Purification and Biophysical Properties of Acute Haemorrhagic Conjunctivitis Virus

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The buoyant density of acute haemorrhagic conjunctivitis virions labeled with either [³H]uridine or [³H]leucine was 1.34 g/ml in CsCl and 1.25 g/ml in sucrose. RNA extracted from the virions gave a sedimentation coefficient of approximately 34S in sucrose, and was found to be sensitive to RNase. Molecular weight of RNA was calculated to be 2.5×10^6 using poliovirus RNA for reference.

Acute haemorrhagic conjunctivitis (AHC) virus is a new RNA virus which in recent years caused a pandemic of acute haemorrhagic conjunctivitis in Africa, India, Asia, and some European countries. It has been reported (3) that the virus is resistant to chloroform, acid stable at pH 3.0, retains full infectivity by heating at 50 C for 1 h in the presence of 1 M MgCl₂, and its growth in tissue culture is not inhibited in the presence of 5-iododeoxyuridine. Electron microscope examination of crude virus preparations revealed that AHC virion is a naked spherical particle, probably cubic in symmetry with a diameter of 29 ± 1 nm. The present investigation was undertaken to determine some physicochemical properties of the virion, and the information presented here provisionally classifies the virus as a member of the enteroviruses.

Experimental

The standard strain of AHC virus, J 670/71, which had been passaged three times in human embryonic kidney cells after being isolated from a patient in Japan, was purified by two successive plaque clonings in human embryonic lung cells, and then grown in cynomolgus monkey kidney (MK) cell cultures to prepare the seed virus. Virus titration was performed in MK cell cultures grown in microplates with 0.5% lactalbumin hydrolysate in Earle buffered salt solution (LE) containing 2% calf serum. Eight wells were inoculated with each serial 10-fold virus dilution and incubated in Eagle minimum essential medium (MEM) at 33 C (optimal temperature). At the end of 5 days the plates were examined for cytopathic effect (CPE).

Prior to labeling viral nucleic acid with radioisotope, we first tested the effect of various concentrations of actinomycin D (0.5 to 10 µg/ml) on incorporation of [³H]uridine into

uninfected MK cells and on replication of AHC virus in the same cells. As was expected, even the highest concentration of actinomycin D (10 µg/ml) did not significantly inhibit the viral replication, whereas the same dose of actinomycin D reduced cellular RNA synthesis by over 98% within 2 h. The failure to inhibit viral replication is consistent with the general property of the picornaviruses. In subsequent experiments, therefore, we used a 10 µg/ml dose of actinomycin D which significantly reduced the incorporation of [³H]uridine into cellular RNA and thus eliminated contamination of viral material with labeled cellular RNA. The second baseline experiment was performed to determine the time for labeling viral nucleic acid. As will be reported in detail elsewhere (K. Miyamura and S. Yamazaki, manuscript in preparation), viral RNA synthesis began within 3 h after infection of MK cells with AHC virus at a multiplicity of infection of approximately 10, increased up to 7 to 8 h, and then leveled off. Under the same experimental conditions, the production of virus particles in infected cells followed a similar time pattern, but most of the virus particles were not readily released into the culture medium even at 8 h after infection when the maximum titer was attained intracellularly. Based on the information from these studies, isotopically labeled AHC virus was prepared as described below.

Monolayer cultures of 2×10^7 MK cells grown in flasks (126-cm² surface) with LE medium containing 2% bovine serum were washed and infected with approximately 10 mean lethal doses (TCD₅₀) per cell. After adsorption at 33 C for 1 h, the monolayers were washed three times and exposed to actinomycin D (10 µg/ml) for 2 h at 33 C. The medium was then aspirated, and the monolayers were incubated at 33 C with 20 ml of MEM without serum, but containing 10

μCi of [^3H]uridine per ml ([$5\text{-}^3\text{H}$]uridine, 27 Ci/mmol; Amersham, England). At about 16 h after infection, when 75% of the cells showed CPE and the virus titer of the culture ranged from $10^{7.5}$ to $10^{8.0}$ TCD₅₀ per ml, the cultures were frozen and thawed three times, and centrifuged at 3,000 rpm for 10 min to remove cell debris. The clarified culture fluids were recentrifuged at $10,000 \times g$ for 15 min; the supernatants were saved and used for subsequent virus purification. An 18-ml volume of the virus-containing material was then layered on a double cushion, consisting of a bottom layer of 3 ml made up of 60% (wt/wt) sucrose and CsCl (15% wt/vol), and an upper layer made of 7 ml of 15% (wt/wt) sucrose in TNE buffer (0.01 M Tris-hydrochloride, pH 7.4, 0.1 M NaCl, and 0.001 M EDTA). Centrifugation was performed in a RPS 25 rotor of Hitachi-55PA ultracentrifuge at 25,000 rpm ($63,500 \times g$) for 2 h at 4°C. A considerable amount of radioactivity was retained in the top layer. However, labeled virus and approximately 60% of the total infectivity was sedimented at interphase. The purified virions, which banded between the 15 and 60% sucrose layers, were collected by fractionation

from the bottom of the centrifuge tubes. Fractions were pooled and then dialyzed in a large volume of TNE buffer for 48 h at 4°C, prior to use for buoyant density determination.

