Dane Particles and Associated DNA-Polymerase Activity in Saliva of Chronic Hepatitis B Carriers

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To investigate further the problem of salivary transmission of type B hepatitis, salivas free of blood contamination from three HBsAg-positive carriers with chronic active hepatitis were examined by CsCl equilibrium density gradients and electron microscopy (EM). In the CsCl gradient HBsAg of whole salivas distributed in a band centered at 1.19 g/ml with a clearly defined shoulder at 1.24 g/ml; the HBsAg was found mainly in the mucous component. On EM examination, fractions from the 1.19 g/ml peak contained spherical HBsAg particles of 22 ± 3 nm diameter, whereas in the 1.24 g/ml shoulder Dane particles 43 nm in diameter with 28 nm cores were found. Specific hepatitis B virus associated DNA-polymerase activity also was found in the 1.24 g/ml shoulder where the Dane particles occurred and was absent from the saliva of healthy controls. When salivas were incubated for three hours at 37°C the total amount of HBsAg diminished and the 1.24 g/ml shoulder disappeared, probably as a result of endogenous degradation of the Dane particles and the free HBsAg. These findings clearly indicate that the hepatitis B viral particle is present in the saliva of chronic HBsAg carriers with active disease and further confirm that saliva is an important vehicle of infection.

Key words: Dane particles, DNA-polymerase, saliva, HBsAg carriers

INTRODUCTION

It is now established that transmission of hepatitis B is not exclusively parenteral. Krugman et al [1967] infected children experimentally by oral administration of patient's serum, nonparenteral intrafamilial transmission has been repeatedly observed [Szmuness et al, 1973; Koff et al, 1977], and contact spread epidemics of hepatitis B have been reported [Villarejos et al, 1972, 1975]. Thus, there is sufficient proof of other than parenteral means of spread of type B hepatitis. However, the exact mechanism of non-parenteral transmission remains unknown. There is evidence implicating saliva as the vehicle of infection; hepatitis B surface antigen (HBsAg) has been detected in the saliva from patients with acute and chronic hepatitis B [Brodersen et al, 1974; Villarejos et al, 1974], and the disease has been transmitted to nonhuman primates by injection of saliva.

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from chronic HBsAg carriers [Alter et al, 1977; Bancroft et al, 1977]. We report here the
detection of Dane particles and high levels of specific DNA-polymerase activity in saliva
samples of patients with chronic active hepatitis B.

PATIENTS AND METHODS

Three essentially asymptomatic carriers who had been consistently HBsAg positive
during the previous two years and in whom a recent liver biopsy showed histopathologic
signs of chronic active hepatitis were selected for this study. None of the patients had been
treated. Immunological and biochemical data are given in Table I.

Three healthy controls matched by sex and age were HBsAg negative by RIA (Aus-
tria II, Abbott, North Chicago, IL), with normal levels of transaminase and anti-DNA titers
below 80 mg/ml [Villarejos et al, 1978].

Collection and Preparation of Saliva Samples

Salivas from the three carriers and the controls under study were collected early in
the morning, before mouth toilet, to prevent blood contamination from gum bleeding; the
specimens were frozen within one to three hours after collection and were kept at -70°C
until examination. After thawing, salivas were homogenized by 20 strokes in a glass-Teflon
Potter homogenizer and tested for occult blood by the o-toluidine method [Fielding and
Langley, 1958].

CsCl Density Gradient Centrifugation

Homogenized saliva samples were brought to 0.02 M Tris-HCl at pH 7.5, and solid
CsCl was added to reach an initial density of 1.20 gm/cm³. Centrifugation was for 60
hours at 18°C and 42,000 rpm in a preparative ultracentrifuge (either Hitachi 65 P with
RPS 65T rotor or Beckman L 50 with SW 50 L rotor). Gradients were collected in 0.2 ml
fractions from the top using an ISCO (Lincoln, NE) Model D gradient fractionator and a
Fluorinert dense chase solution. The density of the individual fractions, as well as of the
initial solution, was calculated from their refractive indices measured at 25°C from 0.05
ml aliquots, using the relationship of Ifft et al [1970]:

\[ \rho = 10.2402 (n^2_0) - 12.6483 (1.00 < \rho < 1.38 \text{ gm/cm}^3) \]

<table>
<thead>
<tr>
<th>TABLE I. Serologic and Biochemical Data on Carriers Studied</th>
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<tr>
<td>Identification number</td>
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<tr>
<td>Sex and age</td>
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<tr>
<td>HBsAg (rephoresis)a</td>
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<tr>
<td>HBeAg (rephoresis)</td>
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<tr>
<td>Anti-HBc (immunoadherence)</td>
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<td>Anti-DNA (CIEP) mg/ml</td>
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<td>SGPT units/ml (colorimetric)</td>
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aAll three carriers subtype adw.
bAnti-HBe positive.
Radioimmunoassay for Hepatitis B Surface Antigen (HBsAg)

Radioimmunoassay (RIA) for HBsAg was carried out on all fractions with the Austria II-125 system of Abbott Laboratories (North Chicago, IL), using 0.1 ml saliva samples diluted to 0.2 ml with saline, with overnight incubation. HBsAg concentration is expressed as the ratio of net $^{125}$I cpm in the sample to mean cpm of the negative controls. There was no significant difference in counts between negative salivas and negative sera; therefore, all ratios were calculated using the negative sera references provided by Abbott.

Electron Microscopy

Fractions from CsCl density gradients were dialyzed against phosphate-buffered saline (PBS). A drop of the dialyzed sample was placed on a 400-mesh carbon-collodion-coated grid, and excess PBA was removed with filter paper without further drying; grids were then stained with phosphotungstic acid (PTA) at pH 7 and examined in a Hitachi HU 12A electron microscope at 40,000 X plate magnification.

DNA-Polymerase Assay

Each saliva sample was centrifuged in triplicate tubes. Identical 0.2 ml fractions from the three equivalent CsCl gradients were pooled; a 0.4 ml aliquot of each pooled fraction was diluted with 5 ml PBS and sedimented for four hours at 4°C and 90,000g max in a Hitachi RPS 65 T or a Beckman SW 50 L rotor. The precipitate was carefully resuspended in 0.2 ml of PBS. The hepatitis B virus-associated DNA-polymerase (HBV-pol) activity was assayed according to the method of Hirschman and Garfinkel [1977]. The standard DNA polymerase reaction mixture contained 0.05 M Tris-HCl (pH 7.5); 0.04 M MgCl$_2$, 0.4 M KCl, 0.3% 2-mercaptoethanol; 0.2% Nonidet-P40; 0.001 M each of dATP, dCTP, and dGTP; and 2.5 μCi of $^{3}H$dTTP (90 Ci/mmole specific activity). To the reaction mixture 50 μl of a polymerase-containing pellet suspension was added (100 μl final reaction volume). For each pool sample, HBV-pol activity was measured in duplicate before and after incubation at 38°C for three hours. Samples were then spotted on 22 mm Whatman 3 MM paper discs and immediately soaked in 5% trichloroacetic acid (TCA), 1% sodium pyrophosphate (10 ml per filter), according to the method of Bollum [1966]. After a minimum of 15 minutes of precipitation, filters were washed twice for 15 minutes in 5% TCA, twice for five minutes in 95% ethanol, and once in absolute ethanol. Filters were dried overnight and counted using standard procedures. Activity is expressed as $^{3}H$ cpm of incubated sample minus $^{3}H$ cpm of non-incubated sample.

Chemicals and Reagents

The unlabeled deoxynucleotide triphosphates, 2-mercaptoethanol, and CsCl were purchased from Sigma Chemical Co. (St. Louis, MO). Tritiated deoxythymidine triphosphate ($^{3}H$dTTP, specific activity 90 Ci/mmole) was obtained from ICN Pharmaceuticals (Irvine, CA), and Nonidet P-40 from Particle Data Laboratories (Elmhurst, IL).

RESULTS

All saliva samples were negative for occult blood when tested by the o-toluidine method.
HBsAg Distribution in CsCl Equilibrium Density Gradients of Saliva

Figure 1 shows an example of the CsCl equilibrium density gradient profile of homogenized whole saliva from one of the three carriers studied. The HBsAg, as measured by RIA, was distributed in a band centered at 1.19 gm/cm³, separated from the bulk of mucous material, which banded from 1.24 gm/cm³ to 1.27 gm/cm³; sometimes mucous material was found pelleted at the bottom of the centrifuge tube. A very clear shoulder was found repeatedly on the heavy side of the HBsAg band at densities from 1.23 gm/cm³ to 1.24 gm/cm³. The densities of 1.19 gm/cm³ and 1.24 gm/cm³ conform with published values for the spherical forms of HBsAg and Dane particles, respectively [Kaplan et al., 1976; Gerin et al, 1971].

Distribution of HBsAg in the Two Main Components of Saliva

To determine if the HBsAg was actually associated with the liquid or the mucous components, these were separated by low-speed centrifugation at 90g max for ten minutes. The sediment was resuspended in saline to the original volume of saliva and homogenized by 20 strokes in a glass-Teflon homogenizer. The supernatant was concentrated five times in a Minicon S-125 (Amicon Corp., Lexington, MA). Both fractions were centrifuged to equilibrium in CsCl gradients (Fig. 2). The mucous phase (Fig. 2A) contained a high concentration of HBsAg, but the level of the antigen found in the liquid phase was too low to show its distribution in the density gradient; it was necessary to make a fivefold concentration before centrifugation to determine the distribution of the latter (Fig. 2B).

