Delta Hepatitis Agent: Structural and Antigenic Properties of the Delta-Associated Particle

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Received 8 June 1983/Accepted 15 November 1983

Delta agent (δ) was serially passaged to a second and third hepatitis B surface antigen (HBsAg) carrier chimpanzee, using as inoculum the peak delta antigen (δAg) serum of an animal previously infected with human serum. The characteristics of serially transmitted δAg were similar to those described in first-passage animals. It was consistently detected before the development of anti-δ, in association with a 35- to 37-nm subpopulation of HBsAg particles and a unique low-molecular-weight (5.5 × 10^5) RNA. RNase susceptibility of the delta-associated RNA and release of δAg activity upon treatment of delta-associated particles with detergent revealed that this particle is organized into a virion-like form with the RNA and δAg as internal components within a coat of HBsAg. Surface determinants of the delta-associated particle were more robust than HBsAg and were not detected by radioimmunoprecipitation experiments, using sera of humans and chimpanzees convalescent from delta hepatitis. The HBsAg-associated particle is the "candidate agent" of delta hepatitis.

ΔAg copurified with 35- to 37-nm particles which contained hepatitis B surface antigen (HBsAg) as well as a small RNA molecule (5.5 × 10^5 molecular weight) (12). These particles had a buoyant density of 1.25 g/cm^3 in CsCl and a sedimentation coefficient intermediate between that of the 22-nm HBsAg forms and the hepatitis B virion (Dane particle) (12). A similar association of serum δAg with HBsAg and RNA was observed in a human HBV carrier with acute δ infection (1). The exact association of delta-associated RNA (DAR), δAg, and the 36-nm HBsAg particle, however, remained unclear. We report here studies on the organization and immunological properties of δ-associated particles purified from plasma of an experimentally infected chimpanzee. DAR and δAg represent internal components of the 36-nm particle (δ particle) which are coated by HBsAg derived from the "helper" HBV infecting the host.

MATERIALS AND METHODS

Source of δAg. The animals inoculated in this study were chimpanzees 57 and 814; they were asymptomatic chronic carriers of HBsAg without serological evidence of prior exposure to δ agent. Both animals had serum hepatitis B antigen (HBeAg) and DNA polymerase activity and intrahepatic hepatitis B core antigen (HBCAg).

The serum containing the peak δAg activity from chimpanzee 800 (1 ml, undiluted) was used as inoculum for the second passage of δ agent to chronic HBsAg (adw) carrier chimpanzee 57. This animal developed intrahepatic δAg 2 weeks postinoculation and hepatitis at week 4. δAg appeared in the serum at week 2, rose to a peak level at week 4, and declined to unmeasurable levels at week 6. Hepatitis resolved and anti-δ seroconversion occurred at week 6 postinoculation. The serum containing peak δAg activity from chimpanzee 57 (1 ml, undiluted) was used as inoculum for a third passage of δ to chronic HBsAg carrier chimpanzee 814. δAg appeared in liver at 2 weeks post-inoculation and in serum at week 3, rose to peak levels at week 4, and declined to undetectable levels with anti-δ seroconversion at week 6. In both animals delta infection resulted in suppression of HBV replication; HBCAg disappeared from hepatocyte nuclei and DNA polymerase activity was no longer detectable in serum. Serum δAg had characteristics identical to those previously reported in the first-passage chimpanzees; it was detected only after detergent (NP-40) treatment of serum, copurified with a 35- to 37-nm subpopulation of HBsAg particles, and was associated with a small RNA molecule (5.5 × 10^5 molecular weight). Further experiments were conducted with δ-associated particles obtained from serum of chimpanzee 814 when δAg was at its highest titer.