The purified, [^3H]uridine-labeled AHC virions were then centrifuged to equilibrium in CsCl at a starting density of 1.33 g/ml. Centrifugation and fractionation were performed as described in the legend to Fig. 1. A 100- μl portion of each fraction was weighed for density determination, and then treated with 5% trichloroacetic acid. The precipitates were washed and collected on GF/C filters (25 mm in diameter; Whatmann, W. & R. Balston Ltd., England). After drying, the filters were submerged in toluene-based fluors, and radioactivity was counted in a Packard scintillation spectrometer. The remaining portion of each fraction was assayed for infectivity in microplates. Figure 1A shows that the peaks of infectivity and radioactivity coincided at a density of 1.34 g/ml. In several subsequent experiments the buoyant density of similarly purified virions varied between 1.32 and 1.35 g/ml. However, if the virions collected from the CsCl viral band were further purified by rate-zonal sedimentation in

FIG. 1. Buoyant density of purified, [^3H]uridine-labeled AHC virions as determined in CsCl and sucrose. (A) [^3H]uridine-labeled AHC virus was purified by differential centrifugation, Daiflon treatment, and sedimentation through a 15% (wt/wt) sucrose barrier onto a 60% (wt/wt) sucrose cushion mixed with CsCl (15% wt/vol). The purified virions, thus collected on a sucrose cushion, were then dialyzed in TNE buffer, and 4.3 ml of the sample was mixed with CsCl to give a starting specific gravity of 1.33 g/ml. Isopycnic centrifugation was performed for 44 h at 35,000 rpm ($100,000 \times g$) in a Hitachi RPS 40 rotor. Fractions of 0.3 ml each were collected from the bottom of the tube, and samples were weighed, assayed for infectivity, and counted for acid-precipitable radioactivity. (B) A 0.5-ml amount of similarly purified AHC virions was layered on a preformed 35 to 60% sucrose gradient and centrifuged for 15 h at 35,000 rpm in the RPS 40 rotor. Symbols: ●, [^3H]uridine; ○, infectivity; —, density.

a sucrose gradient, and then recentrifuged to equilibrium in CsCl, the virus constantly banded at a density of 1.34 g/ml. The buoyant density in sucrose of similarly prepared AHC virions was 1.25 g/ml by equilibrium centrifugation at $100,000 \times g$ for 15 h in a preformed 35 to 60% sucrose gradient (Fig. 1B). Electron microscope examination of the peak fraction from a sucrose gradient revealed virions of cubic symmetry with a diameter of about 30 nm (3).

Homogeneity in size of AHC virus was tested by rate-zonal sedimentation in sucrose of purified AHC virions whose proteins were labeled with [^3H]amino acid. To label viral proteins, the infected, actinomycin-treated MK cells were incubated at 33 C in MEM without leucine, but containing 10 μCi of [4,5- ^3H]leucine per ml (38 Ci/mmol; Radiochemical Center, Amersham, England). As shown in Fig. 2A, the CsCl-purified AHC virions sedimented in a sucrose gradient as one sharp band with 80% recovery of radioactivity in three fractions (fractions 15, 16, and 17), indicating a homogeneous size of the virions labeled with [^3H]leucine. Similar results were obtained with the [^3H]uridine-labeled virions (data not shown). To con-

firm the density, the virions collected from the peak fractions in Fig. 2A were recentrifuged to equilibrium in CsCl. Figure 2B shows that [^3H]leucine-labeled AHC virus also sedimented at a density of 1.34 g/ml.