Electron Microscopy

Fractions from the CsCl equilibrium density gradients of whole saliva from the three carriers were examined in the electron microscope (PTA staining). Fractions from the main peak (1.19 gm/cm³) revealed high concentrations of spherical particles, 22 ± 3 nm in diameter (Fig. 3A); no filaments of any size were seen. Fractions from the heavy shoulder revealed particles with an outer diameter of 43 nm and a core of 28 nm (Fig. 3, B–J), such as described by Dane et al [1970]. Some of the 43 nm particles observed were partially destroyed. This could be due to the preparative procedure or to self-digestion in the saliva (Fig. 3K).

![Figure 1. Distribution of HBsAg in a CsCl density gradient of homogenized whole saliva. (Carrier 208-01 as typical example.) Centrifugation was for 60 hours at 18°C and 42,000 rpm in a Hitachi RPS 65 T rotor. Gradients were collected in 0.2 ml fractions from the top.](image-url)
Changes in HBsAg Distribution in CsCl Gradients After Incubation

Lower levels (Austria II ratios) of HBsAg were found in whole salivas kept for a few hours at room temperature than in the same salivas frozen immediately after collection and stored at -70°C [Villarejos et al, 1974]. To test the possibility that endogenous degradation of HBsAg could account for this decrease as well as for the partial disruption of Dane particles observed in electron micrographs, salivas from the three chronic carriers were pooled, homogenized, and separated into three aliquots; one of them was kept at 4°C, and the other two were incubated at 37°C, one for 30 minutes, and the other for three hours. The three aliquots were then centrifuged to equilibrium in CsCl as described above. Figure 4 shows the distributions of HBsAg obtained. The nonincubated aliquot (Fig. 4A) showed the typical distribution with a peak at 1.19 gm/cm³ and a well-defined shoulder at 1.24 gm/cm³; no significant amount of HBsAg was found in the first fractions. After 30 minutes of incubation at 37°C, the distribution broadened, HBsAg was found in the first fraction, and the heavy shoulder was less evident (Fig. 4B); after three hours of incubation, the total amount of antigen, as judged by the RIA values in contiguous fractions, was lower and the shoulder had completely disappeared (Fig. 4C). Again HBsAg was present in the first fraction, indicating a modification of the density of the antigen particles of incubated samples.

![Diagram](image)

Fig. 2. Distribution of HBsAg in mucous (A) and fluid (B) phases of saliva. Centrifugation conditions as in Figure 1. (Carrier 1020-06 as typical example.)
Fig. 3. Electron microscopy. A. Fractions from the 1.19 gm/cm$^3$ peak, showing 22 nm spherical particles. B. Fraction from the 1.24 gm/cm$^3$ shoulder, showing in the same field three Dane particles (arrows); these three particles are shown enlarged in plates C–E. Plates F–J show different particles found in preparations from the 1.24 gm/cm$^3$ shoulder. K. Disrupted or digested particles. PTA staining; bar represents 100 nm.
Fig. 4. Endogenous degradation of HBsAg in saliva. A. No incubation. B. Incubation for 30 minutes at 37°C. C. Incubation for three hours at 37°C. Centrifugation conditions as in Figure 1.

**Hepatitis B Virus-Associated DNA-Polymerase Activity in HBsAg-Positive Saliva**

The distribution of HBV-pol activity was measured in fractions from the equilibrium density gradients of individual saliva samples from the three chronic hepatitis B carriers and the healthy individuals who served as controls. In the saliva of all three patients one major peak of activity was found at 1.24 gm/cm³, with (³H) incorporation trailing toward higher densities. Figure 5 shows a typical experiment (carrier 916-08 and a healthy control); the density of 1.24 gm/cm³ (Fig. 5A, filled arrow) corresponds to the density of the Dane particles found by electron microscopy in saliva (shoulder in the HBsAg distribution). The level of (³H) cpm incorporation per ml of whole saliva was of the same magnitude as that found in the serum of the same patient. In healthy controls, a small peak of (³H)dTTP incorporation was constantly found at 1.28 gm/cm³ (Fig. 5B, open arrow) but at 1.24 gm/cm³ the (³H) incorporation was always at the background level.
Fig. 5. Hepatitis B virus-associated DNA-polymerase (HBV-pol) activity in HBsAg saliva. A. HBV-pol activity in saliva from an HBsAg carrier with chronic active hepatitis B (Carrier 916-08 as typical example). B. Healthy HBsAg-negative control. Centrifugation conditions as in Figure 1. HBV-pol activity was assayed according to Hirschman and Garfinkel [1977]. Activity is expressed as (\(^3\)H) cpm of incubated sample minus (\(^3\)H) cpm of nonincubated control. The filled arrows indicate the density of 1.24 gm/cm\(^2\) where Dane particles and HBV-pol activity were found; the open arrows indicate the density of 1.28 gm/cm\(^2\) where the nonspecific activity is found.

**DISCUSSION**

The pattern of intrafamilial occurrence of type B hepatitis observed in our epidemiological studies suggested the exchange of saliva, directly between sexual partners (kissing) or indirectly through utensils or chewed objects and candies among children, as the most probable mechanism of transmission; the finding of HBsAg in the saliva of all chronic carriers and 70% of acute cases further pointed to saliva as a vehicle of transmission of hepatitis B [Villarejos et al, 1974]. However, there was a possibility that only the HBsAg was filtered or excreted through the saliva, since Dane particles were not found; if the saliva from HBsAg carriers contained only surface antigen it would probably be noninfective. That it could indeed be infective was demonstrated by the experimental transmission of hepatitis B to chimpanzees and gibbons [Alter et al, 1977; Bancroft et al, 1977] by intravenous injection of HBsAg-positive saliva, although oral inoculation failed.

Reports on the finding of HBsAg in saliva of carriers vary widely [Szmuness et al, 1973; Ward et al, 1972; Vittal et al, 1974], but these variations may only reflect differences in methodology. We have seen that the HBsAg content of the saliva decreases notably as time elapses between collection and examination [Villarejos et al, 1974] and that the antigen is trapped in the mucous, whereas only small amounts are found in the liquid phase.
For optimal results it is critical, then, to examine whole homogenized salivas, and to do so soon after collection. The fact that the HBsAg distribution changes and that HBsAg is found in the top fractions of those CsCl equilibrium density gradients of salivas which have been incubated at 37°C reflects a modification of the surface antigen particles. In CsCl gradients of saliva collected under optimal conditions, fractions from the clearly defined heavy shoulder at 1.24 gm/cm$^3$ examined by electron microscopy contained well-preserved Dane particles with an outer diameter of 43 nm and a core of 28 nm, with morphology similar to that reported by other authors [Dane et al., 1970; Jokelainen et al., 1970].

In the saliva used for their animal experiments, Bancroft et al [1977] found structures suggestive of deformed Dane particles and reported that the DNA polymerase activity of those salivas was of the same order as that measured in the saliva of healthy individuals. In our investigation some of the Dane particles seen were also disrupted, but the majority were complete and well preserved; there was an indication that the total content of HBsAg decreased and the proportion of deformed Dane particles increased in salivas that had been kept at room temperature for six to eight hours before freezing. These observations taken together suggest that the HBsAg may be degraded in the saliva, probably by enzymatic action.

The finding of specific DNA-polymerase activity associated with Dane particles is regarded as evidence of active viral replication [Kaplan et al., 1973; Krugman et al., 1974]. In the three carriers reported herein a high level of specific HBV-pol was detected in those fractions of the density gradients of salivas that contained the Dane particles, together with a variable level of ($^3$H)dTTP incorporation in acid-precipitable material interpreted as nonspecific DNA polymerase activity, which was similar to that observed in normal controls. In nonfractionated samples this nonspecific ($^3$H)dTTP incorporation may obscure the specific HBV-polymerase activity. In CsCl gradients the density difference of 0.04 gm/cm$^3$ found between the two activities is large enough to permit an easy distinction. The facts that no activity was found at 1.24 gm/cm$^3$ in the controls and that the HBV-pol activity was measured as the ($^3$H)dTTP incorporation after subtracting the ($^3$H) cpm in nonincubated samples under conditions where the specific HBV-pol activity is enhanced and other polymerase activities are inhibited [Hirschman and Garfinkel, 1977] lead us to think that the 1.28 gm/cm$^3$ peak present in normal samples may be due to an incomplete inhibition of a nonspecific cellular polymerase activity yet to be characterized. Evidently this nonspecific activity at 1.28 gm/cm$^3$ accounted for the heavy trailing from the specific HBV-pol peak at 1.24 gm/cm$^3$ that was observed in the saliva of the patients with chronic active hepatitis B.

The level of HBV-pol in the salivas of the three patients was as high as that found in their sera, clearly showing that the salivary HBV-pol activity was not due to blood contamination; this was also precluded by the negative results of the o-toluidine tests, which can detect 3 mg of hemoglobin per 100 ml [Fielding and Langley, 1958].

From the finding of intact Dane particles and high levels of HBV-pol activity in saliva, it seems probable that the HBsAg is not just filtered from the serum and excreted in the salivary secretion. We are now investigating the possibility that the complete virus may be produced, or replicated, in tissues of the oral cavity.
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