Buoyant Density of the Hepatitis A Virus-Like Particle in Cesium Chloride

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We recently visualized by immune electron microscopy a virus-like particle in the stools of patients with hepatitis A. The particle measured approximately 27 nm in diameter and morphologically resembled a picornavirus or parvovirus. To further characterize this particle, we have determined its buoyant density in cesium chloride (CsCl) by ultracentrifugation. Hepatitis A particles from three positive stool specimens were isopycally banded in separate experiments, and the gradient fractions were examined for particles by immune electron microscopy by using hepatitis A convalescent sera. In each experiment, the particles were observed in a normal distribution about a peak fraction with a mean density of approximately 1.4 g/cm³. The buoyant density of 1.4 g/cm³ in CsCl together with its morphology and the reported resistance of hepatitis virus to acid, ether, and heat suggest that this particle is parvovirus-like.

We recently described the detection of a virus-like particle in the stool of patients with hepatitis A (HA) (5). By using the technique of immune electron microscopy (IEM) (1), we found this 27-nm particle to be serologically related to both experimentally induced and naturally acquired hepatitis A. Furthermore, the particle was shown not to be antigenically related to either hepatitis B surface or core antigens or the Norwalk particle. The latter was previously identified by Kapikian et al. (10) in a stool filtrate from a patient with acute infectious nonbacterial gastroenteritis.

The hepatitis A particles measured approximately 27 nm in diameter, appeared to have cubic symmetry, and resembled either parvoviruses or picornaviruses morphologically. Marmoset studies by Provost et al. (12), coupled with earlier studies in human volunteers (6), suggest that the hepatitis A virus (HAV) is stable to acid, ether, and heat (60 C for 1 h), characteristics common to the parvoviruses. To further characterize the HA particle, we determined its buoyant density in cesium chloride (CsCl) by utilizing IEM with HA convalescent sera to determine the distribution of particles in fractions of the density gradients.

Stool specimens from volunteers experimentally infected with the MS-1 strain of HAV (2) were supplied as 20% (wt/vol) suspensions in normal saline by D. W. Gibson (Armed Forces

Institute of Pathology). Ten-fold dilutions of these suspensions were filtered and examined by IEM as previously described (5). The virus-like HA particles were found in stool filtrates of five of eight volunteers only during the acute phase of illness. The original 20% stool suspensions from three of these individuals were used as the source of particles in separate experiments for the buoyant density determinations.

The buoyant density in CsCl of the HA particle in one stool was determined by isopycnic banding in a preformed six-step CsCl density gradient (1.1 to 1.6 g/cm³), as described in the legend to Fig. 1.

IEM was performed on the fractions of the CsCl density gradient with minor modifications of the methods of Kapikian et al. for determining the density of the "Norwalk" particle (9). Briefly, 0.3 ml from each gradient fraction was diluted to 0.9 ml in phosphate-buffered saline (0.85% NaCl, 0.01 M phosphate, pH 7.4) and mixed with 0.1 ml of a 1:10 dilution of a convalescent serum from a patient with naturally acquired hepatitis A in American Samoa. This patient had previously been shown to have a specific seroconversion to the HA particle found in a volunteer's acute illness stool (5). The mixtures of antiserum and antigen were incubated for 1 h at room temperature and centrifuged in a 40.2 fixed-angle rotor at 22,000 rpm for 90 min at 4 C by using a Beckman

FIG. 1. Isopycnic banding of the 27-nm hepatitis A particle in a cesium chloride density gradient. A centrifuge tube was loaded with a 10-ml six-step discontinuous density gradient of CsCl in 0.01 M Tris (1.1 to 1.6 g/cm³), then 1 ml of the 20% stool extract that contained the HA particles, and finally, 1 ml of a 0.01 M Tris buffer overlay. The SW41 rotor was accelerated to 35,000 rpm, and the sample was centrifuged for 18 h at 5°C ($88 \times 10^3 \omega^2 T$). The gradient was displaced from the bottom and collected in 15 $\times$ 0.75-ml fractions. Quantitation of particle recovery was by immune electron microscopy as described in text.

L2-65B ultracentrifuge. The supernatant was withdrawn with a Pasteur pipette, and the pellet was resuspended in a drop of deionized water, mixed with an equal volume of 3% phosphotungstic acid, pH 7.2, and placed on a 400-mesh Formvar-carbon grid; the excess fluid was drawn off with filter paper. The grids were examined at an initial magnification of 45,000 with a Siemens Elmiskop 1A electron microscope. All of the particles in 10 good quality grid squares prepared from each sample were counted. Ten additional squares were examined from each fraction in which particles were observed in the initial screening to more precisely determine the peak fraction.