Assays. HBsAg, anti-HBs, anti-HBe, HBCAg, and anti-HBe activities were determined by commercial radioimmunoassays (Austria II, Ausab, Corab, and Abbott-HBe; Abbott Laboratories); dilutions were made in P_NaCl (0.85% NaCl, 0.01 M phosphate buffer, pH 7.4). HBCAg, δAg, and anti-δ were detected by microtitr solid-phase radioimmunoassays, as described previously (3, 8, 10). Pellets and fractions of gradients were tested for δAg activity after dilution in P_NaCl containing 0.3% NP-40. HBsAg-specific DNA polymerase activity was measured as previously reported (4).
Preparations of δ-associated particles. Delta-associated particles were isolated from plasma by isopycnic banding in CsCl and rate zonal sedimentation in sucrose as previously described (1, 12). Briefly, two 5-ml samples of δAg-positive plasma (recalified and clarified at 2,000 rpm for 10 min) were pelleted through a cushion of 20% (wt/wt) sucrose–P2-NaCl and centrifuged for 5 h at 193,000 × g and 4°C. The resulting pellets were resuspended to a combined volume of 0.5 ml with P2-NaCl, layered on top of an 11.0-ml discontinuous gradient (1.2 to 1.5 g/cm³) of CsCl in P2-NaCl, and centrifuged for 24 h at 108,000 × g and 4°C. Fractions were collected by bottom puncture and assayed for HBsAg and δAg. The δAg peak fractions (1.245 g/cm³) were pooled, diluted in P2-NaCl, and centrifuged for 5 h at 193,000 × g and 4°C. The pellet was resuspended in 1 ml of P2-NaCl (P₂) and used as a source of δ-associated particles.

Iodination of δ-associated particles. Delta particle preparations (P₂) were iodinated by the chloramine-T procedure (16); 400 μCi of Na¹²⁵I (New England Nuclear Corp.), 2 nmol of Nai (Fisher Scientific Co.), and 20 μg of chloramine-T (Eastman Kodak Co.) were added to 50 μl of P₂ samples. After 1 min the reaction was stopped by the addition of 40 μl of sodium metabisulfite and 1 μg of KI. Excess reagents were removed by passing the mixture through a Sephadex G-25 column (PD-10; Pharmacia Fine Chemicals, Inc.).

Antisera. Hyperimmune anti-HBs (subtypes ad and ay) guinea-pig sera were those supplied by the Research Resources Branch of the National Institute of Allergy and Infectious Diseases (catalog no. V801-503-558 and V802-501-558, respectively). Antisera monospecific for δAg were obtained from experimentally infected chimpanzees and human HBsAg carriers with intrahepatic δAg. Preincubation sera obtained from chimpanzees and a human (HBsAg, anti-HBc positive, and anti-δ negative) serum were used as controls. Rabbit anti-guinea pig immunoglobulin G (IgG) and goat anti-human IgG, IgA, and IgM sera (Cappel Laboratories Inc.) were used as second antibodies for radioimmunoprecipitation (RIP).

Analysis of DAR. Samples of δ-associated particles pelleted from serum or gradient fractions were digested overnight at 37°C in 500 μl of “lysis solution” (0.2 M NaCl, 0.02 M sodium EDTA, 2% sodium dodecyl sulfate, 0.5 mg of proteinase K, 50 μg of Escherichia coli tRNA “RNase free” [both from Boehringer Mannheim Biochemicals], and 0.05 M HEPES [N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid], pH 7.4). Lysates were extracted with 1 volume of chloroform and 1 volume of neutralized phenol; aqueous phases were precipitated with 2 volumes of ethanol after the addition of potassium acetate (pH 5.75) to 0.2 M. The precipitates were dried, dissolved in electrophoretic buffer (0.04 M Tris, 0.05 M sodium acetate, 0.001 M sodium EDTA, pH 7.8), and electrophoresed overnight at 12.5 mA and 40 V in a 1% agarose horizontal slab (1 by 10 by 20 cm). The gel was stained with 1 μg of ethidium bromide per ml and visualized at 254 nm. Samples of the dried precipitates were dissolved in 0.2 ml of 0.15 M NaCl–0.01 M Tris-hydrochloride, pH 7.4; solutions minus Tris-hydrochloride had been treated with diethyl pyrocarbonate and autoclaved. Pancreatic RNase (heated for 10 min at 90°C before use; Worthington Diagnostics) was added at various concentrations (0.1 to 10 μg/ml). After incubation for 0.5 h at 37°C, the reaction was stopped with lysing solution, preparations were extracted with phenol chloroform, and the aqueous phase was ethanol precipitated. Precipitates were electrophoresed and stained with ethidium bromide as described above.

