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δ agent: Association of δ antigen with hepatitis B surface antigen and RNA in serum of δ-infected chimpanzees

(liver disease/infectious agent)

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ABSTRACT **The hepatitis B virus-associated β antigen was found in the serum of experimentally infected chimpanzees as an internal component of a discrete subpopulation of hepatitis B surface antigen (HBsAg) particles. The 35- to 37-nm particles banded in CsCl at 1.24–1.25 g/cm^3 and sedimented with a mobility intermediate between that of the hepatitis B virion and that of the 22-nm form of HBsAg. The particles contained only indistinct internal structure by electron microscopy and were not unique to δ agent infection, similar particles without δ-antigen activity being observed in the preinfection serum of HBsAg carrier chimpanzees. A small RNA (M_r, 5 × 10^5) was temporally associated with δ antigen in the serum of infected chimpanzees and copurified with the δ-antigen-associated particles. This RNA is smaller than the genomes of known RNA viruses but larger than the viroids of higher plants.**

The δ antigen (δ-Ag), a relatively new specificity, first was detected by immunofluorescence in the liver of human subjects with chronic hepatitis B surface antigen (HBsAg) hepatitis (1). Ultrastructural studies have failed to demonstrate components of hepatitis B virus (HBV) in δ-Ag-positive nuclei (2) and the δ-Ag–anti-δ-Ag system is distinct from the known antigen–antibody systems of HBV (3). Prevalence studies of δ-Ag–anti-δ-Ag in human populations (4, 5) and transmission experiments in chimpanzees (6) indicate that δ-Ag is associated with a transmissible pathogenic agent, δ agent, that is either a HBV mutant with characteristics of a defective interfering particle or a new agent which requires helper functions of HBV for its expression.

After extraction from hepatocyte nuclei with guanidine hydrochloride, δ-Ag was characterized as a protein with a molecular weight of approximately 68,000 (2). Although δ-Ag has not been detected in the sera of patients with intrahepatic δ-Ag, such individuals develop high titers of anti-δ-Ag which might interfere with the available solid-phase radioimmunoassay for δ-Ag. The analysis of serial specimens from chimpanzees to which δ-agent was transmitted revealed δ-Ag in the sera during the acute phase of infection and prior to the development of anti-δ-Ag (6). We report here the association of δ-Ag in serum with a discrete subpopulation of HBsAg and a low molecular weight RNA.

MATERIALS AND METHODS

Source of δ-Ag. Two chronic HBsAg-carrier chimpanzees (nos. 29 and 800) were infected with δ agent by inoculation with serum from a patient with chronic type B hepatitis and intrahepatic δ-Ag. Serum samples and percutaneous liver biopsies were taken from each animal before inoculation and weekly thereafter and analyzed for markers of δ-Ag and HBV. These chimpanzees were part of a transmission study of the δ agent and experimental details are reported elsewhere (6). Serum samples containing δ-Ag activity by radioimmunoassay were used as the source of δ-Ag.

Assays and Reagents. Samples were tested for δ-Ag in duplicate by a microtiter solid-phase radioimmunoassay (2). Briefly, 50-μl aliquots of each test sample were added to the wells of polyvinyl microtiter plates (Cooke Laboratories, Alexandria, VA) previously coated with an optimal dilution (1:1000) of a standard anti-δ-Ag serum. After 24 hr at 4°C, the wells were washed, and 50 μl of ^{125}I-labeled IgG (200,000 cpm) prepared from the standard anti-δ-Ag antiserum was added to each well. After incubation for 3 hr at 37°C, the plates were washed and individual wells were assayed for bound radioactivity. The standard anti-δ-Ag antiserum was obtained from a patient with chronic persistent type B hepatitis and contained a high titer of anti-δ-Ag [1:1000 by immunofluorescence (1); 1:10^5 by a blocking radioimmunoassay (3)], a low titer of anti-HBc (1:600 by radioimmunoassay), and no HBeAg, anti-HBs, or anti-HBe activity by radioimmunoassay. The optimal dilution of the standard anti-δ-Ag serum (1:1000) was previously established by checkerboard titration against a preparation of δ-Ag extracted from a δ-Ag-positive human liver (2). Serum samples were tested before and after treatment with different concentrations of Nonidet P-40. The δ-Ag activity in serum is expressed as the ratio of positive to negative cpm (P/N). The negative value represents the cpm bound by the preinoculation serum. Pellets and fractions of gradients were tested for δ-Ag activity after dilution in 0.01 M phosphate, pH 7.4/0.85% NaCl (P_i/NaCl) containing 0.3% Nonidet P-40. The results are expressed as cpm.