The particles associated with hepatitis A were found in 3 of the 15 fractions in the initial examination of 10 squares from each fraction; particles were again observed in the second 10 squares studied from these three positive fractions. The total number of particles detected in 20 grid squares from positive fractions was determined, and Fig. 1 represents the particle distribution in the density gradient. Of the 121 particles observed, 60 (56%) were observed in fraction 5, which had a mean density of 1.41

g/cm³. The remaining particles were found in fractions 4 and 6, and the relative proportions of particles in these three fractions approximated a normal distribution. Figure 2 is an electron micrograph of a small aggregate of HA virus-like particles observed in the peak fraction.

In another experiment, in which 1 ml of a stool extract from a different volunteer was banded under similar conditions, the results were essentially the same. The particles in the 1.41-g/cm³ fraction were aggregated in separate IEM experiments by the same convalescent serum from the patient with naturally acquired HA and also by the convalescent serum from a volunteer (K). The latter was infected with the MS-1 strain of HAV and developed antibody to the HA particle after illness, as determined by IEM.

In a third experiment, 10 ml of a 20% stool extract from a third patient shown to have a large number of HA particles in his stool were layered on a 28-ml six-step CsCl gradient (1.1 to 1.6 g/cm³) and centrifuged at 25,000 rpm for 21 h at 4°C in an SW27 rotor ($53 \times 10^{10} \omega^2 T$). Twenty fractions were collected, and a sample from each was studied for the presence of the HA particles by IEM by using convalescent serum from volunteer K. A total of 1,973 particles were observed only in fractions 4 through 8 and were distributed in an approximately normal distribution around the peak fraction that had a mean density of 1.39 g/cm³.

The results of separate isopycnic banding experiments with HA particles from the stools

FIG. 2. A small aggregate of hepatitis A virus-like particles observed after incubating 0.3 ml of fraction number 5 (density 1.41 g/cm³) with 0.1 ml of a 1:10 dilution of a convalescent serum from a patient with hepatitis A. The specimen was prepared for electron microscopy as described in Materials and Methods. Magnification, $\times 225,000$.

of three volunteers indicate that the 27-nm hepatitis particle possesses a buoyant density of approximately 1.4 g/cm³ (1.39 to 1.41 g/cm³). This density is consistent with that of a parvovirus or rhinovirus but not an enterovirus. It would appear that the hepatitis A agent is not a rhinovirus because the latter are acid labile (13), whereas Provost et al. (12) showed the agent of HA to be acid stable in studies in marmosets.

Reagents have not been available to make a direct comparison between the HA stool particles from volunteers infected with the MS-1 strain of HA virus and the CR326 strain of HA virus that Provost studied in marmosets. However, in studies with marmosets, Provost et al. (12), using a Costa Rican strain of HAV, and Holmes et al. (8), using an MS-1 derived strain, independently demonstrated neutralization of HAV by convalescent but not preinfection sera from volunteers experimentally infected with MS-1. We demonstrated seroconversions specific for the HA antigen in these same serum pairs.

Somewhat different characteristics were attributed to a serologically distinct virus isolated from a patient with human hepatitis and serially passaged by Deinhardt et al. in marmosets (3). This virus was found to be partially sensitive to ether, inactivated by heat (60 C for 30 min), and to have a buoyant density of 1.21 in CsCl (4). Parks and Melnick (11) provided evidence that this agent was a latent virus of marmosets. However, additional studies must be carried out to determine its true origin.

Although the type of nucleic acid of the HA virus is not known, this virus most closely resembles the parvovirus group. By similar reasoning, the "Norwalk" particle, which was also found in the stool and which has been etiologically related to a form of acute infectious nonbacterial gastroenteritis, was provisionally classified as a parvovirus (9). Furthermore, despite intensive efforts, neither agent has been shown to replicate in an in vitro culture system. It is well known that parvoviruses as a group have often required specialized techniques for their isolation and that the four known parvoviruses of human origin are defective and grow only in the presence of a helper virus (7).

The identification by IEM of a virus-like particle in the stool of patients with hepatitis A has led to the first in vitro serological test for hepatitis A antigen and its antibody. Determination of the buoyant density of the particle in

CsCl has provided additional evidence to that previously reported (5, 12) that hepatitis A virus is parvovirus-like. Knowledge of its density will aid in its purification and further characterization.

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