Electron microscopy. A Hitachi HU-11E electron microscope was used to examine preparations of δ-associated particles after negative staining with 1% phosphotungstic acid.

RESULTS

RIP of purified δ-associated particles. Samples of iodinated δ-associated particles were purified by rate zonal sedimentation on a linear sucrose gradient. Three peaks of radioactivity were obtained (Fig. 1): one was immunoprecipitated by guinea pig anti-HBs and, after 0.3% NP-40 treatment, also by anti-δ; the other two peaks were only precipitated by anti-HBs. No RIP was obtained with anti-HBc serum (Table 1). One or with guinea pig immunoprecipitation serum, used as controls. The radioactivity of the fractions corresponding to δ-associated particles was 90 to 95% precipitable by 10% trichloroacetic acid. Since iodinated particles were precipitated by anti-δ-positive serum only after treatment with 0.3% NP-40 and no significant RIP was obtained without detergent, the preparation was considered to represent intact particles, separated from the bulk of HBsAg.

Intact and disrupted ¹²⁵I-labeled δ-associated particles (5 μl) were used for RIP assays with sera listed in Table 1. Anti-δ-positive sera from humans and chronic HBsAg carrier chimpanzees, convalescent from acute δ hepatitis, failed to precipitate intact particles, whereas significant RIP was obtained with the anti-HBs-positive serum and after disruption of particles with 0.3% NP-40. HBV-susceptible chimpanzees 32 developed an acute type B hepatitis and recovered with seroconversion to anti-δ and subsequently anti-HBs (10). An anti-δ-positive serum from chimpanzee 32, obtained before anti-HBs seroconversion, did not precipitate intact particles, whereas a significant RIP was achieved with another serum, obtained from the same animal, after appearance of anti-HBs (Table 1).

Composition of the purified δ-associated particle. Samples of iodinated and purified δ-associated particles (0.25 ml of
fraction 3, Fig. 1) were treated with detergent (0.3% NP-40) and centrifuged in a CsCl gradient. δAg activity banded at a buoyant density of 1.283 g/cm³, well separated from HBSAg (Fig. 2A), and no HBcAg or endogenous DNA polymerase activities were detected in the preparation. The density of δAg released from the particle was identical to that of antigen extracted from human liver (14). Double-antibody RIP analysis, however, revealed that 50 and 75% of radioactivity was precipitated by anti-HBs and anti-δ sera, respectively, indicating that at this stage of purification δAg remains associated to some extent with HBSAg-reactive material.

DAR. The δ-particle preparation (P₂, 0.5 ml) was sedimented as described for 125I-labeled particles; δAg-positive

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<th>TABLE 1. RIP of the δ-associated particle</th>
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¹ The titer of anti-δ antibody is expressed as the reciprocal log₁₀ dilution which inhibits 50% of binding of iodinated anti-δ IgG probe (14).

² Samples (5 μl) of radiolabeled δ-associated particles (see text) were incubated for 4 h at room temperature with 5 μl of human or chimpanzee sera (1/5 dilution in P₁-NaCl-5% fetal calf serum). Preinoculation sera from chimpanzees and human serum were used as controls. Second antibodies were added overnight in 20-fold excess at 4°C. After centrifugation at 6,000 rpm for 30 min, supernatants were recovered and radioactivity was determined in a 280 UG LKB gamma counter. Specific immunoprecipitation (RIP) was expressed as percentage of total counts per minute precipitated by immune sera minus those precipitated by control sera (% Ppt); values ± 15% were considered positive.

Chimpanzees 814, 57, 800, and 29 were chronic HBV carriers superinfected with δ agent; chimpanzee 32 was an animal that was simultaneously infected with both HBV and δ. The details of these experiments were previously reported (10). The mean anti-HBc titer in all of these sera was 10⁻⁴ (range, 10⁻³ to 10⁻⁶). Only the serum obtained from chimpanzees 32 at week 30 after inoculation was anti-HBs positive with a 10⁻⁷ titer. Pre, Preinoculation serum sample.