To measure the molecular weight of AHC RNA, the sedimentation coefficient of the RNA was estimated by the method of Martin and Ames (4) by using 28 and 18S Vero cell ribosomal RNAs as markers, which were labeled by incubating cells for 18 h with 1.0 μCi of [^{14}C]uridine per ml. For comparison, poliovirus type 1 (strain Mahoney) was grown in MK cells, labeled with [^3H]uridine, and purified in a same way as for AHC virus. The RNAs from purified AHC virus and poliovirus as well as from Vero cells were extracted with phenol at 60 C according to the method of Scherrer and Darnell (6). Figure 3 shows that both AHC RNA and polio RNA sedimented in sucrose at a similar rate with a sedimentation coefficient of 34S (AHC) and 35S (polio), respectively. A molecular weight of approximately 2.5×10^6 was calculated for AHC RNA by the method of Martin and Ames (4) assuming the molecular weight of polio RNA to be 2.6×10^6 (2). The AHC RNA

FIG. 2. Rate-zonal (A) and equilibrium (B) centrifugation of [^3H]leucine-labeled AHC virions. [^3H]leucine-labeled AHC virus was purified by equilibrium centrifugation in CsCl at $147,000 \times g$ for 44 h in a Spinco rotor SW 41. The purified virions were collected from the viral band in CsCl and concentrated in a collodion bag (Sartorius Membranfilters, Göttingen) by pressure dialysis in TNE buffer. (A) A one-ml sample (95,700 counts/min) of this concentrate was layered on a preformed 5 to 30% sucrose gradient and centrifuged at 22,500 rpm ($51,500 \times g$) for 100 min in a Hitachi RPS 25 rotor. Twenty-seven fractions of 1 ml each were collected from the bottom of the tube, and counted for radioactivity. (B) The peak fractions of radioactivity (fraction no. 15, 16, and 17) from A were pooled and dialyzed against TNE buffer to remove sucrose. The sample (4,860 counts/min) was mixed with CsCl to give a starting specific gravity of 1.328 g/ml, and then centrifuged to equilibrium in the RPS 40 rotor (35,000 rpm, 44 h). Thirteen fractions of 0.37 ml each were collected from the bottom of the tube, and assayed for both radioactivity and infectivity. Symbols: ●, [^3H]leucine; ○, infectivity.

FIG. 3. Comparative sedimentation analysis of AHC-RNA and polio-RNA. [^3H]uridine-labeled RNA from CsCl-purified AHC virions and poliovirions was extracted at 60 C for 3 min with TNE-saturated phenol in the presence of 0.1% SDS. The phenol extraction was repeated twice. The extracted aqueous RNA was then precipitated at -20 C with two volumes of ethanol in the presence of unlabeled Vero cell RNA as carrier. The RNA precipitate was collected after 20 h by centrifugation and dissolved in TNE buffer solution; AHC-RNA and polio-RNA solutions contained 432,000 counts/min/ml and 368,000 counts/min/ml, respectively. Samples of 1.0 ml each were mixed with 0.3 ml (254,000 counts/min) of [^{14}C]uridine-labeled RNA which had been extracted from a cytoplasmic fraction of Vero cells, and layered over a 15 to 30% sucrose gradient in TNE buffer containing 0.5% SDS. After centrifugation in a Hitachi RPS 25 rotor at 19,000 rpm ($36,700 \times g$) for 15 h at 20 C, fractions were collected from the top of tubes and trichloroacetic acid-precipitable radioactivity of each 1-ml fraction was determined. Radioactivity counts per minute for both AHC-RNA and polio-RNA were plotted in the same figure by relative distance to Vero cell ribosomal RNA. Symbols: —, [^3H]uridine; ----, [^{14}C]uridine.

was completely digested by incubation at 37 C for 30 min with 20 μg of RNase A (Sigma Chemical Co.), suggesting that AHC virus contains a single-stranded RNA.

In respect to buoyant density of intact virion

and molecular weight of viral RNA, AHC virus was found to be similar to enteroviruses and/or cardioviruses (1). The virus was clearly distinguished from the acid-sensitive picornaviruses such as rhinoviruses and foot-and-mouth disease viruses which have densities in CsCl of 1.38 to 1.41 g/ml and 1.43 g/ml, respectively (5). Moreover, our preliminary experiments revealed that AHC virus was stable when it was incubated for 60 min at 37 C in the presence of 0.1 M NaCl in distilled water, pH 6.0. In contrast, members of the cardioviruses (e.g., mengo virus and ME virus) have been reported, unlike the enteroviruses, to be unstable in the pH range 5 to 7 in the presence of 0.1 M chloride ion.

The data presented in this report, together with other information (3), suggest that AHC virus is a single-stranded RNA virus with no envelope and possesses, as far as examined, the general properties of the enteroviruses. However, serological studies have shown that the virus is antigenically different from any types of previously identified enteroviruses (3). From the properties so far examined, AHC virus might be considered to be a new type species of the enteroviruses, but definitive classification should await analytical studies on the structural proteins, which are now in progress.

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