B-like virus, WHV, can provide the requisite helper functions for replication of δ agent. δ agent of human origin was experimentally transmitted on first passage to three of three WHV-carrier woodchucks. Analysis of sera and livers of first and second passage δ infections in WHV-carrier woodchucks, most of them healthy (i.e., without significant liver lesions), showed that the virologic and pathologic features of δ infection of woodchucks and chimpanzees are similar. The concentration of WHV genomic DNA in serum was diminished during δ antigenemia and the expression of liver WHcAg was inhibited for up to 5 wk after the decrease in serum and liver δ Ag. The δ Ag was located primarily in nuclei of woodchuck hepatocytes but it was also found in the cytoplasm of a few such cells. The nuclear localization of δ Ag is similar to that observed in primates. In contrast, hepatitis B core antigen in primates is predominantly nuclear whereas WHcAg is mainly in the cytoplasm (ref 23; unpublished work). Thus, the helper function of different hepadnaviruses for replication of δ agent appears to depend on metabolic functions that are not related to differences in morphogenesis.

The second passage of δ agent in woodchuck 86 provided additional evidence that the helper functions are provided by WHV. The greater amounts of δ Ag expressed in the liver and serum of this woodchuck may be the result of successful adaptation of the δ agent to the helper functions of WHV,
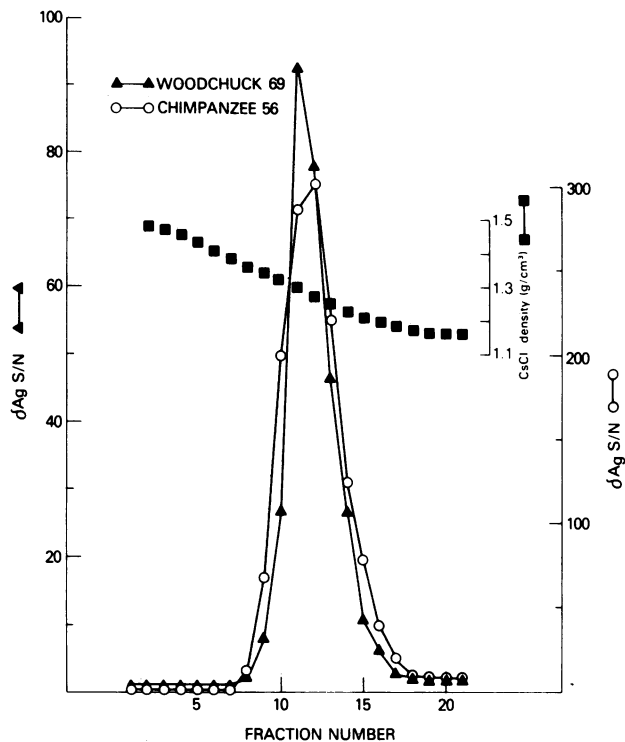
FIG. 7. Isopycnic banding in CsCl of δ antigen from woodchuck and chimpanzee liver homogenates. Hepatic tissue was obtained from a woodchuck (no. 69) and a chimpanzee expressing intrahepatic δ Ag. Livers had been perfused with saline several times at collection, frozen in liquid nitrogen, and stored at −70°C. Livers were thawed and minced 1:10 (wt/vol) in 0.85% NaCl/0.01 M phosphate buffer, pH 7.4, homogenized in a Sorvall Omnimixer, passed through surgical gauze, and centrifuged (10 min, 2000 rpm, IEC PR-6 centrifuge). Two-ml portions of the δ Ag supernates were mixed with 3 ml of a CsCl solution in 0.01 M Tris·HCl (pH 7.4) at a density of 1.48 g/cm³ and centrifuged (69 hr, 45,000 rpm, 4°C, Beckman SW50.1 rotor). Fractions were collected by bottom puncture and assayed for δ Ag at a 1:25 dilution by RIA. CsCl density (■) was determined by refractometry.

including possible encapsidation with WHsAg. In chimpanzees and humans, the circulating δ particle is 35–37 nm in diameter; similar morphologic forms were found during second passage of the δ agent in woodchucks. The requirement for detergent treatment to detect δ Ag in woodchuck serum, the slight difference in buoyant density between δ-containing particles from woodchucks and chimpanzees [Fig. 5; comparable with the small density difference between WHsAg and HBsAg (9)], the presence of WHsAg but not HBsAg in acute-phase serum, and the partial precipitation of δ Ag by WHsAg-specific antibody indicates that δ Ag in woodchucks circulates as an internal component of a particle encapsidated with WHsAg. The encapsidation of δ Ag with surface antigen contributed from the host infection has been well established in the chimpanzee model of δ infection (4). We have not entirely excluded the possibility that the helper functions for δ replication are provided by HBV in the δ inoculum. Although the host range of the hepadnaviruses appears to be quite restricted (9), the HBV susceptibility of woodchucks has not yet been determined.

Histologic analysis of serial liver biopsies from woodchucks experimentally infected with δ agent revealed acute hepatitis in association with intrahepatic expression of δ Ag; the WHV-carrier animals had an almost normal preinoculation appearance except for Victoria blue-positive (i.e., WHV-specific antigen-carrying) hepatocytes. On first passage of δ agent from chimpanzee material in woodchuck 35, the lesion developed after 1 wk, declined within 2 wk, and

was characterized by mainly cytoplasmic (cytopathic?) degeneration of hepatocytes and macrophagic reaction. On second passage of δ in woodchuck 86, similar alterations started within 3 wk and had a less severe but prolonged course, while the liver δ Ag expression was more conspicuous. A second hepatitic reaction, associated with less-degenerative and macrophagic changes and accompanied by the reappearance of WHV replication, developed after the δ-associated hepatitis in these two originally healthy WHV carriers. Repeated cycles of lobular hepatitis resulting from the balance of suppression and reexpression of hepadnaviruses may be usefully followed in the woodchuck model, especially to characterize the reactivation after the suppression.

In conclusion, we have established the woodchuck as a second animal model for study of the δ agent. The model should be useful for study of the mechanisms of δ replication, of the pathogenesis of δ-induced liver disease, and of the development of prophylactic and therapeutic strategies for the control of this pathogenic agent in humans.

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