³ Serum 1 (anti-δ, anti-HBc, and anti-HBe positive) was obtained from a chronic active hepatitis and intrahepatic δAg. Sera 2 and 3 (anti-δ, anti-HBc, and anti-HBe positive) were from two chronic HBsAg carriers who recovered from acute δ hepatitis, and serum 4 (anti-HBe positive at 10⁻⁸ titer. HBeAg, anti-δ negative) was from a patient with chronic active hepatitis and intrahepatic HBeAg.

* N/A. Not applicable.

fractions were pelleted and resuspended to 0.25 ml in P₁-NaCl. Spherical 35- to 37-nm particles were observed by electron microscopy. Purified δ-associated particles were treated with detergent and banded in a discontinuous CsCl gradient as described above for labeled particles; vanadyl ribonucleoside complex (Bethesda Research Laboratories Inc.) was added to inhibit RNase (10 mM final concentration). Fractions (0.5 ml) were collected by top aspiration and assayed for δAg and HBsAg by radioimmunoassays (Fig. 2B). The peak δAg activity contained a small amount of HBSAg, and no "corelike" structures were observed by electron microscopy.

Fractions from three regions of the gradient were pooled:
(i) the peak δAg region (fractions 6 to 11), (ii) fractions 12 to 20 containing minimal amounts of δAg and corresponding to
ethidium bromide from buffer with 1 to 6 h.
DNA of the text.

The pellets were resuspended and the electrophoresis gradient (Fig. 2B) resulted in the appearance of anti-8 in the serum. Surface determinants of the 8-associated particle other than HBsAg were not detected by RIP experiments, using sera of humans and chimpanzees convalescent from 8 hepatitis. Since anti-8 sera do not precipitate intact particles, the rapid clearance of 8Ag and DAR from serum remains unexplained, and mechanisms other than immune clearance mediated by anti-8 must be involved in the disappearance of 8Ag from serum. Intracellular IgG has been reported in the liver of experimentally infected chimpanzees only after the appearance of anti-8 in the serum (11). In view of this, anti-8 antibody may play a major role in inhibiting or possibly blocking the release of 8Ag and DAR from infected hepatocytes.

8Ag and DAR were shown to be internal components of the 8-associated particle as their detection requires disrup-

the top of the gradient, and (iii) the bottom of the gradient (fractions 1 to 5). The pellet from the gradient was resuspended to 0.25 ml of 0.02 M NaCl and 0.01 M sodium EDTA-0.05 M HEPES, pH 7.4. Each fraction was dialyzed for 18 h at 4°C in a large volume of the same buffer. The three pools and the resuspended pellet were then analyzed for DAR by electrophoresis (Fig. 3). DAR was not detected in the gradient fractions but was recovered in the resuspended pellet (1.5 g/cm in CsCl). These data indicate that detergent treatment of the particle resulted in release of the DAR.

To evaluate the stability of this particle, purified particles from a sucrose gradient were assayed for 8 activity by RIP after 12, 24, 48, and 72 h of storage in 30% sucrose-P,NaCl at 4°C. Within 48 h 8Ag activity increased to the value of the NP-40-treated control preparation. In the same time period, however, the control preparation showed a decrease which stabilized at approximately 50% of the initial value (Fig. 4). At each time point the preparation was examined by electron microscopy; particles were randomly photographed and the percentage of empty particles was determined. At 12 h, 10% of the particles appeared empty (Fig. 5A), whereas the percentage rose to 75% after 72 h (Fig. 5B). DAR was detected by electrophoresis in the initial preparation and in lower quantity after 48 h, but was not detectable at 72 h. These data are consistent with the gradual opening of the 8-associated particles under these storage conditions and the accessibility of DAR to environmental RNase. To analyze the integrity of the serum-derived particles, 8-associated particles were subjected to a graded series of RNase conditions. An equivalent amount of DAR was observed with each concentration of RNase (Fig. 6), indicating that DAR of the serum-derived particle is not accessible to RNase. DAR was not recovered after RNase treatment of particles disrupted by detergent.

