Demonstration of Subpopulations of Dane Particles

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Two populations of Dane particles were isolated from the plasma of individuals carrying hepatitis B surface antigen. These populations had densities in CsCl of 1.22 and 1.20 g/ml. Endogenous DNA polymerase activity was found to be associated only with the heavier of these two populations. Using a positive stain, electron microscopic examination of these particles suggested that the heavier particle contained nucleic acid in its core whereas the lighter particle appeared empty. Cores isolated from Dane particles with densities of 1.22 and 1.20 g/ml banded in CsCl at densities of 1.36 and 1.30 g/ml, respectively. Endogenous DNA polymerase activity was associated only with the higher density core particles.

Three particulate forms of hepatitis B surface antigen (HB₄Ag) can be identified in the blood of individuals who are either undergoing an acute infection or have become carriers of hepatitis B virus (2, 9). The first of these forms is a roughly spherical particle averaging approximately 22 nm in diameter. These particles, which are the most predominant form in serum, apparently contain no nucleic acid (8). Secondly, a heterogeneous population of filamentous particles, all with a diameter of about 22 nm, have been identified (1, 2, 8). These filaments are thought to be variant forms of the 22-nm spherical particles (1). Finally, a more complex spherical particle referred to as the Dane particle has been observed (6). This particle is 42 nm in diameter and contains a 28-nm core structure. This core is antigenically distinct from the surface antigen and has been designated as hepatitis B core antigen (HB₄Ag) (3, 13, 20, 26, 28). A circular double-stranded DNA with a molecular weight of 1.6 × 10⁹ has been isolated from Dane particle cores (27). Since the Dane particle contains nucleic acid and has a morphology consistent with that of a virus, it appears as the most likely candidate to be the infectious agent of hepatitis B. This conclusion is further supported by the epidemiological association of DNA polymerase-containing Dane particles and the development of anti-HB₄Ag with clinical disease (17, 21).

A DNA polymerase activity has been demonstrated in Dane-rich concentrates of HB₄Ag (18). This enzymatic activity was dependent on the presence of four deoxyribonucleoside triphosphates and MgCl₂. The activity was enhanced when the preparation was treated with the nonionic detergent Nonidet P-40. The polymerase activity was thought to be DNA dependent because it could be inhibited with low levels of actinomycin D. Although DNA dependent, the enzyme was not stimulated by exogenous DNA templates. The DNA synthesized by this enzyme was homogeneous and sedimented at a rate of 15S. This polymerase product was associated with a structure that sedimented at a rate of 110S. Both denaturation and density studies of this nucleic acid indicated that it had a guanosine plus cytosine content of approximately 49% (27). Experiments have also been conducted that have definitively demonstrated that the enzymatic activity, as well as the DNA product, is associated with the core of the Dane particle. Both the polymerase product and the enzymatic activity in Nonidet P-40-treated preparations were specifically precipitated by anti-HB₄Ag (28). Enzymatic activity prior to detergent treatment was precipitated with anti-HB₄Ag.

In the initial studies of this enzymatic activity (18), it was shown that in sucrose gradients HB₄Ag activity and DNA polymerase activity banded at slightly different densities. From these data it was suggested that the enzymatic activity was associated with a subpopulation of core particles. In this communication we demonstrate that two populations of Dane particles are present in serum, only one of which has
associated DNA polymerase activity. Further, from these two populations, core particles of different densities can be obtained.

**MATERIALS AND METHODS**

**Materials.** [methyl-3H]thymidine 5'-triphosphate (53.7 Ci/mmole) was purchased from New England Nuclear Corp. Deoxyadenosine 5'-triphosphate, deoxyctydine 5'-triphosphate, and deoxyguanosine 5'-triphosphate were purchased from P. L. Biochemicals, Inc. Nonidet P-40 was a gift of the Shell Oil Co. Austria II kits (23) used for radioimmunoassay of HBsAg were purchased from Abbott Laboratories.

**Dane particle preparations.** Two DNA polymerase-positive plasma units (767932 and 715271) were used in this study. A Dane-rich concentrate of 767932 was prepared by centrifugation of the plasma in a type 21 rotor at 20,000 rpm for 4 hr at 4°C in a model L ultracentrifuge as previously described (18, 26). Each pellet was resuspended to the original volume in phosphate-buffered saline (pH 7.4) and recentrifuged as described above. The resulting second pellet was resuspended in phosphate-buffered saline to 1/20 the original volume and stored at -70°C. Plasma sample 715271 was concentrated in a similar manner to that described above; however, the initial pelleting was through a layer of 20% (wt/vol) sucrose. After the second pelleting the material was resuspended in phosphate-buffered saline to 1/50 its initial volume and stored at -70°C. Two milliliters of each of these preparations were then layered on top of discontinuous 20%-over-65% (wt/vol) sucrose density gradients containing 0.01 M Tris-hydrochloride (pH 7.5), 0.10 M NaCl, and 0.5 mg of bovine serum albumin per ml (TNB). Centrifugation was carried out for 3.5 hr at 35,000 rpm at 4°C in an SW41 rotor. The material at the interface between 20 and 65% sucrose was collected and dialyzed overnight against TNB buffer. The resultant material was used in the experiments described in the text.

**Assays.** Assays for DNA polymerase activity in CsCl density gradient fractions were conducted in the manner previously described (18). Either 25 or 50 μl of each fraction was assayed. The Austria II system was used to assay gradient fractions for HBsAg. Since in undiluted form the concentrations of HBsAg present would saturate the solid-phase radioimmunoassay, several dilutions of each of the gradient fractions were tested for activity. The data presented represent the lowest dilution tested at which none of the gradient fractions saturated the assay. The dilutions used are described in the figure legends. The radioimmunoassay used to detect HBsAg has been previously described (26). The test was modified to shorten the incubation of the antigen with immobilized antibody from 40 to 16 hr.

**Electron microscopy.** Gradient fractions were scanned for Dane particles using negative staining procedures. Briefly, 1 drop of the fraction was placed on a carbon-coated grid and allowed to stand for approximately 2 min. Excess material was absorbed on filter paper, and the grids were washed in glass-distilled water. The material was stained with 1% phosphotungstic acid (pH 7.0) for 1 min and then examined in a Hitachi HU-11E-1 electron microscope. Selected fractions were also examined after positive staining with uranyl acetate (14, 16). Material was placed on grids for 15 s. Excess material was then absorbed onto filter paper and the grids were washed. The preparations were stained for 1.5 to 2.0 hr in a humidified chamber with a solution of 0.2% uranyl acetate. In some cases, prior to staining, the material was fixed with 1% glutaraldehyde; however this fixation did not seem to affect the staining patterns or morphology of Dane particles.

**RESULTS**

Density gradient analysis of the Dane particle preparation. Dane particle preparation 767932 was layered on top of a 1.15 to 1.25-g/ml continuous CsCl density gradient with a 1.30-g/ml cushion and centrifuged at 35,000 rpm for 4 hr at 4°C in an SW41 rotor. Fractions were collected and assayed for DNA polymerase, HBsAg, and HBcAg (Fig. 1). A single peak of DNA polymerase activity was found at a density of 1.22 g/ml (fraction 10). Assays for HBsAg, however, showed two peaks of this antigen, one of which seemed to be associated with the polymerase activity. The major proportion of the core antigen was associated with material with a density of approximately 1.20 g/ml. Electron microscopic examination of fractions indicated that the difference in HBcAg titers in the two areas of the gradient reflected different quantities of Dane particles. Most of the HBsAg was observed at a lower density than the Dane particles. This probably reflected size rather than density differences since, under the conditions of centrifugation used, most of the HBsAg would not have sedimented to its buoyant density.

**Isolation of individual Dane particle populations.** Experiments were conducted to further substantiate the presence of two populations of Dane particles. Fractions 9 and 10 and 12 and 13 from the gradient shown in Fig. 1 were separately pooled and dialyzed overnight against TNB buffer. This material was then banded in CsCl in the manner used for their initial isolation. Fractions were again collected and examined for DNA polymerase, HBcAg, and HBsAg. The results of this experiment are presented in Fig. 2. Material isolated from the denser region of the first gradient (fractions 9 and 10, Fig. 1) banded sharply at a density of approximately 1.22 g/ml (Fig. 2A). Unlike the initially isolated material, upon recentrifugation the positions in the gradient of DNA polymerase, HBcAg, and HBsAg all coincided. A slight shoulder of HBsAg and HBcAg was present, which cor-
related with the position of lower density Dane particles. The patterns obtained with material pooled from the lighter area of the primary gradient (fractions 12 and 13, Fig. 1) are presented in Fig. 2B. A sharp peak of HBcAg was observed at a density of approximately 1.20 g/ml. There was little if any DNA polymerase activity associated with this material. A sharp peak of HBsAg was seen which tailed toward the top of the gradient. This tailing was probably due to the short time of centrifugation.

These data suggested that there were two populations of Dane particles, the majority of which, in this preparation, had a density of 1.20 g/ml. These particles did not have DNA polymerase activity associated with them. A minor proportion of the particles, however, banded at a density of approximately 1.22 g/ml and had DNA polymerase activity.

**Electron microscopic examination of isolated Dane populations.** Fractions in both gradients shown in Fig. 2 were examined in the electron microscope. In both gradients the quantity of HBsAg correlated well with the relative number of Dane particles observed. Uptake of uranyl acetate by Dane particle cores is probably due to the presence of nucleic acid (15). Based on this, particles in the peak fractions of each gradient were examined to determine their staining patterns using a positive staining procedure. Approximately 100 particles were randomly photographed from each gradient, and the percentage of particles that had electron-dense cores was determined. Over 60% of the particles seen in the peak fraction from the gradient shown in Fig. 2A appeared to take up the uranyl acetate used as the positive stain (Fig. 3A). On the other hand, in the peak fraction from the gradient containing the lower density Dane particles (Fig. 2B) more than 85% of the particles appeared empty. The appearance of these empty particles can be seen in Fig. 3B.

**Isolation of cores from the two populations of Dane particles.** Electron microscopic examination of the two populations of Dane particles

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**Fig. 1.** Density gradient analysis of preparation 767932. One milliliter of virus was layered on top of a 1.15 to 1.25-g/ml continuous CsCl density gradient with 1.0 ml of a 1.30-g/ml CsCl cushion. The CsCl contained 0.01 M Tris-hydrochloride (pH 7.5) and 0.5 mg of bovine serum albumin per ml. The material was then centrifuged in an SW41 rotor at 35,000 rpm for 4 h at 4°C. Fractions were collected and tested for DNA polymerase activity (25 μl) (O) and HBsAg (25 μl) (△). An assay for HBsAg (●) was also carried out, using 200 μl of each fraction after it had been diluted 1:100. Densities (■) were determined by refractometry.

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**Subpopulations of Dane Particles**

Vol. 17, 1976

Page 887
suggested a difference in the nucleic acid content of their cores. If this were the case, it would be expected that core structures isolated from these two populations would differ in density.

Dane particle preparation 715271 was banded in a CsCl density gradient in a manner similar to that previously described. Fractions were collected and assayed for DNA polymerase, HB_{Ag}, and HB_{sAg}. They were also examined by electron microscopy. The results of this experiment are presented in Fig. 4. Again, it was apparent that the DNA polymerase activity was associated only with the denser of the two major peaks of Dane particles, as indicated by HB_{sAg} titers. With preparation 715271 (unlike 767932, Fig. 1) a minor peak at a density of 1.24 g/ml could be detected. This material had HB_{sAg}, HB_{Ag}, and DNA polymerase activity. Examination of this material by electron microscopy indicated the presence of aggregates of Dane particles and filamentous particles. This aggregation was probably due to the presence of low levels of anti-HB_{sAg} in the preparation. This is further suggested by the unusually high ratio of HB_{Ag} to HB_{sAg} obtained with this material as compared to that seen in the major peak of DNA polymerase-containing Dane particles. Further, particle-associated antibody would shift the density of aggregates, making them more dense.

Fractions 8 and 9 and 12 and 13 were separately pooled, adjusted to 1% Nonidet P-40 and 0.3% 2-mercaptoethanol, and incubated for 30 min at 37 C. This material was then layered on

Fig. 2. Density gradient analysis of isolated Dane particle populations. Fractions 9 and 10 and 12 and 13 (Fig. 1) were dialyzed overnight against TNB buffer and centrifuged as described in Fig. 1. Fractions from the gradient obtained using fractions 9 and 10 (A) were assayed for DNA polymerase (50 μl) (○), HB_{Ag} (25 μl) (●), and HB_{sAg} (1:4) (●). Material from the gradient prepared using the fraction 12 and 13 pool (B) was assayed in a similar manner, using a 1:40 dilution of each fraction to assay HB_{sAg}.
top of discontinuous CsCl density gradients and centrifuged at 40,000 rpm for 18 h at 4°C in an SW50.1 rotor. Fractions were collected and assayed for DNA polymerase, HB₃Ag, and HBcAg. Figure 5A depicts the results obtained with cores isolated from the dense, DNA polymerase-containing Dane particles (fractions 8 and 9, Fig. 4). It can be seen that all of the DNA polymerase activity banded at a density of 1.355 g/ml. Most of the HBcAg activity was also localized at this density. A significant amount of HBcAg was also observed at a density of 1.30 g/ml. No DNA polymerase activity was associated with this material. Assays for HBcAg indicated that, after the Nonidet P-40–2-mercaptoethanol treatment, this antigen could be detected only at the top of the gradient (data not shown). Cores isolated from the lighter density Dane particles (fractions 12 and 13, Fig. 4) banded at a density of 1.30 g/ml (Fig. 5B). This area of the gradient was totally devoid of DNA polymerase activity. The DNA polymerase activity present in this gradient was found at a density of 1.355 g/ml, a position which coincided with a small peak of HBcAg.

These data indicated that Dane particles that band at 1.22 g/ml in CsCl contained core particles that band at a density of 1.36 g/ml and had DNA polymerase activity. On the other hand, Dane particles with a density of 1.20 g/ml contained core particles with a density of 1.30 g/ml, which appeared to be free of DNA polymerase activity.

**DISCUSSION**

These data clearly demonstrate that in the preparations tested there are two distinct populations of Dane particles, which can be separated on the basis of their densities in CsCl. The endogenous DNA polymerase is completely as-
Fig. 3. Positively stained particles in isolated Dane particle populations. Particles were examined in the peak fractions shown in Fig. 2A (A) and Fig. 2B (B). x200,000.

Fig. 4. Density gradient analysis of preparation 715271. One milliliter of virus was layered on a 1.15 to 1.25-g/ml continuous CsCl density gradient and centrifuged as described in Fig. 1. Fractions were collected and assayed for DNA polymerase (50 μl) (○), HBsAg (25 μl) (△), and HbsAg (1:40) (∗).

associated with the denser of these populations. Based on electron microscopic and density analyses, it appears that there is significantly more nucleic acid in the cores of the heavy Dane particles that have associated DNA polymerase activity.

Gerin (7) previously reported the existence of two populations of Dane particles that banded
in CsCl at 1.20 and 1.25 g/ml, respectively. Examination of several Dane-rich concentrates revealed a close correlation between the presence of the denser species of Dane particles and the level of DNA polymerase. Moritsugu et al. (24) described the isolation of two species of cores with densities of 1.36 and 1.30 g/ml from Dane particles. Both of these populations had DNA polymerase activity. They found that the lighter core population had human immunoglobulin, presumably anti-HBcAg, associated with it, which they felt shifted its density. They noted that the lower density cores had much less enzymatic activity associated with them than the higher density core particles. Based on this observation they speculated that, in addition to antibody-associated DNA polymerase-containing core particles, this lower density material also contained particles without DNA polymerase activity. Our data indicate that this is indeed the case.

There are a number of possible explanations for the inability to detect DNA polymerase in the lower density Dane particles. One possibility is that the enzyme is not in the core of these particles. Another possibility is that the enzyme is present but the particles do not contain a template DNA, and therefore no endogenous DNA polymerase activity would be detectable. It is also possible that light Dane particles have neither the enzyme nor its nucleic acid template. Since all attempts to demonstrate DNA polymerase activity using exogenous templates or to solubilize the enzyme have failed, it is impossible to determine if the enzyme is present in these low-density Dane particles. The data, however, do indicate that the cores within these particles contain little or no nucleic acid.

If there are small amounts of DNA in these particles it could be of two origins, viral or cellular. Since DNA polymerase activity cannot be demonstrated, it seems likely that the DNA in these particles, if there is any, lacks an initiation site for the enzyme. If this were true, it would seem unlikely that the DNA could be of viral origin, since if polymerase activity is essential for viral replication such deletions would be strongly selected against. All naturally occurring or artificially created deletion mutants of simian virus 40 contain one or more copies of the nucleotide sequence involved in the initiation of DNA replication (5, 19, 22). We feel the most likely explanation for the data obtained is that the cores of these lighter Dane particles are empty. With no template present, the particles would have no demonstrable DNA
polymerase activity irrespective of whether the enzyme were present. The existence of such particles would certainly not be unexpected since most virus preparations do contain variable numbers of empty particles (10, 11, 15, 25).

Previously we studied the appearance of DNA polymerase in the serum of people undergoing an acute infection of hepatitis B (17, 21). It was observed that the DNA polymerase activity reached a peak and declined prior to the onset of clinical disease and at a time when HBAg titers were still high. It may be important to determine if, during the course of an acute infection, this decrease in DNA polymerase activity reflects a drop in the number of Dane particles or a shift in the ratio of full-empty Dane particles.

Unlike acutely infected individuals, most carriers of HBAg have only low levels of DNA polymerase activity in their blood (24). We have examined serum samples from HBAg carriers who have large numbers of Dane particles yet very little DNA polymerase activity, suggesting that most of the particles in these preparations are empty. Core particles have been isolated from the nuclei of liver cells from an individual infected with hepatitis B. These particles had a density of 1.30 g/ml and, when tested, showed no endogenous DNA polymerase activity, which suggested that they were empty (12). If full rather than empty Dane particles are infectious, then demonstrable DNA polymerase activity should correlate better with infectivity than either HBAg or HBcAg.

There are, potentially, three interfering particles in preparations of HBAg, which must be dealt with in any attempt to grow this virus in vitro: 22-nm particles, filaments, and empty Dane particles. Use of heavy Dane particles (polymerase containing) free from these potentially interfering forms of HBcAg may aid attempts to cultivate the agent of hepatitis B.

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LITERATURE CITED