HBsAg was determined by a commercial radioimmunoassay (Ausria II, Abbott Laboratories); dilutions were made in P_i/NaCl. The Dane particle-associated HBcAg activity was measured by a solid-phase radioimmunoassay as described (7); sample dilutions were made in P_i/NaCl containing 0.1% Nonidet P-40 and 0.1% 2-mercaptoethanol. A 20-times concentrate of Dane particles from human serum was used as the positive control. HBsAg-specific DNA polymerase activity was assayed by the method of Kaplan *et al.* (8). Intrahepatic δ-Ag was measured by immunofluorescence (6). The hyperimmune guinea pig anti-HBs/*ad* serum was a reagent prepared for the Research Resources Branch, National Institute of Allergy and Infectious Diseases; cat. no. V801-503558. The preparation of monospecific antibodies to the *a*, *d*, and *y* determinants of

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Abbreviations: δ-Ag, δ antigen; HbsAg, hepatitis B surface antigen; HBV, hepatitis B virus; P_i/NaCl, 0.01 M phosphate, pH 7.4/NaCl.

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HBsAg by using hyperimmune guinea pig antisera was as described (9). Dane particles were purified from the serum of a chronic HBsAg carrier as described (10); the preparation contained neither δ-Ag nor anti-δ-Ag by radioimmunoassay.

Electron Microscopy and Immunoelectron Microscopy. A Hitachi HU-11E electron microscope was used to examine samples after negative-staining with 1% phosphotungstic acid. Immunoelectron microscopy was performed by a modification of a procedure devised by J. D. Almeida and Y. Bradburne (personal communication). The sample (5 μl) was added to carbon/Formvar grids and drained after 30 sec. A drop of P_i/NaCl containing Nonidet P-40 at 0.05–0.5% was added for 1 min and the grid was washed by repeated application of distilled water and then drained. Grids were inverted and allowed to float for 5 min on a drop of either anti-δ-Ag, anti-HBs, or anti-HBc antiserum, washed, and stained with 1% phosphotungstic acid. Various dilutions of preparations of the δ-Ag-associated particles were incubated with dilutions of the standard anti-δ-Ag antiserum, IgG isolated from the standard anti-δ-Ag antiserum or a guinea pig anti-HBs/*ad* antiserum. The Dane particle preparation and human serum containing high-titered anti-HBc (1:10^6 by Corab; Abbott Laboratories) but negative for anti-HBs, anti-HBe, and anti-δ-Ag by radioimmunoassays were used as controls. The reactions were performed in P_i/NaCl or P_i/NaCl containing one of various concentrations (0.05%–0.5%) of Nonidet P-40 followed by washing in P_i/NaCl. In additional experiments, the preparation of purified δ-Ag-associated particles was layered onto and immobilized on grids as described above, treated with various concentrations of Nonidet P-40, washed with P_i/NaCl and treated with antisera.

Immunoprecipitation of δ-Ag-Associated Particles with Anti-HBs. Samples of sera or preparations of δ-Ag-associated particles from gradient fractions were incubated with an optimal dilution, in normal guinea pig serum, of guinea pig antiserum to HBsAg or the *a, d,* or *y* determinant of HBsAg. Purified and iodinated HBsAg (11) (5000 cpm) was added before the reaction and the mixture was incubated overnight at 4°C. Rabbit anti-guinea pig IgG in 20-fold excess to the guinea pig serum was added, followed by incubation at room temperature for 4 hr and centrifugation at 5000 × *g* for 20 min. The supernatant was removed and the percentage precipitation of ^{125}I-labeled HBsAg was calculated from the cpm in the precipitate and the combined cpm of the supernatant and precipitate (9). An aliquot of the supernatant was analyzed for δ-Ag activity by the solid-phase radioimmunoassay.

Isolation and Characterization of Nucleic Acid. Samples of δ-Ag-associated particles pelleted from serum or gradient fractions were resuspended in 0.5 ml of 0.2 M NaCl/0.01 M sodium EDTA/2% $NaDodSO_4$/0.05 M Tris·HCl, pH 8.2; 0.5 mg of proteinase K (Boehringer Mannheim) was added and the sample was incubated overnight at 37°C. Immunoprecipitates of δ-Ag-associated particles and anti-HBs were digested similarly except that an additional 0.5 mg of proteinase K was added after the overnight incubation and digestion was continued for an additional 8 hr at 37°C. One volume of lysate was extracted with 2 vol of neutralized phenol/chloroform, 1:1 (vol/vol); the aqueous phase was precipitated with 2 vol of ethanol after the addition of potassium acetate (pH 5.75) to 0.2 M and *Escherichia coli* tRNA to 50 μg. The precipitates were dried, dissolved in electrophoresis buffer (0.04 M Tris/0.05 M sodium acetate/1 mM sodium EDTA, adjusted to pH 7.8 with glacial acetic acid) plus bromphenol blue and glycerol, added to wells in 1 × 10 × 20 cm 1% agarose horizontal slabs and electrophoresed overnight at 12.5 mA and 40 V. The gel was removed, stained with ethidium bromide (1 μg/ml in electrophoresis buffer), and examined with 254-nm illumination.

A sample of the dried precipitate was dissolved in 0.5 ml of 0.15 M NaCl/0.01 M Tris·HCl, pH 7.4, previously treated with diethyl dioxydiformate and autoclaved. Aliquots were digested with either DNase I (Worthington, RNase-free) at 100 μg/ml with added $MgCl_2$ (4 mM) or RNase A (Worthington, heated for 10 min at 90°C before use) at 10 μg/ml; a control sample contained no enzyme. Incubation (37°C) was 60 min for the DNase and control samples and 30 min for the RNase sample. Before electrophoresis, all samples were subjected to further digestion with proteinase K, extraction, and ethanol precipitation as described above.

RESULTS

Detection of δ-Ag in the Serum. Serial samples of serum from δ-agent infected chimpanzees were analyzed for δ-Ag activity by radioimmunoassay before and after treatment with various concentrations of Nonidet P-40. No δ-Ag activity was detected in the absence of detergent or at concentrations below 0.05%; the δ-Ag activity was optimal at 0.3% and was lower at concentrations between 0.3% and 0.05%. The activity of δ-Ag in the serum and expression of δ-Ag in the liver of chimpanzees nos. 29 and 800 during the infection are shown in Fig. 1. Each serum sample was positive for HBsAg but negative for Dane particle-associated HBcAg and endogenous DNA polymerase activities. δ-Ag activity was first detected in the serum of each animal 4 weeks after inoculation, and peak levels occurred at weeks 5 and 6; subsequent samples were negative for δ-Ag.

Buoyant Density and Sedimentation Properties of δ-Ag in Serum. A portion (7 ml) of each serum sample from both chimpanzees taken at weeks 5 and 6 was layered onto a 4-ml cushion of 20% (wt/wt) sucrose in P_i/NaCl and centrifuged at 4°C for 5 hr at 193,000 × *g* in a Beckman SW 41 rotor. The supernatants were removed, the pellets were resuspended in 1/20 vol of P_i/NaCl, and both fractions of each serum were assayed for δ-Ag in the presence of 0.3% Nonidet P-40. δ-Ag activity was demonstrated only in the pellets at concentrations 10- to 30-fold higher than in the starting sera. The pellets contained no detectable HBcAg or DNA polymerase activities. A pellet from the week 5 serum of each chimpanzee was banded in a CsCl density gradient. For comparison, a sample of a purified Dane particle preparation was centrifuged in a companion tube and fractions from each gradient were assayed for HBsAg, HBcAg, and δ-Ag activity. The δ-Ag activity banded with a peak at 1.24–1.25 g/cm^3 in CsCl, a buoyant density slightly less than that of the Dane particles marker (HBcAg) and higher than the bulk of the HBsAg activity in the chimpanzee

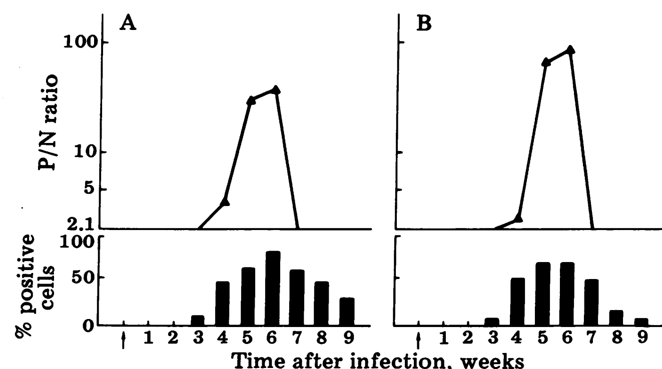

FIG. 1. Transmission of δ agent to HBsAg-carrier chimpanzees 29 (*A*) and 800 (*B*) inoculated (arrow) with serum from a patient with δ-positive chronic hepatitis. The δ activity of serum was analyzed by a solid-phase radioimmunoassay after the addition of Nonidet P-40 to 0.3% and is expressed as P/N (▲). δ-Ag in hepatocyte nuclei was detected by direct immunofluorescence (5) using fluorescein-labeled human anti-δ IgG.

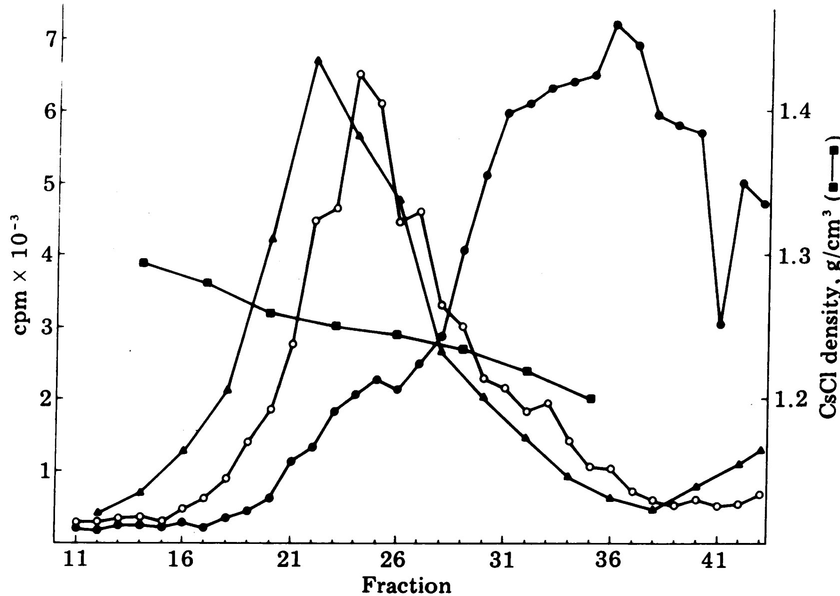

FIG. 2. Isopycnic banding of δ activity in CsCl. A sample (7 ml) of week 5 serum from chimpanzee 800 was pelleted for 5 hr at 193,000 × *g* and 4°C through 20% (wt/wt) sucrose in P_i/NaCl. The resuspended pellet (5.5 ml in P_i/NaCl) and an equal volume of a Dane particle preparation were each mixed in separate cellulose nitrate tubes with 5.5 ml of 1.5 g/cm³ CsCl in P_i/NaCl and centrifuged at 160,000 × *g* and 4°C for 36 hr. Fractions (250 μl) were collected by bottom puncture and assayed for δ (1:11 dilution; ○), HBsAg (1:4000 dilution; ●), and HBcAg (1:11 dilution; ▲) by solid-phase radioimmunoassays. CsCl density (■) was determined by refractometry.

sera; data from chimpanzee 800 are shown in Fig. 2. Fractions with δ-Ag activity from the CsCl gradients were pooled, diluted, and pelleted through 20% (wt/wt) sucrose as above. Each of the resuspended pellets was sedimented in a 10–30% (wt/wt) sucrose gradient; the preparation of Dane particles was centrifuged in a companion tube. The distribution of the Dane particle marker is superimposed on the HBsAg and δ-Ag markers from one gradient in Fig. 3. The δ-Ag activity from each preparation sedimented as a discrete population of particles with a sedimentation coefficient intermediate between that of the predominant 22-nm form of HBsAg and that of the Dane particle marker.

Electron microscopic examination of pellets of the sucrose gradient fractions with δ-Ag activity revealed particles of heterogeneous appearance (Fig. 4), the predominant form being 35–37 nm in diameter; particles of similar size and shape were occasionally observed in the preinoculation (δ-Ag-negative) sera of the chimpanzees.

Immunoprecipitation and Immunoelectron Microscopy. In both experiments above (Figs. 2 and 3) the δ-Ag activity comigrated with a subpopulation of the HBsAg activity of the sample. This association was analyzed by double-antibody immunoprecipitation using preinoculation or hyperimmune anti-HBs guinea pig sera as the first antibody. The analysis of week 5 serum (Fig. 1) from chimpanzees nos. 29 and 800 revealed that 89% and 86% of the δ-Ag activity, respectively, was precipitated by anti-HBs compared to the preinoculation control, as was 86% of trace quantities of ^{125}I-labeled HBsAg added to the test samples as an internal control. The association of δ-Ag activity with HBsAg was further confirmed by the analysis of week 5 serum of chimpanzee 800 by using monospecific antibodies to the *a*, *d*, and *y* determinants of HBsAg (Table 1). The δ-Ag activity was completely precipitated by anti-*a* and anti-*d* sera. Partial precipitation of δ-Ag was also obtained with anti-*y* in this experiment but anti-*y* failed to precipitate δ-Ag in other experiments.

The δ-Ag associated particles were analyzed by immunoelectron microscopy with serum containing anti-HBs, anti-HBc, or anti-δ-Ag. Immune aggregation occurred after incubation with anti-HBs (Fig. 4 *Inset*) but not with anti-HBc or anti-δ-Ag. Except for complexes of the 22-nm HBsAg forms with anti-HBs, no distinct immune aggregates were observed when Nonidet

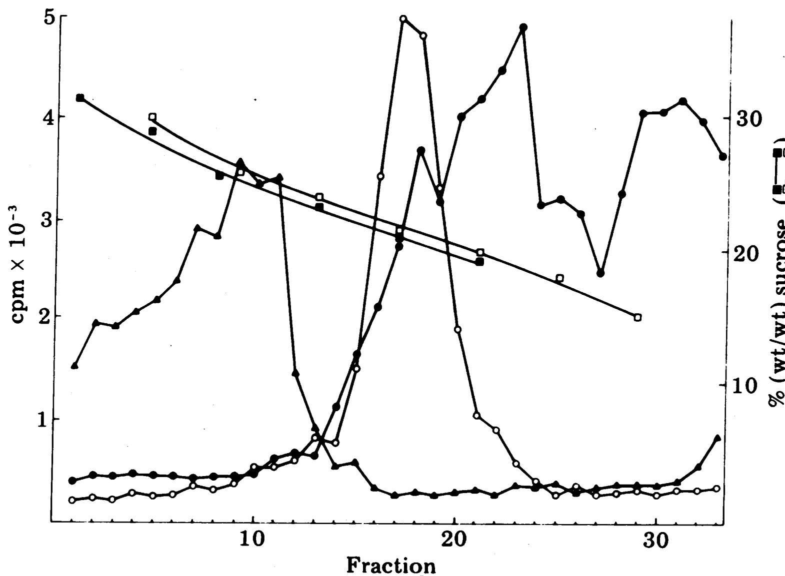

FIG. 3. Rate zonal sedimentation of δ activity in a sucrose gradient. Fractions from the gradients in Fig. 2 (δ and HBcAg, respectively) were pooled, diluted in P_i/NaCl and centrifuged for 5 hr at 193,000 × *g* and 4°C. Each pellet was resuspended in 400 μl of P_i/NaCl and layered onto a linear 10–30% (wt/wt) sucrose gradient (11 ml) with a 0.5-ml 65% (wt/wt) sucrose cushion. The gradients were centrifuged at 193,000 × *g* and 4°C for 110 min and collected by bottom puncture. Fractions of 350 μl were assayed for δ (1:5 dilution; ○), HBsAg (1:500 dilution; ●), and HBcAg (1:5 dilution; ▲) by solid-phase radioimmunoassays. Sucrose concentrations for the δ-positive (□) and Dane particle (■) gradients were determined by refractometry.

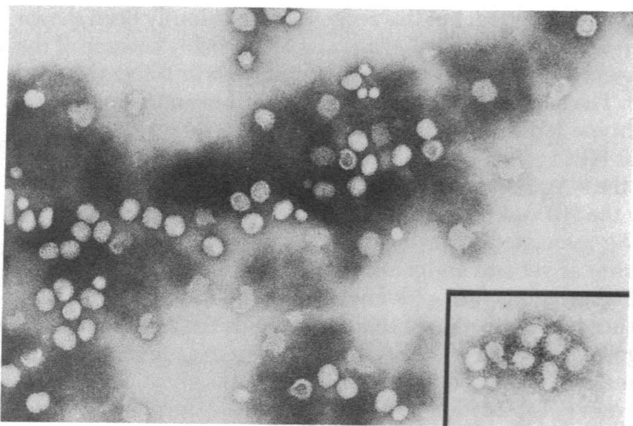

FIG. 4. Electron microscopy of δ-associated particles. δ-Ag-associated particles isolated from the week 5 serum of chimpanzee no. 800 were negatively stained with 1% phosphotungstic acid. The predominant form was a 35- to 37-nm particle; a few 22-nm forms of HBsAg were also observed. (×70,500.) (*Inset*): Immune aggregate of δ-Ag-associated particles with guinea pig anti-HBs serum; the immune complex also contains 22-nm forms of HBsAg. (×70,500.)

P-40-treated samples of δ-Ag-associated particles in suspension or fixed to the grid were exposed to the different antisera. Under the same conditions, incubation of Dane particles with anti-HBc resulted in immune aggregation of the core particles.

Detection of Low Molecular Weight RNA in the δ-Ag-Associated Particles. Weekly blood samples of the chimpanzees were pelleted through 20% (wt/wt) sucrose as described above; pellet digests were extracted and analyzed for nucleic acid by agarose electrophoresis. A discrete band of ethidium bromide fluorescence was observed in those samples that contained δ-Ag activity, and the intensity was proportional to the concentration of δ-Ag in the serum of both animals; the gels for weeks 4, 5, 6, and 7 of chimpanzee no. 29 are shown in Fig. 5. A sample (1 ml) of the week 6 serum of chimpanzee no. 29 was incubated for 1 hr at 37°C with 0.2 ml of either preinoculation or hyperimmune anti-HBs guinea pig antiserum. After overnight incubation with 3.3 ml of rabbit anti-guinea pig IgG, the samples were pelleted for 30 min at 5000 × *g*. The pellets were repeatedly washed in P_i/NaCl, and pellet digests were extracted and analyzed for nucleic acid. The discrete band of fluorescence

Table 1. Association of δ-Ag with HBsAg; coprecipitation of δ-Ag and HBsAg by guinea pig monospecific antibodies

Antigen	Guinea pig antiserum			
	Preserum	Anti-*a*	Anti-*d*	Anti-*y*
δ-Ag*	7009	271	646	ND
^{125}I-Labeled HBsAg/*ad*†	(9.3)	(88.4)	(71.1)	ND
δ-Ag*	7153	250	ND	3031
^{125}I-Labeled HBsAg/*ay*†	(7.8)	(79.0)	ND	(82.8)

Replicate 5-μl samples of ^{125}I-labeled HBsAg were mixed with 5 μl of the δ-Ag preparation from week 5 serum of chimpanzee no. 800. Guinea pig anti-*a* or anti-*d* (each diluted 1:40 in 1:5 guinea pig preserum) or anti-*y* (1:5) serum (5 μl) was then added and the mixtures were incubated overnight at 4°C; rabbit anti-guinea pig IgG (20 μl) was added to each tube. After incubation and centrifugation, 20 μl of each supernatant was assayed for δ-Ag by solid-phase radioimmunoassay; the pellets and supernatants were assayed for ^{125}I. ND, not determined.

* Expressed as cpm. Values were 6808 cpm for an aliquot of δ diluted in P_i/NaCl as a control and 240 cpm for the blank control.

† Shown as % of precipitated cpm. In a parallel experiment without δ-Ag, 17.4% of ^{125}I-labeled HBsAg/*ad* was precipitated by anti-*y* and 11.2% of ^{125}I-labeled HBsAg/*ay* was precipitated by anti-*d* at the antibody dilutions shown in the legend.

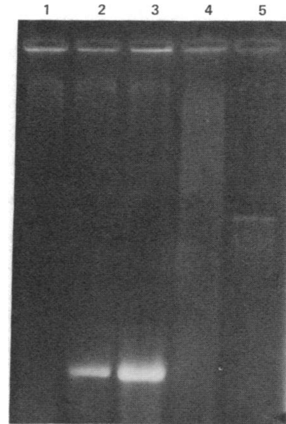

FIG. 5. Temporal association of a low molecular weight nucleic acid with δ-Ag activity in serum of chimpanzee no. 29. Samples (2 ml) of week 4, 5, 6, and 7 sera, obtained at the onset, peak, and disappearance of δ-Ag in the serum (Fig. 1), were pelleted for 5 hr at 193,000 × *g* and 4°C through 20% (wt/wt) sucrose. The pellets were digested, extracted, and analyzed for nucleic acid by agarose electrophoresis. Lanes 1–4 correspond to the weekly serum samples; lane 5 contains woodchuck hepatitis virus DNA (12) (3.27 kilobase pairs) as a marker. A discrete band of fluorescence was observed in lanes 3 and 4.

shown in Fig. 5 was found in the pellet of the sample incubated with guinea pig anti-HBs antiserum but not that of the guinea pig preinoculation control.

The δ-Ag-associated particles were isolated from serum by isopycnic banding in CsCl and rate sedimentation in sucrose as described above in Figs. 2 and 3. A sample (12 ml) of week 6 serum from chimpanzee no. 29 was pelleted through 20% (wt/wt) sucrose and the resuspended pellet was banded to equilibrium in CsCl (Fig. 2). Fractions from three regions of the gradient were pooled: the δ-Ag region (fractions 18–30) containing the bulk of the δ-Ag activity, a region (fractions 33–41) with minimal amounts of δ-Ag activity, and a region devoid of δ-Ag (fractions 11–15). Each pool was diluted in P_i/NaCl and pelleted as described above; the pellet digests were extracted and analyzed for nucleic acid. By comparison with a sample of the initial pellet electrophoresed on the same gel, full recovery of the fluorescent band was obtained in the gradient and the degree of fluorescence paralleled the relative concentration of δ-Ag in the sample (data not shown).

A sample of the pellet from the major δ-Ag pool was subjected to rate zonal sedimentation in a sucrose gradient as in Fig. 3; δ-Ag activity again sedimented faster than the bulk of the HBsAg and in association with a subpopulation of HBsAg particles. The gradient was divided into four regions (Fig. 3): fractions 27–33 (pool A), 21–26 (pool B), 13–20 (pool C), and 1–12 (pool D). Each pool was diluted in P_i/NaCl and pelleted; the pellets were examined by electron microscopy. The pellets of pools A, B, C, and D contained 22-nm particles, small fila-

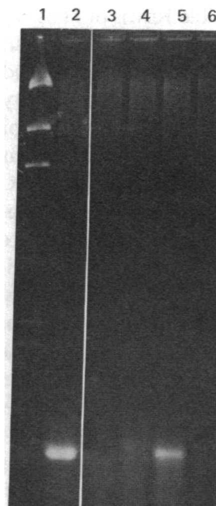

FIG. 6. Analysis of δ-associated particles for nucleic acid. The sucrose gradient (Fig. 3) was divided into four pools as described in the text and analyzed for nucleic acid by agarose electrophoresis. Lanes 3, 4, 5, and 6 correspond to pools A, B, C, and D, respectively. The peak of ethidium fluorescence (lane 5) corresponds to the peak of δ-Ag-activity (pool C) in the sucrose gradient. Lane 2 contained nucleic acid extracted from a sample of the starting material of the rate zonal centrifugation; lane 1 contained a *Hin*dIII digest of λ DNA with double-stranded fragments of 23.72, 9.46, 6.67, 4.26, 2.25, and 1.96 kilobase pairs (top to bottom).

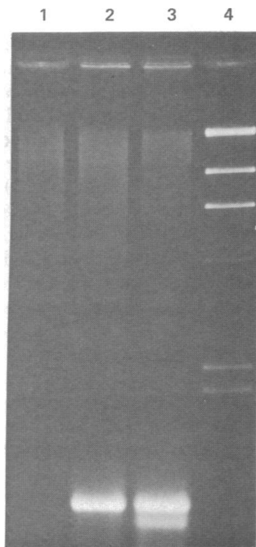

FIG. 7. Susceptibility of the δ-Ag-associated nucleic acid to nucleases. A sample (6 ml) of week 6 serum of chimpanzee no. 29 was pelleted and the pellet digest was extracted for nucleic acid and incubated with either RNase A (lane 1) or DNase I (lane 3). Lane 2 contained the undigested control and lane 4 contained a λ DNA *Hin*dIII digest as in Fig. 6. The nucleic acid was completely digested by RNase; the small additional band in the DNase-digested sample may be due to trace contamination of the DNase with RNase.

ments and 25- to 28-nm particles, 35- to 37-nm δ-Ag-associated particles, and amorphous aggregates, respectively. A sample of each pellet and the starting sample of the sucrose gradient was digested, extracted, and analyzed for nucleic acid by agarose electrophoresis (Fig. 6). Again, good recovery of the fluorescent band was observed in the gradient and the intensity of fluorescence was proportional to the concentration of δ-Ag activity in the pool. Therefore, the low molecular weight nucleic acid copurified with the δ-Ag-associated particle on the basis of both buoyant density and size.

The nucleic acid was digested by RNase A but not by DNase I (Fig. 7). Based on the migration of the λ DNA *Hin*dIII fragments (Figs. 6 and 7) and tRNA (bottom of gels but deleted from Figs. 5, 6, and 7), the δ-Ag-associated RNA was estimated to be about molecular weight 5×10^5. However, a precise determination will require appropriate markers and consideration of the structure of the RNA.

DISCUSSION

Approximately 3 weeks after inoculation with serum from a δ-Ag-positive patient, chronic HBsAg-carrier chimpanzees developed hepatitis coincident with intrahepatic and circulating δ-Ag (6). Physical characterization of δ-Ag activity in serum demonstrated that it was associated with a discrete subpopulation of HBsAg particles with a buoyant density of about 1.25 g/cm^3 in CsCl and a sedimentation coefficient intermediate between that of the 22-nm and Dane particle forms of HBsAg. The predominant form observed by electron microscopy in δ-Ag-positive fractions was a 35- to 37-nm particle that shared determinants of HBsAg with the 22-nm form. The δ-Ag activity of the serum was precipitated by anti-HBs and detection of δ-Ag required detergent treatment, indicating that δ-Ag was an internal component of this subpopulation of HBsAg. Extensive examination by electron microscopy and immunoelectron microscopy of detergent-treated particles failed to reveal a definite structure reactive with anti-δ-Ag; the association of δ-Ag with HBsAg, therefore, appears to differ from that between HBcAg and Dane particles (13). Although δ-Ag was found only in association with the 35- to 37-nm forms, these particles were not unique to δ-agent infection; similar particles were observed in preinoculation sera and sera from δ-Ag-negative HBsAg carriers.

The immunoprecipitation experiments with anti-*d* and anti-*y* revealed that the δ-Ag-associated particles were fully precipitated by anti-*d*. Partial and variable precipitation with anti-*y* was also observed, but this reagent has previously been shown to react with some HBsAg/*ad* samples probably because of the presence of antibody to HBsAg determinants (*w*) other than *d*/*y* (9). The δ-agent inoculum was derived from an Italian carrier, subtype HBsAg/*ay*; the subtype of the recipient chimpanzee was HBsAg/*ad*. Therefore, it appears that the HBsAg surface of the δ-Ag-associated particle was contributed for the most part by the HBV of the host animal and not that of the donor. The δ-Ag-associated forms have not been detected in the donor serum as yet and antigenic analysis is not possible. However, HBsAg/*ayw* is the predominant subtype in Italy and no evidence for coinfection of this patient with HBV of the heterologous *ad* subtype was observed. The δ agent appears to require functions of HBV for its expression and replication. The apparent encapsidation of δ agent with HBsAg could represent one such helper function of HBV to provide this agent a mode of transmission and access to susceptible hepatocytes.

A RNA species of low molecular weight was temporally associated with δ-Ag in the sera of infected chimpanzees. This RNA copurified with the δ-Ag-associated subpopulation of HBsAg particles throughout the isopycnic banding and rate sedimentation procedures and was specifically immunoprecipitated with δ-Ag by anti-HBs. Several laboratories have reported the association of RNA with HBsAg (14–16) but these reports have not been confirmed in the general literature (17). Possibly, some of these observations were due to infection of the carriers used as the source of HBsAg with the δ-Ag agent or a similar agent. Although further conclusions must await the results of the studies now in progress, it is tempting to speculate that the RNA represents genetic material of the δ agent. If so, it appears to be smaller than the genomes of the known RNA viruses but larger than the viroids of higher plants (18).

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