**DISCUSSION**

The 8 agent was serially passaged in a second and third HBsAg carrier chimpanzee, using as inoculum the serum obtained at the peak of 8Ag from another animal infected with human serum. 8Ag was found in the blood of these animals during a relatively narrow time span of about a month before the appearance of anti-8 and was associated with 35- to 37-nm particles of HBsAg and a low-molecular-weight (5.5 × 10^3) RNA.

The titers of 8Ag and 8-associated RNA (DAR as detected by ethidium bromide fluorescence) were positively correlated and both quickly declined with the appearance of anti-8 in the serum. Surface determinants of the 8-associated particle other than HBsAg were not detected by RIP experiments, using sera of humans and chimpanzees convalescent from 8 hepatitis. Since anti-8 sera do not precipitate intact particles, the rapid clearance of 8Ag and DAR from serum remains unexplained, and mechanisms other than immune clearance mediated by anti-8 must be involved in the disappearance of 8Ag from serum. Intracellular IgG has been reported in the liver of experimentally infected chimpanzees only after the appearance of anti-8 in the serum (11). In view of this, anti-8 antibody may play a major role in inhibiting or possibly blocking the release of 8Ag and DAR from infected hepatocytes.

8Ag and DAR were shown to be internal components of the 8-associated particle as their detection requires disrup-

![FIG. 3. Analysis of CsCl fractions for DAR. Pellet and fractions from the CsCl gradient (Fig. 2B) were digested with proteinase K, extracted with chloroform-phenol, and ethanol precipitated as described in the text. Precipitates were dissolved in the electrophoretic buffer and run overnight at 12.5 mA and 40 V in a 1% agarose horizontal slab (1 by 10 by 20 cm). The gel was stained with 1 μg of ethidium bromide per ml and visualized at 254 nm. Lane D contains the pellet of the gradient. Lanes A, B, and E contain pooled fractions 1 to 5, 6 to 11, and 12 to 20, respectively. Lane C contains a λ-HindIII restriction enzyme digest with double-stranded fragments of DNA: 23.7, 9.5, 6.7, 4.3, 2.3, and 2.0 kilobase pairs (top to bottom).](http://iai.asm.org/)

![FIG. 4. RIP of 125I-labeled 8-associated particles after storage in 30% sucrose. Purified 125I-labeled particles were stored in 30% sucrose-P,NaCl at 4°C and immunoprecipitated with human anti-8-positive serum (no. 1, Table 1). The results are expressed as percentage of total counts per minute obtained by RIP with (●) or without (▲) the addition of 0.3% NP-40.](http://iai.asm.org/)
FIG. 5. Electron microscopy of δ-associated particles. Delta particle preparations in 30% sucrose-P,NaCl were examined by electron microscopy (×150,000) after negative staining with 1% phosphotungstic acid. The predominant forms were spherical particles averaging approximately 36 nm, some empty (10%) and others with internal structure (A); after 72 h at 4°C, >75% of particles appeared empty and partially disrupted (B).

FIG. 6. Integrity of serum-derived δ-associated particles. The serum pellet (P1) obtained, as described above, from 2 ml of δAg-positive serum was resuspended to 0.25 ml of P,NaCl and mixed with 0.80 ml of 10× standard sodium citrate (SSC = 1.5 M NaCl, 0.15 M trisodium citrate) and 0.60 ml of diethyl pyrocarbonate-treated water. The mixture was equally divided into four tubes, and pancreatic RNase was added in graded concentrations (2.0, 1.0, and 0.1 μg/ml) to three of them. After incubation at 37°C, the mixtures were analyzed by electrophoresis for DAR as described in the text. Lane A in the gel contained the RNase untreated control sample; lanes B, C, and D contained samples treated with 0.1, 1.0, and 2.0 μg of RNase per ml. The far left lane contains a λ-HindIII restriction enzyme digest as described in the legend to Fig. 4.
ACKNOWLEDGMENTS

We thank Eugenie Ford for the electron microscopy and Judy Nelson for technical assistance.

This work was supported by contract NOI-AI-22665 between Georgetown University and the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED